Modulation of Ca$^{2+}$ Efflux from Heart Mitochondria

By ERIC J. HARRIS

Department of Biophysics, University College London, London WC1E 6BT, U.K.

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The efflux of Ca$^{2+}$ from rat heart mitochondria has been examined by using Ruthenium Red to inhibit active uptake after predetermined loadings with Ca$^{2+}$. The efflux is proportional to the internal Ca$^{2+}$ load; it is increased by Na$^{+}$ applied when the mitochondria are respiring and this effect is inhibited by oligomycin. The efflux of Ca$^{2+}$ is diminished by ATP and by ADP, with the latter the more effective. Both active uptake and efflux of Ca$^{2+}$ are slowed by bongkrekic acid; this action has a time lag. The lower efflux found with the nucleotides and with bongkrekic acid seems to correspond to the more condensed state seen in the electron microscope when these agents are applied [Stoner & Sirak (1973) J. Cell Biol. 56, 51–64, 65–73]. The results are discussed in relation to the less-permeable state being contingent upon nucleotide binding to the membrane.

The mechanisms by which mitochondria may participate in the control of the free Ca$^{2+}$ concentration in the cytosol are being elucidated by studies of the uptake and release of Ca$^{2+}$ by mitochondria as influenced by cytosolic components and certain specific inhibitors (Spencer & Bygrave, 1973; Pozzan et al., 1977; Crompton et al., 1976a, b, 1977; Harris, 1977). The present study is concerned with the movements of free Ca$^{2+}$; it is still uncertain whether mitochondria possess the property of exporting Ca$^{2+}$ in ‘packets’, as discussed by Lehninger (1970) with respect to the calcification process. Ca$^{2+}$ is usually rapidly accumulated by energized mitochondria but such accumulation is accompanied by a slow leak. This leak or efflux is now readily measurable, thanks to the possibility of specific inhibition of further influx with Ruthenium Red (Vasington et al., 1972; Crompton et al., 1976a, 1977). The effect on the efflux of previously accumulated Ca$^{2+}$ of factors such as Na$^{+}$ concentration, nucleotides and certain inhibitors can thus be conveniently investigated. In the present study it is shown that the increased efflux associated with Na$^{+}$ (Crompton et al., 1976a, 1977) is diminished by oligomycin. The Ca$^{2+}$ efflux occurring in the absence of added sodium salt is inhibited by adenine nucleotides and by bongkrekic acid. The latter substance promotes tight binding of the nucleotides to the membrane (Klingenber et al., 1970). The results fit in with the observations of a condensation of matrix material, ascribed to contraction of the inner membrane, in response to the same agents (Stoner & Sirak, 1973a, b).

Methods

Rat heart mitochondria were prepared as described before (Harris & Zaba, 1977; Harris, 1977), with the final washing in a medium without chelating agent other than 0.1% defatted albumin. The final suspension was made in 300mM-mannitol/5mM-KCl/10mM-Tris/chloride, pH 7.2. After a sample was taken for protein determination by a biuret method, an addition was made of 2mg of serum albumin/ml.

The Ca$^{2+}$ movements were observed as before by recording changes in the differential absorbance of the metallochrome dye Arsenazo III with an Amino DW 2 spectrophotometer. The wavelength pair 685–665nm was used, which provides a higher sensitivity than the 685–675nm pair used before. Uptake and efflux experiments were made in a medium having either KCl or NaCl at 120mM (or a mixture as specified), and Tris chloride, pH7.2 (20mM), Tris phosphate (1.2mM), Tris succinate (3mM), rotenone (1μg/ml) and sufficient purified Arsenazo III to provide $A_{520}$ 1.5. The usual procedure was to dilute the mitochondria with medium to obtain about 0.5mg of protein/ml. A measured addition of CaCl$_2$ was made to provide the desired loading. The uptake was followed (as illustrated in Fig. 1) and was nearly complete after about 2min unless bongkrekate had been added. The active influx was stopped by adding Ruthenium Red (0.4μM); a trial showed that a further similar addition had no effect. The efflux revealed when the influx had been stopped was recorded before and after various additions.

