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Stimulation of Mitochondrial Calcium Ion Efflux by Thiol-Specific Reagents and by Thyroxine

THE RELATIONSHIP TO ADENOSINE DIPHOSPHATE RETENTION AND TO MITOCHONDRIAL PERMEABILITY

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Respiring rat heart mitochondria were loaded with Ca^{2+} and then treated with Ruthenium Red. The factors affecting the subsequent Ca^{2+} efflux were studied. Addition of rotenone or antimycin led to a decline of efflux except at pH values above 7.2, provided the load was less than about 80 nmol per mg of protein. Oligomycin reversed the effect of the respiratory inhibitors. Independently of respiration, efflux was stimulated by the uncoupler trifluoromethyltetrachlorbenzimadazole, by mersalyl and by thyroid hormones. The stimulated efflux could be diminished by ADP, with Mg^{2+} as cofactor if efflux was rapid. With respiration in progress, efflux could be stimulated by *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoate). The effects of mersalyl and of thyroid hormones could be diminished with dithiothreitol. In the absence of stimulating agents, the Ca^{2+} efflux was proportional to the load up to some critical amount, this critical amount was decreased by the agents. Thyroxine and mersalyl caused not only loss of Ca^{2+}, but also simultaneous, but not necessarily proportional, loss of internal adenine nucleotides. Both efflux rates were kept at a low value by bongkrekic acid added before the stimulating agent. It is concluded that Ca^{2+} efflux is a measure of a permeability controlled by the binding of ADP (and Mg^{2+}) to the inner membrane, and that this in turn depends on the maintenance of certain thiol groups in a reduced form by a reaction that uses NADH and ATP and the energy-linked transhydrogenase.

When energized mammalian mitochondria have taken up Ca^{2+}, the consequences depend on the size of the Ca^{2+} load and on the presence or absence of one or more of a series of unrelated compounds in the supporting medium. Drahota *et al.* (1965) showed that, as the load is increased, there is a tendency for the Ca^{2+} to be released after a Ca^{2+}-dependent delay; however, merely by using the protein at a higher concentration permitted a given loading per mg of protein to be better retained, and adding ATP to the medium had a protective action. Although under some conditions the release of the accumulated Ca^{2+} was associated with a permanent enhancement of respiration (similar to uncoupling), there could instead ensue a change to diminished respiration associated with loss of NAD^+ (Vinogradov *et al.*, 1972). The presence of Mg^{2+} as well as ATP protects against the uncoupling effect of the exposure to high Ca^{2+} (Rossi & Lehninger, 1964). An unidentified cytoplasmic component has also been reported to protect against Ca^{2+}-induced uncoupling accompanied by loss of mitochondrial Mg^{2+} (Lee *et al.*, 1971; Binet & Volfin, 1974). The latter authors used conditions under which ADP was required as a cofactor to the cytoplasmic factor. Although ATP has a protective action, the latter authors (Binet & Volfin, 1974), and Leblanc & Clauser (1974*a*) agree in the finding that ADP is the more effective nucleotide.

The damage resulting from exposure to high Ca^{2+} appears to ensue as the Ca^{2+} emerges after its transient accumulation; this can be inferred from the respiration-rate traces of Drahota *et al.* (1965). In the Ca^{2+}-damaged state there appears to be a non-specific increase of permeability; for example, sucrose penetrates the matrix (Hunter *et al.*, 1976). Moreover, the swelling associated with the damaged condition appears to be of colloid osmotic origin, because it can be prevented by adding colloid to the medium (Baum & Al-Shaikhaly, 1977), and in this respect resembles the swelling inducible by thyroxine (Lehninger *et al.*, 1958). Similarly, after exposure of the mitochondria to a high load of Ca^{2+}, the transport of P_i is no longer sensitive to poisoning with mercurials (Leblanc & Clauser, 1974*b*). Both the specific mercurial-sensitive permeability to P_i and the

Abbreviation used: Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

impermeability to sucrose are restored under these conditions by adding Mg^{2+} and adenine nucleotide. The altered permeability properties are accompanied by visible changes of appearance in the electron micrographs of fixed specimens (Hunter *et al.*, 1976). It is worth remarking that mitochondrial shrinkage in response to ADP (often requiring Mg^{2+} as well) has been described in the literature for many years. Probably some of the earlier reports were really describing the reversal of Ca^{2+}-induced swelling, but more recently, in a careful study of both volume and electron-microscopic appearances, Stoner & Sirak (1973*a*,*b*) showed that there are changes caused by ADP without prior exposure to Ca^{2+}. Stoner & Sirak (1973*b*) noted that high concentrations of bongkrekic acid simulated the effect of ADP. Since bongkrekic acid even at low concentrations causes tight binding of ADP to specific sites on the mitochondrial adenine nucleotide carrier, they proposed that, at the high concentrations necessary, there was an action on internal ADP, causing it to bind to additional membrane sites. Bongkrekic acid has been found to protect against the loss of adenine nucleotides in response to high Ca^{2+} (Out *et al.*, 1971), and it also protects against release of Ca^{2+} by uncoupling agents (Peng *et al.*, 1977), so there is circumstantial evidence for it acting by reason of its induction of nucleotide binding. Al-Shaikhaly & Baum (1979) have reported a relation between losses of Ca^{2+} and of nucleotide, with both losses being diminished by bongkrekic acid.

