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 bongkreikic acid; this action has a time lag. The lower efflux found with the nucleotides and with bongkreikic 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 bongkreikic acid. The latter substance promotes tight binding of the nucleotides to the membrane (Klingenberg *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 300 mM-mannitol/5 mM-KCl/10 mM-Tris/chloride, pH 7.2. After a sample was taken for protein determination by a biuret method, an addition was made of 2 mg 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 Aminco DW 2 spectrophotometer. The wavelength pair 685–665 nm was used, which provides a higher sensitivity than the 685–675 nm pair used before. Uptake and efflux experiments were made in a medium having either KCl or NaCl at 120 mM (or a mixture as specified), and Tris chloride, pH 7.2 (20 mM), Tris phosphate (1.2 mM), Tris succinate (3 mM), rotenone (1 $\mu\text{g}/\text{ml}$) and sufficient purified Arsenazo III to provide A_{520} 1.5. The usual procedure was to dilute the mitochondria with medium to obtain about 0.5 mg 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 2 min unless bongkreikate had been added. The active influx was stopped by adding Ruthenium Red (0.4 μM); 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 butan-1-ol. 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 1–2 μM - 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 $10\text{ }\mu\text{M}$ -ATP the sensitivity to $1\text{ }\mu\text{M}$ - Ca^{2+} was decreased to 94% of the control value, and albumin at $3\text{ }\mu\text{M}$ (0.2 mg/ml) decreased it to 93%; the corresponding value for $6\text{ }\mu\text{M}$ -albumin was 89%. Since the sensitivity depends on the ratio of free Ca^{2+} to chelating agents test calibrations were made in most efflux experiments by using an addition of 0.4 or $0.8\text{ }\mu\text{M}$ - Ca^{2+} .

Analyses of the Ca^{2+} 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 bongkreikic 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 Ca^{2+}

In a previous paper (Harris, 1977) it was shown that oligomycin would reverse the net release of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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).

Fig. 1. Comparison of time courses of uptake for Ca^{2+} added to suspensions of heart mitochondria in NaCl and KCl media, and the effect of oligomycin

An addition of CaCl_2 to provide a concentration of $14\text{ }\mu\text{M}$ was made in each case. The course of the removal of the Ca^{2+} (a) from the NaCl medium and (b) from the KCl medium (as monitored with Arsenazo III; see the Methods section) is shown. An addition of oligomycin ($2\text{ }\mu\text{g/ml}$) was made late in the experiment of curve (a) at the point marked OL. In two other experiments made in the respective media both supplemented with oligomycin at $2\text{ }\mu\text{g/ml}$ the uptake courses were both on curve (c). The media were as described in the Methods section with mitochondrial protein at 0.85 mg/ml ; the temperature was 20°C .

When Ruthenium Red was added after nearly all the Ca^{2+} 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 Ca^{2+} . 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 Ca^{2+} -loaded mitochondria in NaCl media as compared with KCl media is associated with a higher efflux of Ca^{2+} in the former. In the absence of Ruthenium Red to block active re-uptake of Ca^{2+} the higher leak leads to more energy dissipation by recycling of Ca^{2+} . The interaction between Ca^{2+} 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 Ca^{2+} uptake with oligomycin.

Fig. 2. Effects of some reagents on Ca^{2+} efflux measured after addition of Ruthenium Red

Mitochondria were loaded with 65 nmol of Ca^{2+} /mg of protein before addition of Ruthenium Red (RR) at 0.4 μM . (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 bongkreic acid (BKA) at 90 nmol/mg of protein or atractylate (ATR) at 10 μM accelerate the efflux. The values shown are the 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 0.77 mg/ml for (a) and (b) and 0.92 mg/ml for (c) and (d).