The Arsenazo III (Aldrich, Milwaukee, WI, U.S.A.) was purified by dissolving the commercial material in 10 times its weight of water and adding 10 times its weight of Dowex 50 (H$^+$ form). The acid dye was then successively extracted with 100 times its weight of butanol. The solid was recovered by allowing evaporation of the butanol at room temperature. A solution of the solid was neutralized with Tris and passed through Dowex 50 (Tris form); the effluent still contained 1–2μM-Ca$^{2+}$, and it was used to prepare the test medium described above.
The sensitivity of the dye + medium was diminished by chelating agents such as ATP and albumin. With 10 μM-ATP the sensitivity to 1 μM-Ca²⁺ was decreased to 94% of the control value, and albumin at 3 μM (0.2 mg/ml) decreased it to 93%; the corresponding value for 6 μM-albumin was 89%. Since the sensitivity depends on the ratio of free Ca²⁺ to chelating agents test calibrations were made in most efflux experiments by using an addition of 0.4 or 0.8 μM-Ca²⁺.

Analyses of the Ca²⁺ content of the various solutions were made with an atomic absorption spectrophotometer (Techtron AA 100). The KCl and NaCl were recrystallized from water with 1 mM-EDTA present. The bongkrekic acid (as ammonium salt) was a gift made in part to Professor H. Baum of Chelsea College, London, and in part directly to me from Professor H. Berends of Delft Technological University, The Netherlands. The Ruthenium Red was obtained from BDH Ltd. (Poole, Dorset, U.K.); it was purified and its concentration was determined as described by Luft (1971).

Results

Effect of oligomycin on influx and efflux of Ca²⁺

In a previous paper (Harris, 1977) it was shown that oligomycin would reverse the net release of Ca²⁺ from respiring mitochondria when it has been induced by making a small addition of a sodium salt to the suspension. This release might have been due to any combination of increased efflux with decreased influx. Carafoli & Crompton (1976) observed that the influx was slower from NaCl- than from KCl-containing media, a difference that also pertains between curves (a) (for NaCl) and (b) (for KCl) in Fig. 1. If oligomycin is added to the suspension in the NaCl medium the Ca²⁺ uptake is accelerated, typically by a factor of 1.25. This effect is indicated on the addition of oligomycin to the experiment shown in curve (a). However, because of the exponential character of the uptake curve the effect is better shown in curve (c), which represents the uptake from an NaCl medium supplemented throughout with oligomycin. In this series of experiments uptake in the NaCl medium plus oligomycin followed the same course as held for the KCl medium plus oligomycin; curve (c) is almost superimposable on curve (b) for the KCl medium alone. The equality between rates in NaCl + oligomycin and in KCl + oligomycin was not always seen, but in general the uptake of Ca²⁺ from the NaCl medium was accelerated by oligomycin. An exception was provided by the 'fluffy' material usually discarded when cardiac mitochondria are prepared; a suspension of this material did not exhibit respiratory control by ADP and it took up Ca²⁺ from KCl- and NaCl-containing media, with or without oligomycin, at the same rate. A similar heterogeneity of mitochondria has been described by Bygrave et al. (1978).

When Ruthenium Red was added after nearly all the Ca²⁺ had moved into the mitochondria the active uptake process was halted and the slow efflux could be recorded. It was technically convenient to increase the sensitivity so that several readings of efflux could be made during a relatively small loss of the total mitochondrial Ca²⁺. Figs. 2(a) and 2(b) illustrate the efflux to NaCl- and KCl-containing media respectively. The efflux is higher to the NaCl medium and is slowed by oligomycin; in contrast the efflux to KCl is slightly accelerated by oligomycin. These results show that the higher respiratory rate of Ca²⁺-loaded mitochondria in NaCl media as compared with KCl media is associated with a higher efflux of Ca²⁺ in the former. In the absence of Ruthenium Red to block active re-uptake of Ca²⁺ the higher leak leads to more energy dissipation by recycling of Ca²⁺. The interaction between Ca²⁺ and the mitochondria appears to involve ATP specifically, since it is inhibited by oligomycin. The slight acceleration of efflux seen when oligomycin is added to suspensions in KCl media can perhaps be attributed to a combination of loss of the chelating contribution of internal ATP with the loss of availability of ATP itself as a supplementary energy supply. Brand & Lehninger (1975) showed that the latter effect played an important role in slowing active Ca²⁺ uptake with oligomycin.
Fig. 2. Effects of some reagents on Ca\(^{2+}\) efflux measured after addition of Ruthenium Red
Mitochondria were loaded with \(65\text{nmol} / \text{mg}\) of protein before addition of Ruthenium Red (RR) at \(0.4\mu\text{M}\). (a) and (b) Experiments comparing the efflux to NaCl (a) and KCl (b) media show that oligomycin (OL) added at 4\(\mu\text{g} / \text{ml}\) decreases the rate of Ca\(^{2+}\) release to the NaCl medium and accelerates it to the KCl medium. (c) and (d) Experiments made with a different preparation show that successive additions of either bongkrekic acid (BKA) at 90\(\text{nmol} / \text{mg}\) of protein or atracylolate (ATR) at 10\(\mu\text{M}\) accelerate the efflux. The values shown are Ca\(^{2+}\) efflux (nmol/min per mg of protein). In (a) the medium was the NaCl-based mixture and for (b), (c) and (d) it was the KCl-based mixture. The protein concentrations were 0.77mg/ml for (a) and (b) and 0.92mg/ml for (c) and (d).