Ca^{2+}-induced damage to non-energized liver mitochondria is enhanced by exposure to either mercurials or thyroxine (Al-Shaikhaly & Baum, 1979). The present paper describes the effects of the same agents on the efflux of energetically accumulated Ca^{2+} from rat heart mitochondria. Another thiol-specific reagent, namely *N*-ethylmaleimide, has also been reported as inducing a loss of Ca^{2+} from energized mitochondria (Lofrumento & Zanotti, 1978; Chen *et al.*, 1978). These effects of mercurials and *N*-ethylmaleimide suggest that free thiol groups are required to maintain the membrane in a non-leaky state, and it is this that we have investigated.

Methods

Rat heart mitochondria were prepared as described previously (Harris, 1977, 1978) from hearts pre-perfused with 0.9% NaCl to remove blood. The tissue was minced in a Climpex stainless-steel mincer. The mince was digested for 15 min at 0°C after a brief homogenization in 20 ml (per heart) of supporting medium (75 mM-KCl, 150 mM-mannitol, 1 mM-Tris/EGTA, 5 mM-Tris/HCl, pH 7.2, and 0.05% purified serum albumin), containing, in 20 ml, 1 mg of trypsin (crystalline; Koch–Light) and of collagenase (type V; Sigma). During digestion, brief homogenizations were carried out at 5 min intervals. Finally the liquid was made up to 100 ml with a solution containing 300 mM-mannitol 1 mM-Tris/EGTA, 5 mM-Tris/HCl, pH 7.2, and 0.05% serum albumin. The homogenate was then centrifuged by accelerating up to 2000 rev./min in an angle rotor ($r_{max.}$ 10 cm). The supernatant was filtered through nylon gauze (0.2 mm mesh) and the filtrate centrifuged for 60000*g*-min to recover the mitochondria. The pellet was resuspended in the same solution as used to dilute the digest (20–30 ml) and resedimented. A further resuspension was done in a medium similar but lacking EGTA, and the mitochondria were again sedimented. The pellet was finally suspended in a solution containing 300 mM-mannitol and 10 mM-Tris/HCl, pH 7.2, about 0.5 ml/heart being used to obtain about 30 mg of protein/ml. A sample was taken for protein estimation by a biuret method (Layne, 1957) (standardized with serum albumin) and then serum albumin was added to provide a concentration of 2 mg/ml of suspension.

The movements of Ca^{2+} were measured as before (Harris, 1977, 1978) with an Aminco DW2 spectrophotometer, with the metallochrome Arsenazo II in the medium. In the presence of Ca^{2+} the dye changes from red to blue, and, by measuring the differential absorbance between the wavelengths 685 and 665 nm, a sensitive indication of Ca^{2+} concentration is obtained that is relatively independent of swelling of the particles (Vallieres *et al.*, 1975).

The medium used to suspend the mitochondria when observing Ca^{2+} movements contained 120 mM-KCl, 5 mM-Tris/HCl, pH 7.2 (unless specified otherwise), and 3% poly(ethylene glycol); the latter was included to lessen colloid osmotic swelling. The experiments were carried out with respiratory substrate included in the medium, usually pyruvate and malate at 3 mM each with 1.2 mM-Tris/phosphate and at 22°C. An addition of $CaCl_2$ was made to a portion of the stock suspension (about 30 mg of protein/ml) dispersed in 2.5 ml of medium in a cuvette to give a final protein concentration of around 0.5 mg/ml, and the difference in absorbance between 685 and 665 nm was read out. The initial absorbance increase in response to the Ca^{2+} salt rapidly declined as the Ca^{2+} was removed into the mitochondria. When this uptake was complete, an addition of Ruthenium Red was made (0.4 or 0.8 μM). The rate at which Ca^{2+} was emerging from the particles could then be read. (For this it was convenient to increase the recorder sensitivity.) After the basal efflux rate had been determined, various additions were made to measure the leak under the influence of the additives. The sensitivity of the measuring system to free Ca^{2+} was calibrated during each of such experiments, because it depends on the ratio of the freed Ca^{2+} to the chelating substances present, and it is also diminished by Mg^{2+} ions.