Effects of atractylate and bongkreikate on Ca^{2+} movements

Atractylate and bongkreikate both inhibit adenine nucleotide exchange but their mechanisms of action differ. Atractylate displaces carrier-bound nucleotide (Klingenberg, 1970) whereas bongkreikate 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 atractylate. If the atractylate 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 bongkreikate 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 bongkreikate on Ca^{2+} movements depended upon when the addition was made and on the order of additions. If added after Ruthenium Red the bongkreikate behaved initially like atractylate and stimulated the Ca^{2+} efflux (Fig. 2c). This stimulation was abolished if the medium had been supplemented with ADP (10 μM) before addition of the bongkreikate. If the bongkreikate was added before the Ruthenium Red and before the addition of Ca^{2+} to the mito-

chondria 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 bongkreikate 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 bongkreikate 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 bongkreikate 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 bongkreikate 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 bongkreikate, appeared to depend upon the Ca^{2+} load, and irrespective of load the efflux was inhibited by the bongkreikate (Table 1).

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

Fig. 3. Time course of removal of Ca^{2+} from the medium by mitochondria after different times of preincubation with bongkreikic 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 bongkreikate (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 bongkreikate, was identical with the curve marked 15 s. The KCl-based medium was used, with protein at 0.4 mg/ml.

words the Na^+ -dependent efflux was not abolished by bongkreikate 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 bongkreikate (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

Fig. 4. Effect of bongkreikic acid at different concentrations on Ca^{2+} removal from the medium by the mitochondria and on the subsequently measured efflux seen after Ruthenium Red addition

The bongkreikate was added to the suspension 90 s before the CaCl_2 (to provide 39 nmol/mg of protein, or initially 20 μM). The control (a) without bongkreikate followed the same uptake course as the experiment with 27 nmol of bongkreikate/mg of protein, but the efflux seen after the Ruthenium Red addition was slower in the latter case (a') than in the control (a). It is relevant that time elapsing during the uptake part of the experiment would have allowed the slowly acting bongkreikate to take effect. With the higher concentrations of bongkreikate both the uptake and efflux rates were decreased. The concentrations were: (b) 54 nmol/mg of protein; (c) 108 nmol/mg of protein; (d) 216 nmol/mg of protein. In all experiments the recording rate and sensitivity were changed after addition of Ruthenium Red in order to obtain suitable deflexions. The values shown are the Ca^{2+} efflux in nmol/min per mg of protein. The KCl-based medium was used, with protein at 0.52 mg/ml.

Table 1. Dependence of Ca^{2+} efflux on load of Ca^{2+} before and after addition of bongkreikate

Bongkreikate (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 μM).

Ca^{2+} load (nmol/mg)	Efflux after Ruthenium Red additions (nmol/min per mg)	
	Without bongkreikate	With bongkreikate
30	0.91	0.61
55	1.96	1.02
105	3.36	1.68

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 $10\mu\text{M}$ was made. Over about 30s 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 Ca^{2+} by the ATP

(see the Methods section). A pair of efflux values measured directly in $120\mu\text{M}$ -NaCl medium are also included in Fig. 5(a).

Further trials of the nucleotides revealed that oligomycin did not prevent ATP from lessening the Ca^{2+} 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 Ca^{2+} efflux. Values were measured sequentially and a linear correction was applied to allow for the effect of the changing Ca^{2+} load on the efflux.

The observation that ADP is more effective than ATP as a means of diminishing efflux of Ca^{2+} finds a parallel in the observation made by other authors on

Fig. 5. Comparison of the rates of Ca^{2+} release from the mitochondria as a function of the Ca^{2+} 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 Ca^{2+} before addition of the Ruthenium Red. The control values of the efflux (curve c) were measured and then alternatively either (curve b) additions of 2.4mM -NaCl were made or (curve d) an addition of ATP (to $10\mu\text{M}$) was made. The ATP caused the efflux to diminish over about 30s and it is the value after this time that is plotted on (d). Two values of efflux measured in 120mM -NaCl medium are shown at (a). The media are as described in the Methods section. The protein concentration was 0.6mg/ml .