**Effects of atracylolate and bongkrekate on Ca\(^{2+}\) movements**

Atracylolate and bongkrekate both inhibit adenine nucleotide exchange but their mechanisms of action differ. Atracylolate displaces carrier-bound nucleotide (Klingenberg, 1970) whereas bongkrekate causes a tight association between ADP and the carrier (Klingenberg et al., 1970; Weidemann et al., 1970).

Asimakis & Sordahl (1977) describe a triggering of Ca\(^{2+}\) release from Ca\(^{2+}\)-loaded mitochondria on the addition of atracylolate. If the atracylolate is added after Ruthenium Red, so that active uptake is blocked, then the rate of Ca\(^{2+}\) leakage can be seen to increase (Fig. 2d). This effect on Ca\(^{2+}\) efflux can reasonably be ascribed to an opening of pores or a series of sites along which the Ca\(^{2+}\) leaks out. The possibility of some agents having a contrary effect, namely that of keeping the pores or sites closed, is indicated by the observations by Peng et al. (1977), who found that bongkrekate slows the loss of Ca\(^{2+}\) from liver mitochondria after their energy supply has been dissipated by uncoupling with dinitrophenol.

In the present series of experiments the effect of bongkrekate on Ca\(^{2+}\) movements depended upon when the addition was made and on the order of additions. If added after Ruthenium Red the bongkrekate behaved initially like atracylolate and stimulated the Ca\(^{2+}\) efflux (Fig. 2c). This stimulation was abolished if the medium had been supplemented with ADP (10\(\mu\text{M}\)) before addition of the bongkrekate. If the bongkrekate was added before the Ruthenium Red and before the addition of Ca\(^{2+}\) to the mitochondria its effect was to slow both the active uptake of Ca\(^{2+}\) and the efflux seen after Ruthenium Red addition. The effect takes some minutes to develop, recalling that seen on oxidative phosphorylation (Henderson & Lardy, 1970). Fig. 3 illustrates the progressively lower rates of uptake obtained with a fixed amount of bongkrekate applied at increasing intervals before the CaCl\(_2\). The control rate and the rate found after 15 s exposure were identical. The delay in taking effect may explain why this action of bongkrekate has not been noticed before. It implies that either the active influx of Ca\(^{2+}\) itself or the active ion movements necessary to provide energy for the process are impeded. Fig. 4 compares the consequences on the rate of Ca\(^{2+}\) uptake of applying different concentrations of bongkrekate at a fixed time before the CaCl\(_2\). In the same experiments, after altering the scales of the recorder, the efflux was evaluated after addition of Ruthenium Red. With each bongkrekate concentration tested the Ca\(^{2+}\) efflux was substantially decreased compared with the control results, but always to about the same value, which is noted on the curves. In another series of experiments with different preliminary loadings with Ca\(^{2+}\) the efflux found in the presence of the Ruthenium Red, either with or without prior treatment with bongkrekate, appeared to depend upon the Ca\(^{2+}\) load, and irrespective of load the efflux was inhibited by the bongkrekate (Table 1).