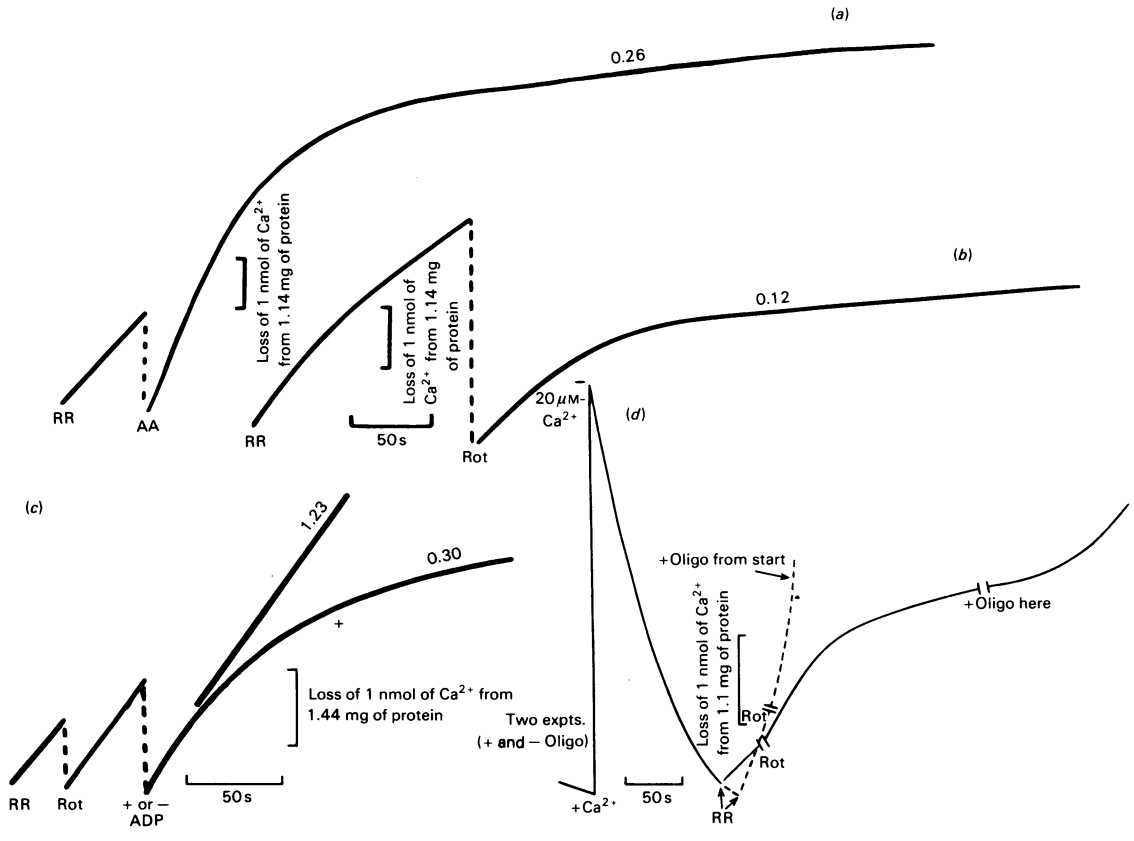

Fig. 1. *(a and b) Inhibition of Ca^{2+} efflux from previously Ca^{2+}-loaded mitochondria by inhibitors of respiration, (c) inhibition of Ca^{2+} efflux from previously Ca^{2+}-loaded mitochondria by ADP after inhibition of respiration, and (d) action of oligomycin on the efflux when respiration is inhibited*

(*a* and *b*) The deflection is a measure of the free Ca^{2+} in the suspension as measured by forming a complex with Arsenazo II (see the Methods section). Before the part of the record shown, the mitochondria had been loaded with 40nmol of Ca^{2+}/mg of protein. At 'RR', Ruthenium Red (0.8 μM) was added to stop active uptake of Ca^{2+}; in consequence the existence of the efflux can be observed. In record (*a*), antimycin (1 μg/ml) was added at 'AA', and at 'Rot' on record (*b*) rotenone was added (1 μg/ml). These agents respectively inhibit oxidation of the substrates that were present, namely, succinate (3 mM) in the medium for (*a*) and pyruvate and malate (3 mM each) in the medium for (*b*). The recorder was reset (broken lines). Over about 2min the Ca^{2+} efflux diminished to the low values (in nmol/min per mg of protein) noted on the curves. Different preparations were used for the two experiments, with the protein concentrations respectively 0.46 and 0.56mg/ml in (*a*) and (*b*). (*c*) Some preparations with a given pre-loading of Ca^{2+} did not respond to respiratory inhibition in the way shown in (*a*) and (*b*). The preparation used for this experiment was aged for 24h after Expt. (*b*) and efflux did not diminish until an addition of ADP (10 μM) had been made. The other conditions were as in (*b*). (*d*) This diagram is a composite from two experiments. The mitochondria in each were first loaded with Ca^{2+} as before with 50nmol per mg of protein, but one sample was supplemented with oligomycin ('Oligo'; 1 μg/ml). At 'RR', Ruthenium Red additions were made and the sensitivity was increased. Efflux in the presence of oligomycin (shown as a broken line) was more rapid than from the control [in agreement with Harris (1979*a*)]. On adding rotenone (Rot) at 1 μg/ml, the control efflux gradually diminished as in (*a*), but efflux from the oligomycin-supplemented suspension tended to increase rather than diminish. When oligomycin (1 μg/ml) was added later to the control suspension, it led to a delayed increase of efflux. The medium used was described in the Methods section, with Tris/pyruvate and Tris/malate at 3 mM each and Tris/phosphate at 1.2 mM. The temperature was 22°C. The protein concentration was 0.44mg/ml.

To measure the efflux of adenine nucleotide during the progress of the Ca^{2+} efflux, the mitochondria were loaded with ^{14}C-labelled nucleotide during their preparation. For this, the volume of the first wash medium was decreased to 10ml and to it 0.5 μCi of [^{14}C]ADP was added; the particles were dispersed

in this solution and kept at 0–4°C for 30min before sedimenting them and proceeding with a second wash in the normal medium and the final resuspension. The specific activity was measured by assaying the ADP-loading medium (after incubation) for ADP and ATP together with a radioactivity measurement, as well as by assaying an acid extract of a portion of the loaded mitochondria together with radioactivity measurement. The two agreed to within 5%, indicating that the specific activities of the two nucleotide pools had equilibrated. During the efflux experiments an initial volume of medium-plus-mitochondria of 4ml was taken in order to permit eight samples (0.25ml each) to be taken while the spectrometer record was still operative. The samples were centrifuged in Coleman Microfuge centrifuges as they were taken and the supernatants were removed for radioactivity measurement to find the increment of activity due to nucleotide movement from the particles into the medium.