Fig. 6. Diminution of Ca^{2+} efflux caused by addition of ADP

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

Table 2. Effects on Ca^{2+} efflux of the addition of ADP or ATP with or without oligomycin after the preliminary reading of efflux after addition of Ruthenium Red

The Ca^{2+} load before the Ruthenium Red was 77nmol/mg of protein.

Control efflux measured after Ruthenium Red (nmol/min per mg of protein)	Subsequent additions	Concn. of nucleotide ...	% of control efflux		
			$2\mu\text{M}$	$6\mu\text{M}$	$10\mu\text{M}$
1.85	ATP		60	49	35
2.17	Oligomycin ($2\mu\text{g/ml}$) + ATP		66	46	33
2.24	Oligomycin ($2\mu\text{g/ml}$) + ADP		46	32	14
2.40	Hexokinase (1 unit/ml) + 2.3mM glucose + ADP		43	17	12

Fig. 7. Comparison of inhibition of Ca^{2+} efflux by ATP, ADP and albumin

Mitochondria were loaded with 30 nmol of Ca^{2+} /mg for the albumin and ADP experiments; loads of either 45 nmol/mg (○) or 170 nmol/mg (□) were used for the ATP experiments. As before, the efflux of Ca^{2+} was measured after addition of Ruthenium Red, and then increasing amounts of the agents were added; sufficient time was allowed after each addition for the efflux to come to a steady value. The ADP was used in presence of a hexokinase+glucose trap. The KCl medium was used with mitochondrial protein at 0.6 mg/ml.

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 ADP, 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 80 nmol 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 24 nm-tetrachlorotrifluoromethylbenzimidazole 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 40 μM, 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-Shaikhly & 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 100 μM unless it was supplementary to ADP already present. For example: two successive additions of MgCl_2 at 100 μM each had no effect when applied after Ruthenium Red, but, if a first addition of ADP at 2 μM was made (decreasing the efflux to just over 60% of the post-Ruthenium Red control value), then the further addition of 100 μM- 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 ($2\mu\text{g/ml}$) after the initial reading of efflux after Ruthenium Red addition. Up to loads of 80nmol 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 $2\mu\text{M}$ -ADP was increased on adding antimycin only by a factor of 1.2 (Table 3). The further addition of an uncoupler (tetrachlorotri-fluoromethylbenzimidazole at $1.2\mu\text{M}$) 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 $10\mu\text{M}$ -ADP after oligomycin decreased the respiration in the KCl medium to 0.57 times the pre-ADP rate. Addition of albumin at $3\mu\text{M}$ 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, bongkreic 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 ADP 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 150nmol of Ca^{2+}/mg of protein; the change corresponded to all of the water space becoming sucrose-accessible. When subsequently treated with $\text{ATP} + \text{Mg}^{2+}$ 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 $\text{ATP} + \text{Mg}^{2+}$. The requirement for Mg^{2+} along with nucleotide if mitochondria are to retain loads of 100nmol 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 3. Immediate effect of a respiratory inhibitor on Ca^{2+} efflux after addition of Ruthenium Red with and without the presence of $2\mu\text{M}$ -ADP and glucose + hexokinase trap throughout
After 2–3 min efflux diminishes to a low value not shown here.

Ca ²⁺ load (nmol/mg of protein)	Efflux (nmol/min per mg of protein)			
	Without ADP		With $2\mu\text{M}$ -ADP	
	Before antimycin	After antimycin ($2\mu\text{g/ml}$)	Before antimycin	After antimycin ($2\mu\text{g/ml}$)
40	0.58	1.00	0.45	0.73
55	0.74	1.54	0.52	0.73
80	0.82	1.91	0.54	0.61
100	1.11	6.66	1.01	4.44

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 ADP 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 bongkreic 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-Shaikhly & 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-Shaikhly, 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).

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