In other experiments (not shown) the efflux remaining in the presence of bongkrekate was found still to increase in response to an addition of NaCl and to diminish again in response to oligomycin. In other

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Fig. 3. Time course of removal of Ca\(^{2+}\) from the medium by mitochondria after different times of preincubation with bongkrekic acid

The curves show the time courses of movement of the added Ca\(^{2+}\) (50 nmol/mg of protein) into the mitochondria. The mitochondria were exposed to the bongkrekate (45 nmol/mg of protein) for the times (s), before addition of CaCl\(_2\), marked on the curves. The control curve, obtained without addition of bongkrekate, was identical with the curve marked 15s. The KCI-based medium was used, with protein at 0.4 mg/ml.

words the Na\(^{+}\)-dependent efflux was not abolished by bongkrekate under conditions where the basal efflux had been considerably reduced.

Effects of Na\(^{+}\) and adenine nucleotides on Ca\(^{2+}\) efflux at various Ca\(^{2+}\) loads

The efflux, whether measured in the absence or presence of bongkrekate (Table 1), appears to show an approximately linear dependence on the Ca\(^{2+}\) load that has been introduced. Fig. 5 illustrates this dependence for several conditions; there is some efflux even without preliminary loading, on account of the presence of endogenous Ca\(^{2+}\) (6–9 nmol/mg of protein). After measurement of the efflux to the KCl medium in the presence of Ruthenium Red (Fig. 5c) a

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Table 1. Dependence of Ca\(^{2+}\) efflux on load of Ca\(^{2+}\) before and after addition of bongkrekate

Bongkrekate (279 nmol/mg of protein) was added 5 min before the Ca\(^{2+}\). The Ca\(^{2+}\) loading was carried out before the addition of Ruthenium Red (0.4 \(\mu\)M).

<table>
<thead>
<tr>
<th>Ca(^{2+}) load (nmol/mg)</th>
<th>Without bongkrekate</th>
<th>With bongkrekate</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.91</td>
<td>0.61</td>
</tr>
<tr>
<td>55</td>
<td>1.96</td>
<td>1.02</td>
</tr>
<tr>
<td>105</td>
<td>3.36</td>
<td>1.68</td>
</tr>
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</table>
further measurement was made after addition of NaCl to obtain the higher efflux plotted in curve (b) of Fig. 5. In another set of experiments, after measurement of the efflux to the KCl medium alone, an addition of ATP to 10 μM was made. Over about 30 s the efflux diminished to a lower value and this is plotted in curve (d). A small correction was made for the decrease in the concentration of free Ca²⁺ by the ATP (see the Methods section). A pair of efflux values measured directly in 120 μM-NaCl medium are also included in Fig. 5(a).

Further trials of the nucleotides revealed that oligomycin did not prevent ATP from lessening the Ca²⁺ efflux and that ADP, whether applied with a glucose + hexokinase trap or with oligomycin to inhibit its phosphorylation, was still more effective (Table 2). For making further tests with ADP the hexokinase + glucose trap was preferred because of the slight increase of efflux induced by oligomycin alone when added to the KCl medium. Figs. 6 and 7 illustrate the influence of ADP concentration on the Ca²⁺ efflux. Values were measured sequentially and a linear correction was applied to allow for the effect of the changing Ca²⁺ load on the efflux.

The observation that ADP is more effective than ATP as a means of diminishing efflux of Ca²⁺ finds a parallel in the observation made by other authors on

![Graph](image-url)

**Fig. 5. Comparison of the rates of Ca²⁺ release from the mitochondria as a function of the Ca²⁺ content in control media and media with addition of either sodium salt or ATP**

The values plotted are the efflux measured after Ruthenium Red addition (as in Fig. 2). The mitochondria were prepared by loading them with different quantities of Ca²⁺ before addition of the Ruthenium Red. The control values of the efflux (curve c) were measured and then alternatively either (curve b) additions of 2.4 mM-NaCl were made or (curve d) an addition of ATP (to 10 μM) was made. The ATP caused the efflux to diminish over about 30 s and it is the value after this time that is plotted on (d). Two values of efflux measured in 120 mM-NaCl medium are shown at (a). The media are as described in the Methods section. The protein concentration was 0.6 mg/ml.