Results

Effect of inhibiting respiration at various pH values

The inhibition of respiration after an active uptake of Ca^{2+} eventually leads to there being no source of energy to maintain the active process, so that the net efflux becomes detectable. However, on account of the presence of endogenous ATP there is a time lag of several minutes when the Ca^{2+} loading has been moderate (e.g. 50nmol per mg of protein). For this reason the measurements of efflux were made with Ruthenium Red present to inhibit the active influx, and on this account the efflux could be measured while respiration was still active. The appropriate inhibitor of respiration was then added, rotenone to media with pyruvate and malate as substrates, and antimycin to media with succinate as substrate. Tests with KCN as inhibitor showed that this agent produced similar results. The immediate consequence of respiratory inhibition was usually to temporarily increase the efflux, as noted by Harris (1979*a*,*b*), but after a few minutes the efflux usually declined spontaneously to a value lower than before applying the inhibitor (Figs. 1*a* and 1*b*). If the efflux did not decline spontaneously it could be diminished by making an addition of ADP, as shown in Fig. 1(*c*). The differing behaviour is likely to be determined by the content of endogenous nucleotide, since the change from the result of Fig. 1(*b*) to that of Fig. 1(*c*) (−ADP) was obtained by storage of the preparation.

Without additions other than the respiratory inhibitor, the measured efflux depended on the prior Ca^{2+} loading and on the pH of the medium. Table 1 gives the values of efflux found with a particular preparation before and after the respiratory inhibition. At the highest pH tested, there was no spontaneous downturn of efflux on stopping respiration, but addition of ADP diminished the efflux. A possible explanation is that binding of ADP is less at the high pH, but another factor is likely to be the pH-dependence of Ca^{2+}-activated phospholipase, which has its optimum at pH 9.5 (Wojtczak & Lehninger, 1961). Evidently, to obtain the downturn of Ca^{2+} efflux, it is necessary to avoid pH above 7.2. A further factor was found to be the amount of the prior loading with Ca^{2+}; at higher loads the downturn did not take place without ADP addition.

Oligomycin, if present initially, prevents the decrease of efflux in response to respiratory inhibition. If added after the efflux has declined owing to rotenone addition, oligomycin causes a gradual upturn of efflux (Fig. 1*d*). These are important observations that suggest an explanation of the decrease of efflux in response to respiratory inhibition. Clearly, we are not dealing here simply with the interruption of energy supply, or oligomycin, by blocking the use of endogenous ATP, would reinforce the effect of stopping respiration. Instead it would seem that the retention of Ca^{2+} is favoured by an energy supply (endogenous ATP) and a reduced state of the early respiratory carriers.

In a state of respiratory inhibition with low Ca^{2+} efflux (0.17nmol/min per mg in a particular example) the successive addition of the uncoupling agent (trifluoromethyltetrachlorbenzimidazole) to 0.12, 0.6 and 1.2μM increased the Ca^{2+} efflux rate to 0.24, 0.78 and 1.2nmol/min per mg respectively. This increase may be attributable to the abolition by the uncoupler of any energy-dependent processes mediated by endogenous ATP.

Table 1. *The effect of pH on the Ca^{2+} efflux from mitochondria before and after inhibition of respiration*

During respiration a load of 36nmol of Ca^{2+}/mg of protein was accumulated; Ruthenium Red was then added (0.4μM) to inhibit active Ca^{2+} influx. The efflux was observed; its value after about 30s is shown in the first set of efflux rates; rotenone was then added (2μg/ml). After an initial more rapid efflux a new low value became established (see Fig. 1), except at the highest pH.

	Ca^{2+} efflux (nmol/min per mg of protein)	
pH	Before respiratory inhibitor	2min after respiratory inhibitor
6.50	3.17	0.08
6.68	1.72	0.08
7.00	1.25	0.16
7.22	0.96	0.16
7.48	2.15	4.0 (fell to 0.36 with 2μM-ADP)

In view of the similar behaviour of strontium towards mitochondria, with the difference that Sr^{2+} is more readily retained and causes less colloid osmotic swelling (Carafoli, 1965; Carafoli *et al.*, 1965*a*,*b*), we decided to compare its behaviour in experiments of the kind described here. It was observed (results not shown) that Sr^{2+} efflux was also diminished in response to inhibition of respiration. The effects of mersalyl and of thyroxine on Sr^{2+} efflux are described below.

Effects of thiol reagents and of thyroid hormones on Ca^{2+} efflux, and reversal of these effects

Tests of the effects of various agents on Ca^{2+} efflux were made on mitochondria that had first been loaded with different amounts of Ca^{2+} (before addition of Ruthenium Red) and either without or with subsequent addition of a respiratory inhibitor. It was convenient to titrate in the agent so as to obtain a series of values of efflux, but in performing