![Graph](image-url)

**Fig. 6. Diminution of Ca²⁺ efflux caused by addition of ADP**

The efflux values for different Ca²⁺ loads measured after the addition of Ruthenium Red but before the ADP are shown on curve (a). Addition of ADP to 2 μM decreased each value after about 30 s to the corresponding value plotted on curve (b); further additions of ADP to 6 μM and 10 μM led respectively to the values plotted on curves (c) and (d). The ADP was maintained by use of a hexokinase (1 unit/ml) + glucose (2.3 mM) trap. The KCl medium was used with mitochondrial protein at 0.4 mg/ml.

**Table 2. Effects on Ca²⁺ efflux of the addition of ADP or ATP with or without oligomycin after the preliminary reading of efflux after addition of Ruthenium Red**

<table>
<thead>
<tr>
<th>Control efflux measured after Ruthenium Red (nmol/min per mg of protein)</th>
<th>Subsequent additions</th>
<th>Conc. of nucleotide</th>
<th>% of control efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.85</td>
<td>ATP</td>
<td></td>
<td>2 μM</td>
</tr>
<tr>
<td>2.17</td>
<td>Oligomycin (2 μg/ml) + ATP</td>
<td>60</td>
<td>49</td>
</tr>
<tr>
<td>2.24</td>
<td>Oligomycin (2 μg/ml) + ADP</td>
<td>46</td>
<td>32</td>
</tr>
<tr>
<td>2.40</td>
<td>Hexokinase (1 unit/ml) + 2.3 mM glucose + ADP</td>
<td>43</td>
<td>17</td>
</tr>
</tbody>
</table>
the effects of the nucleotides on the retention of high loads of Ca$^{2+}$ (Binet & Volfin, 1974; Leblanc & Clauser, 1974a,b) as well as with the effects on mitochondrial volume (Stoner & Sirak, 1973a). Atractylate, if added before the ATP, prevented its action on Ca$^{2+}$ efflux, which, however, could then be restored with bongkrekate. Controls for this experiment confirmed that the addition of both ADP and bongkrekate was more effective than that of bongkrekate alone.

Since respiring mitochondria normally carry a preponderance of their internal adenine nucleotide as ATP it would seem likely that conversion of this endogenous ATP into ADP might affect the Ca$^{2+}$ efflux. Gunter et al. (1978) found that low concentrations of uncoupler did prolong the retention of a load of 80nmol of Ca$^{2+}$/mg of protein by liver mitochondria, in contrast with the rapid release induced by higher concentrations. In the present work measurement of the rates of Ca$^{2+}$ release after Ruthenium Red addition were made with sequential increases of uncoupler concentration. In an experiment where the initial efflux after Ruthenium Red was 1.03 nmol/min per mg, the presence of 24nm-tetrachlorotrifluoro-methylbenzimidazole decreased the efflux to 0.39 nmol/min per mg. With further uncoupler the efflux increased again. Hence there seem to be two effects operating, one inhibitory and due probably to the production of internal ADP, and the other stimulatory and presumably due to an opening of the structure by the uncoupler.

Trials of the guanosine nucleotides, which are effective as re-coupling agents on brown-fat mitochondria (Hohorst & Rafael, 1968; Nicholls, 1974), indicated that they were relatively ineffective in inhibiting Ca$^{2+}$ efflux from heart mitochondria. At 40µM, GDP decreased the Ca$^{2+}$ efflux to half.

Effect of albumin

Low concentration of albumin decrease the steady concentration of extramitochondrial Ca$^{2+}$ maintained by respiring heart mitochondria (Harris, 1977). In Fig. 7 albumin is shown to diminish the efflux of Ca$^{2+}$. This result corresponds to an observation by Pozzan et al. (1977) obtained with liver mitochondria. Al-Shaikhaly & Baum (1979) showed that fatty acids accelerated the Ca$^{2+}$ efflux from liver and heart mitochondria, so that part, at least, of the albumin effect may arise from its sequestration of fatty acids.

Effect of Mg$^{2+}$

Mg$^{2+}$ inhibits Ca$^{2+}$ uptake (Crompton et al., 1976b). In efflux experiments in the conditions I have used it had no effect or slight effects at concentrations up to 100µM unless it was supplementary to ADP already present. For example: two successive additions of MgCl$_2$ at 100µM each had no effect when applied after Ruthenium Red, but, if a first addition of ADP at 2µM was made (decreasing the efflux to just over 60% of the post-Ruthenium Red control value), then the further addition of 100µM-MgCl$_2$ decreased efflux to 28% of the original value. Hence the effect resembles that obtainable by increasing the ADP concentration. This relatively slight effect of Mg$^{2+}$ contrasts with the Mg$^{2+}$ requirement seen if ADP is to restore coupling and respiratory control that have been lost owing to exposure to high Ca$^{2+}$ concentrations (Leblanc & Clauser, 1974a,b; Hunter et al., 1976).
Effect of inhibition of respiration and of uncouplers

The question whether the low efflux of Ca\(^{2+}\), which is seen in the presence of added ADP, is related to energy production or is solely a measure of membrane leakiness was tested by adding antimycin (2 \(\mu\)g/ml) after the initial reading of efflux after Ruthenium Red addition. Up to loads of 80 nmol of Ca\(^{2+}\)/mg of protein the respiratory inhibitor transiently doubled the rate in the absence of ADP, whereas the lower efflux rate found with 2 \(\mu\)M-ADP was increased on adding antimycin only by a factor of 1.2 (Table 3). The further addition of an uncoupler (tetrachlorotri fluoromethylbenzimidazole at 1.2 \(\mu\)M) increased the efflux to 3 times the original value in the ADP-free medium, but it had little or no effect if ADP was present (not shown in Table 3). The observation that uncoupler is effective in stimulating efflux (in the absence of ADP), even though respiration is inhibited, is suggestive of a specific action on the membrane. The increased efflux after inhibition of respiration changes in 2–3 min to a decreased efflux (Harris, 1979).

Effects of the Ca\(^{2+}\)-efflux modulators on respiration

If the substances that decrease Ca\(^{2+}\) efflux act by making the membrane generally less permeable, then wasteful ion cycling would be reduced and resting respiration diminished. This would contrast, for example, with the enhanced respiration seen in response to Na\(^+\) (Crompton et al., 1976a). In the present work the respiratory stimulation in response to Na\(^+\) was confirmed and it was also seen that adding oligomycin after the sodium salt decreased the respiration to about the value holding before the addition of the salt. Addition of 10 \(\mu\)M-ADP after oligomycin decreased the respiration in the KCl medium to 0.57 times the pre-ADP rate. Addition of albumin at 3 \(\mu\)M decreased the respiration (in the absence of oligomycin) to 0.8 times the control value.

Discussion

The efflux of Ca\(^{2+}\) from Ca\(^{2+}\)-loaded mitochondria, seen when Ruthenium Red has been added to inhibit the active uptake process, can operationally be divided into (1) a residual ADP-insensitive part, which appears to be unaffected by respiratory activity, (2) a part that is capable of being inhibited by ADP, bongkrekic acid and albumin and (3) a part that is Na\(^+\)-dependent. The third requires a supply of ATP, since it is inhibited by oligomycin and is absent when respiration is stopped or uncoupler is present (Crompton et al., 1976b). I observed that there was no stimulation of efflux by Na\(^+\) after antimycin had been added to inhibit respiration. The present study does not provide an explanation of the Na\(^+\)- and ATP-requiring efflux.

The distinction between the components (1) and (2) above may be artificial since a property such as permeability can have a span of values without reaching a complete cut-off condition. It is then plausible to regard the Ca\(^{2+}\) efflux, which is proportional to the internal Ca\(^{2+}\) concentration, as a measure of an unspecific leakiness of a structure whose properties are altered by the binding of adenine nucleotides, with ATP as the more effective agent. This corresponds to conclusions reached by Stoner & Sirak (1973a,b) from the nucleotide-dependent volume changes and the related differences in electron-microscopic appearance. Evidence for an unspecific permeability change is provided by the study by Hunter et al. (1976), who found that the sucrose-accessible space increased after the mitochondria were exposed to 150 nmol of Ca\(^{2+}\)/mg of protein; the change corresponded to all of the water space becoming sucrose-accessible. When subsequently treated with ATP + Mg\(^{2+}\) a sucrose-inaccessible part, amounting to 15% of the total water space, reappeared. Since the pellet water in the experiments of Hunter et al. (1976) was only 70% accessible to inulin, the result points to a change of sucrose space within the outer membrane, from 15% of the pellet water to 30% in response to Ca\(^{2+}\), and its reversal by ATP + Mg\(^{2+}\). The requirement for Mg\(^{2+}\) along with nucleotide if mitochondrion are to retain loads of 100 nmol of Ca\(^{2+}\)/mg of protein or more, appears in the reports by both Hunter et al. (1976) and Leblanc & Clauser (1974a). There is, however, no indication that the leakage of Ca\(^{2+}\) from