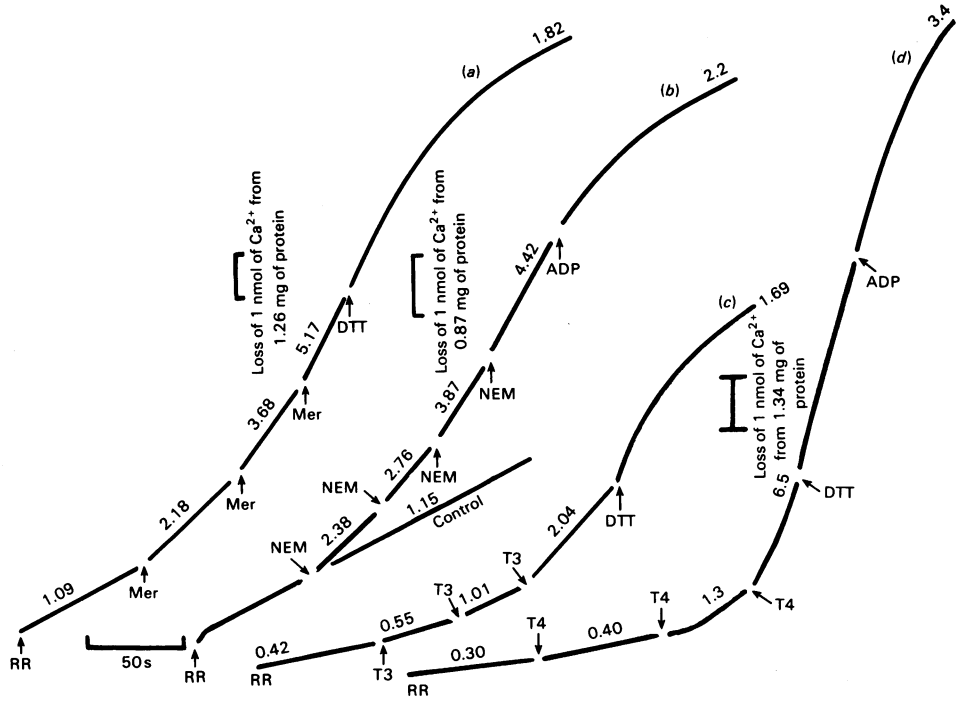

Fig. 2. *Examples of modulation of Ca^{2+} efflux*

(*a*) Increase of efflux obtained in response to mersalyl and its subsequent diminution by dithiothreitol. The mitochondria were first loaded with 30nmol of Ca^{2+}/mg of protein and then Ruthenium Red was added at 'RR'. After the efflux had been observed, three successive additions of mersalyl (Mer) were made (to give 20, 40 and 60μM); these progressively increased efflux. Then dithiothreitol (DTT, 200μM) was added, which, after a delay, caused efflux to diminish. Values on the curve are efflux in nmol/min per mg of protein. The protein concentration was 0.50mg/ml. (*b*) Increase of efflux obtained in response to *N*-ethyl maleimide and its subsequent diminution by ADP. The mitochondria were first loaded as in (*a*), and Ruthenium Red was added. The efflux of Ca^{2+} was observed after four successive additions of *N*-ethylmaleimide (NEM) to give 40, 50, 120 and 160μM. The increased efflux is shown to diminish in response to an addition of ADP to 10μM. A control without the *N*-ethylmaleimide additions shows the constancy of efflux under these conditions. The protein concentration was 0.35mg/ml. (*c*) Increase of efflux obtained with tri-iodothyronine and its subsequent diminution by dithiothreitol. The mitochondria were first loaded with 67nmol of Ca^{2+}/mg and then Ruthenium Red was added (0.4μM). The efflux of Ca^{2+} was observed, and then three successive additions of tri-iodothyronine (T3) were made to give 20, 40 and 60μM. The values of efflux in nmol/min per mg of protein are indicated. Finally, an addition of dithiothreitol (DTT) to 200μM was made, this diminished efflux after a delay. The protein concentration was 0.56mg/ml. (*d*) Increase of efflux obtained with thyroxine and its subsequent diminution after adding dithiothreitol and ADP. The mitochondria were first loaded as in experiment (*c*), and the Ruthenium Red was added. The efflux of Ca^{2+} was observed, and then three successive additions of thyroxine (T4) were made to give 10, 20 and 30μM. As above, the efflux is given in nmol/min per mg of protein. Finally, dithiothreitol (200μM) and ADP (10μM) were added, which led to a diminished efflux. The protein concentration was 0.56mg/ml.

this it was necessary to curtail the periods at any particular concentration. Over short periods the efflux was usually linear, unless either high Ca^{2+} loadings or high concentrations of thiol-specific reagent or thyroid hormone had been added.

The effects of the thiol-specific reagents mersalyl and *N*-ethylmaleimide on the Ca^{2+} efflux from respiring mitochondria are shown in Figs. 2(*a*) and 2(*b*). Nbs_2 acted similarly and results are not illustrated. The efflux increased progressively with increasing mersalyl or Nbs_2 and could be diminished again with dithiothreitol (Fig. 2*a*); since the action of *N*-ethylmaleimide is to alkylate thiol groups, its effect is not reversed with dithiothreitol, but the Ca^{2+} efflux can still be diminished again with ADP (Fig. 2*b*). The thyroid hormones have similar effects on Ca^{2+} efflux [cf. Herd (1978) for tri-iodothyronine applied to liver mitochondria]. The effects of tri-iodothyronine and thyroxine are shown in Figs. 2(*c*) and 2(*d*).

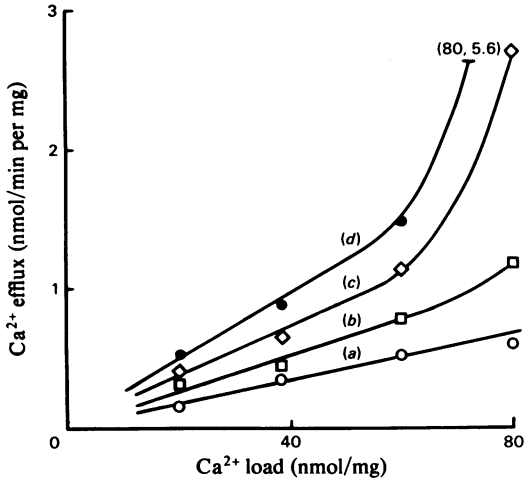

Fig. 3. *Effects of mersalyl and Ca^{2+} load on the Ca^{2+} efflux*

The mitochondria were loaded with chosen amounts of Ca^{2+} and then Ruthenium Red was added to inhibit influx. The efflux was measured and then successive additions of mersalyl were made to give concentrations of 20, 40 and 60μM. The efflux rates after each addition were measured. The control values without addition are plotted along curve (*a*) and with the respective mersalyl concentrations along curves (*b*), (*c*) and (*d*). Efflux, after previous results (Harris, 1979*a*) is assumed to vary linearly with the Ca^{2+} load, so the effect of the diminution of Ca^{2+} has been allowed for when plotting these results by making a proportional correction to observed values. The medium was as described in the Methods section with 3mM each Tris/pyruvate and Tris/malate, 1.2mM-Tris/phosphate, pH7.2. The temperature was 22°C and the protein concentration was 0.54mg/ml.

The stimulated efflux could be lessened by dithiothreitol alone when efflux was moderate, but it was necessary to add ADP as well if efflux was rapid.

Results of similar experiments in which either mersalyl or thyroxine was titrated into respiring suspensions with a series of different preliminary Ca^{2+} loads are shown in Figs. 3 and 4. Over the lower range of Ca^{2+} loads the effect of mersalyl is additive to that of the Ca^{2+} load, but at the highest load used in the series there is a more-than-proportional stimulation of efflux. With thyroxine (Fig. 4) at the highest concentration tested, the non-linear dependence of efflux on the load was apparent even at small Ca^{2+} loads. Similar results with mersalyl and the thyroid hormones were obtained with mitochondria that after loading had been treated with rotenone and allowed to develop the low-efflux condition; examples are shown in Fig. 5. In the non-respiring condition the action of dithiothreitol seemed to be less than when the mitochondria were respiring. Neither *N*-ethylmaleimide nor Nbs_2 was effective at stimulating efflux in the non-respiring condition (results not shown).

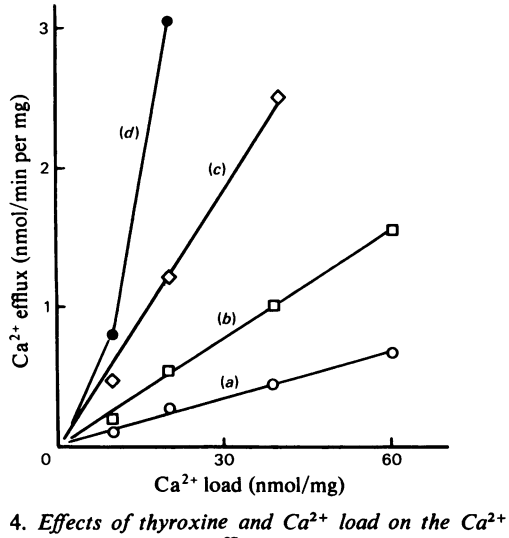

Fig. 4. *Effects of thyroxine and Ca^{2+} load on the Ca^{2+} efflux*

The mitochondria were loaded with chosen amounts of Ca^{2+} and then Ruthenium Red was added to inhibit influx. The efflux was measured and then a series of additions of thyroxine were made to give 10, 20 and 30μM. The efflux without addition is plotted along curve (*a*) and values after thyroxine additions are respectively on curves (*b*), (*c*) and (*d*). As described for Fig. 3, the efflux values have been corrected for the diminished Ca^{2+} contents at each measurement. The experimental conditions were as used in the experiments of Fig. 3.

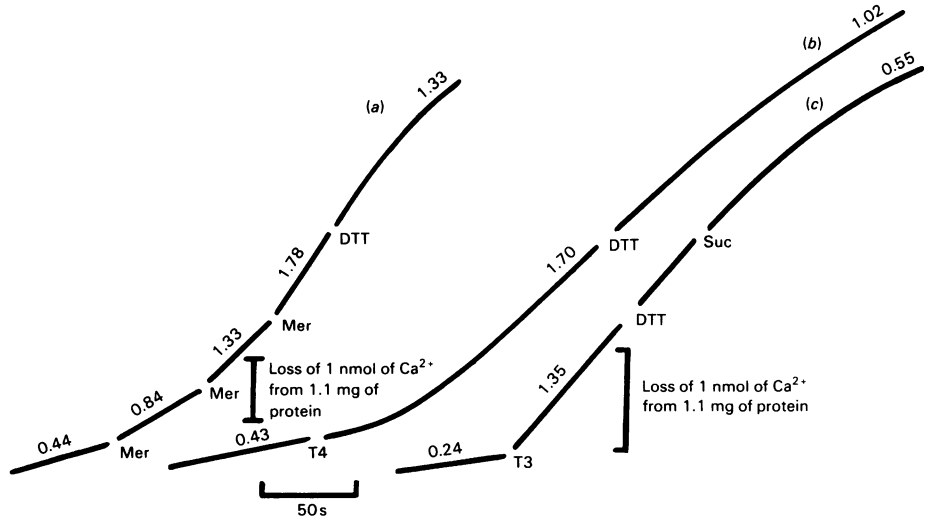

Fig. 5. *Stimulation of efflux of Ca^{2+} from rotenone-treated mitochondria by mersalyl or by thyroid hormones*
The mitochondria were loaded as above with 40nmol of Ca^{2+} per mg of protein while respiring with pyruvate and malate as substrates. Active influx was then inhibited with Ruthenium Red (0.4μM) and respiration was inhibited with rotenone (1μg/ml) just as in the experiment shown in Fig. 1(*b*). The efflux declined to the values noted on the commencements of the respective traces (the units are nmol of Ca^{2+}/min per mg of protein). Addition of either mersalyl (Mer) at successively 40, 80 and 120μM (curve *a*), or thyroxine (T4) at 30μM (curve *b*), or tri-iodothyronine (T3) at 30μM (curve *c*) led to increased efflux. Subsequent addition(s) of dithiothreitol (DTT) to 100μM, followed in (*c*) by succinate (Suc) at 0.6mM, led to diminution of the efflux. The preparation used for (*a*) and (*b*) was different from that used for (*c*), but the protein concentrations were the same, namely 0.44mg/ml. The KCl/Tris medium was used with pyruvate and malate at 3mM each and phosphate at 1.2mM.