<table>
<thead>
<tr>
<th>Ca(^{2+}) load (nmol/mg of protein)</th>
<th>Without ADP</th>
<th>With 2 (\mu)M-ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before antimycin</td>
<td>After antimycin (2 (\mu)g/ml)</td>
</tr>
<tr>
<td>40</td>
<td>0.58</td>
<td>1.00</td>
</tr>
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<td>55</td>
<td>0.74</td>
<td>1.54</td>
</tr>
<tr>
<td>80</td>
<td>0.82</td>
<td>1.91</td>
</tr>
<tr>
<td>100</td>
<td>1.11</td>
<td>6.66</td>
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</table>
mitochondria with relatively low Ca\(^{2+}\) loads is associated with a Mg\(^{2+}\) requirement. Such requirement seems to arise only after the autoaccelerated discharge of the total content of Ca\(^{2+}\), which ensues spontaneously at some time after a load of 100 or more nmol of Ca\(^{2+}\)/mg of protein has been given (Hunter et al., 1976; Binet & Volfin, 1974).

Evidence that the leakage observed in the presence of Ruthenium Red is a measure of what is in progress in the absence of this inhibitor, though masked then by simultaneous re-uptake, can be drawn from comparisons between the effects of agents applied in the presence and absence of Ruthenium Red. For example, ATP and albumin both lower the ambient free Ca\(^{2+}\) concentration, which pertains for some time in a medium carrying respiring mitochondria, whereas sodium salts raise the concentration (Harris, 1977). This indicates some combination of raised influx and diminished efflux with ATP and albumin and conversely for Na\(^{+}\). The influx is not seen to increase with low concentrations of ATP as used here, though it is diminished by the sodium salts (Fig. 1). The efflux is seen to diminish with ATP and with albumin (Fig. 7) and to increase with Na\(^{+}\) (Fig. 5). A corollary of the greater decrease of efflux obtained with ATP than with ATP is that the impression is thereby given that efflux is energy-linked. That this is not so is shown by the facts that adding oligomycin does not alter the leak in presence of ATP and that the leak is less in the presence of ATP than with no added nucleotide.

The requirement for external nucleotide to decrease the leak, and the effect of bongkrekic acid on the leak, point either to a specific leakage path for Ca\(^{2+}\) including bound ADP as an obstacle or at least to a path that is closed up by bound ADP. The effect of albumin in decreasing the leak may in part stem from its complement of thiol groups; E. J. Harris, M. Al-Shaikhaly & H. Baum (unpublished work) have observed that dithioerythritol removes all the leakage inducible by the mercurial compounds and some of that inducible by thyroxine (Baum & Al-Shaikhaly, 1977). Taking into account the Mg\(^{2+}\) requirement described by other authors, the suggestion may be made that the membrane permeability is controlled by a complex of thiol groups, Mg\(^{2+}\) and ADP. This concept allows an explanation to be offered for a recent report by Lehninger et al. (1978) of the Ca\(^{2+}\) release being controlled by the redox state of the nicotinamide nucleotides, because the reduction of the thiol groups will depend upon the supply of reducing equivalents from NADH to NADPH and thence for reduction of oxidized sulphide groups. It also accords with the Ca\(^{2+}\) release inducible with N-ethylmaleimide (Lofrumento & Zanotti, 1978).

My thanks are due to Professor H. Baum for discussion of the results and help with their presentation, to Dr. M. C. W. Evans of the Botany Department, University College London, for use of the DW 2 spectrophotometer and to the British Heart Foundation for a grant for expenses. I am also grateful to Professor H. Baum and Professor H. Berends for gifts of bongkrekic acid.

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1979