Effects of the agents on efflux from strontium-loaded mitochondrias

Experiments made with the same procedure, but with the preliminary loading of strontium (40nmol/mg of protein), which would be additional to endogenous Ca^{2+} (not more than 10nmol/mg of protein), showed that no additional efflux was induced by mersalyl at up to 80μM. However, the Sr^{2+} efflux was accelerated 2-fold by thyroxine at 10μM, and 7-fold at 20μM. The inference is that the mersalyl effect depends to a considerable extent on co-operative action with internal Ca^{2+} to sweep away the Mg^{2+} and ADP that maintain impermeability, whereas thyroxine is effective even in co-operation with the less potent Sr^{2+} ion, with a likely contribution made by endogenous Ca^{2+}.

Correlation between leakage of Ca^{2+} and of adenine nucleotides

By using mitochondria whose adenine nucleotides (ADP+ATP) had been labelled by pretreatment with [^{14}C]ADP, the movement of the label could be used to measure the nucleotide release during the progress of the Ca^{2+} efflux. The samples taken during the experiment were treated as described in the Methods section to obtain a value for the amount of nucleotide transferred from the particles to the medium. The release of the Ca^{2+} was deduced from the spectrophotometer recording as described above. The results of some experiments, all made on one preparation, are shown in Figs. 6(*a*) and 6(*b*).

Fig. 6(*a*) shows that thyroxine stimulates losses of both Ca^{2+} and adenine nucleotides, and that prior addition of bongkrekic acid (□, ■) strongly inhibits the loss of nucleotide and considerably inhibits the Ca^{2+} loss. To the control (○, ●) an addition of dithiothreitol leads to a slowing of both Ca^{2+} and adenine nucleotide losses (although the latter effect is difficult to assess precisely, because of rapid depletion of the total endogenous adenine nucleotide pool). In Fig. 6(*b*) the two effluxes are stimulated by mersalyl (the decline in adenine nucleotide loss here corresponding to loss of total adenine nucleotide content). Another experiment not illustrated showed that in the presence of $MgCl_2$ added at 25μM, the adenine nucleotide loss induced by thyroxine was reduced to about the same amount as that illustrated for bongkrekic acid in Fig 6(*a*) (■), whereas the loss of Ca^{2+} was intermediate between the control and +bongkrekate values in the same experiment. Hence there appears to be a quite variable ratio between the

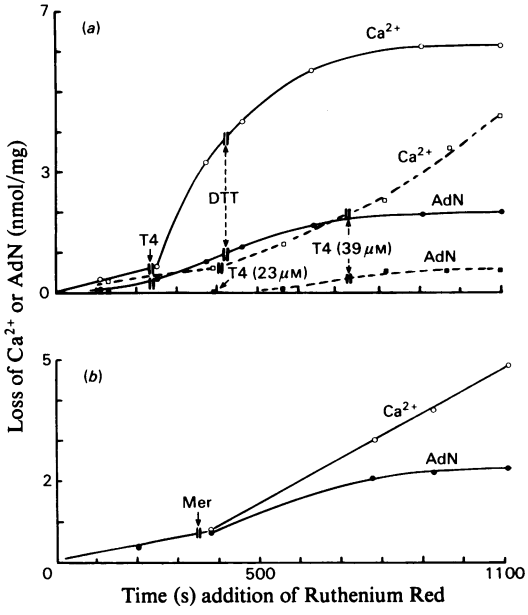

Fig. 6. *Movements of Ca^{2+} and adenine nucleotides induced by thyroxine and mersalyl*

(*a*) The detailed procedure for the sampling to measure the efflux of adenine nucleotide (AdN) accompanying the Ca^{2+} from previously Ca^{2+}-loaded mitochondria is given in the Methods section. The amounts of Ca^{2+} lost from the mitochondria at the times at which samples were separated are plotted as open symbols; the corresponding amounts of adenine nucleotide lost are plotted as closed symbols. In the control experiment (——) the loss of Ca^{2+} and adenine nucleotide initiated by thyroxine (applied at 36 μM at 'T4') was slowed by a later addition of dithiothreitol (DTT) (167 μM). In the experiment made with an addition of bongkrekic acid (– – – –) at 31 nmol/mg of protein, after the loading with Ca^{2+} at 60 nmol/mg of protein, two successive additions of thyroxine were made, the first was to 23 μM and the second to 39 μM (total). (*b*) The effect of an addition of mersalyl to 150 μM on the efflux of adenine nucleotide and Ca^{2+} from similarly loaded mitochondria.

losses of adenine nucleotide and of Ca^{2+} depending on the conditions.

Discussion

Our observation that inhibition of respiration favours retention of mitochondrial Ca^{2+} corroborates a recent observation of Lehninger *et al.* (1978) that moving the redox state of the mitochondrial nicotinamide nucleotides toward reduction lessens Ca^{2+} efflux. The pH-dependence of the effect underlines the necessity to control pH in order to ensure reproducibility. It also finds a parallel in some recent observations by Dr. A. Williams (Cardiothoracic Institute, London) (personal communication) that the net Ca^{2+} efflux from rabbit heart mitochondria increases as pH is increased. The pH-dependence may reflect an effect on the binding of ADP and Mg^{2+} to the membrane proteins.

The effect of the state of reduction of the mitochondrial NAD^+ will be to alter the provision of substrate to the transhydrogenase, which will produce NADPH so long as ATP or an energy source is present. Assuming for a moment that NADPH is necessary to retain Ca^{2+}, the requirement for ATP when respiration is inhibited can account for our observations of the efflux promoting effect of oligomycin illustrated in Fig. 1 (*d*). The requirement for an active transhydrogenase provides a locus for attack by mercurials and thyroid compounds; Hoch (1976) has shown that thyroid compounds lessen NADPH production in thyroidectomized rats. The NADPH-dependence can be explained by the participation of this compound in the reduction of oxidized glutathione to the reduced state so as to provide a source of new thiol groups.

The retention of Ca^{2+} is favoured by exogenous ADP (Drahota *et al.*, 1965; Harris, 1979*a*), and under some conditions of high Ca^{2+} movement (with loads of 100 nmol/mg or more) Mg^{2+} ions are also required (Leblanc & Clauser, 1974*a*,*b*). Loss of Ca^{2+} is paralleled by loss of internal nucleotide and eventually of endogenous Mg^{2+} (Binet & Volfin, 1974). We see (Fig. 6*a*) that retention of Ca^{2+} and of nucleotide is favoured by bongkrekic acid, which is known to bind ADP to the translocase (Weidemann *et al.*, 1970). In contrast, atractylate displaces ADP and potentiates release of Ca^{2+} (Harris, 1979*a*). Oleyl-CoA has similar effects to atractylate (Al-Shaikhaly & Baum, 1979), perhaps also by reason of an effect on the nucleotide translocator. This effect might also be the basis of the potentiation of Ca^{2+} release induced by long-chain fatty acids and by prostaglandins. Such effects are inhibited by ADP or bongkrekic acid.

Excepting the energized accumulation of Ca^{2+} salts such as the acetate, which exert a direct osmotic effect, the usual Ca^{2+}-induced swelling of mitochondria seems to be colloid osmotic in nature. It is not instantaneous, but follows a sequence of Ca^{2+} uptake and spontaneous loss. It is this swelling that can be prevented by colloid solutes in the medium, e.g. poly(ethylene glycol) (which does not affect Ca^{2+} movements). The swelling can alternatively be prevented by inhibiting Ca^{2+} uptake by prior addition of Ruthenium Red, or by protecting against Ca^{2+} loss by adding ADP or bongkrekic acid. Facilitation of Ca^{2+} efflux and of the associated loss of ADP by agents such as thyroxine, mercurials or fatty acids accelerates subsequent swelling. Thus any endogenous Ca^{2+}-activated phospholipase activity will

amplify the effects of even low concentrations of free Ca^{2+}. Albumin, by acting as a scavenger of endogenously generated fatty acids (Wojtczak & Lehninger, 1961) and possibly as a source of thiol groups, maintains the membrane in its relatively impermeable state (Harris, 1979*a*), so that ADP is not lost and can protect against the development of the swelling.

The maintenance of the osmotic integrity of the inner membrane seems to depend on the tight binding of MgADP, which in turn is affected by the oxidation state of certain crucial thiols and by the adenine nucleotide carrier exerting a structural role. Alternatively, other ADP sites are involved that affect the membrane and are sensitive to atractylate, bongkrekate and acyl-CoA. The displacement of ADP (along with Mg^{2+}) and the concomitant increase of non-specific permeability (manifested as swelling in the absence of colloid in the medium) may be achieved by Ca^{2+}, alone if the load is sufficiently high, or by attack on the thiol groups alone. It is perhaps pertinent in this connection that the mercurials increase K^+ permeability as do a series of inorganic ions (Zn^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}; Brierley, 1976) that share the properties of forming insoluble sulphides and phosphates at alkaline pH. Also relevant is the observation by Vignais & Vignais (1972) that certain thiol groups that may be connected to the adenine nucleotide translocator itself have an ADP-dependent accessibility to attack.

In summary, the combination of accumulated Ca^{2+} with those agents that affect membrane binding of ADP can lead to loss of ADP and Mg^{2+} along with the Ca^{2+} and hence to a general increase of permeability, perhaps accentuated by Ca^{2+} stimulation of phospholipase. These phenomena may be of direct physiological importance. For example, foetal mitochondria have low contents of nucleotides and are highly permeable to K^+, they have poor respiratory control and have unusual substrate permeabilities. The properties are shifted to resemble those of normal mitochondria by adding adenine nucleotide (Pollak, 1977).

It is possible that the Ca^{2+} leak sets the magnitude of the overall metabolic activity by controlling both nucleotide translocation and substrate flux. Herd (1978) showed that thyroid hormone stimulates the translocase of the mitochondria obtained from thyroidectomized rats, and co-movement of substrate anions with Ca^{2+} is known to take place (Harris & Berent, 1969; Harris, 1978).

Another set of observations, made on effects of glucagon, also appear to be explicable as being due to enhanced Ca^{2+} turnover; these are: the enhancement of nucleotide translocase activity (Bryła *et al.*, 1977), the higher maximal respiration obtainable, so giving a higher rate of Ca^{2+} uptake (Yamazaki, 1975) and a higher rate of Ca^{2+} efflux (Bygrave & Tranter, 1978).

The observations of the discharge of Ca^{2+} in response to the addition of uncoupling agents, whether or not respiration has been inhibited, show that the action is here either on the membrane directly, or indirectly because of the abolition of energized transhydrogenase activity. The so-called 'high-affinity' binding of Ca^{2+} by non-respiring mitochondria has now been attributed by three groups of workers to the presence of residual energy-yielding materials (Southard & Green, 1974; Åkerman *et al.*, 1974; Reed & Bygrave, 1975); this consensus would agree with our proposal that a supply of ATP is necessary (in absence of respiration) to energize the transhydrogenase to keep up the supply of NADPH to feed to glutathione reductase for the supply of -SH groups; these in turn keep the membrane in the impermeable condition by ensuring the binding of MgADP.

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