Respiration-Dependent Efflux of Magnesium Ions from Heart Mitochondria

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Energy-linked respiration causes a net movement of Mg^{2+} between rat heart mitochondria and the ambient medium. When the extramitochondrial concentration of Mg^{2+} is less than about 2.5 mM the net movement of Mg^{2+} constitutes an efflux, whereas a net influx of Mg^{2+} occurs when the external concentration of Mg^{2+} is greater than this. Both the efflux and the influx are induced to only a very small degree by externally added ATP. Evidence suggests that P_i may be required for the respiration-induced efflux of Mg^{2+} .

Nearly all of the mitochondria that have been studied are able to accumulate Ca^{2+} , Sr^{2+} and Mn^{2+} by processes that are supported by respiration or by hydrolysis of ATP (see Lehninger *et al.*, 1967). Mitochondria from heart tissue are the only ones that are known to accumulate large amounts of Mg^{2+} , in addition to the other bivalent cations, by a respiration-dependent process (Brierley *et al.*, 1962, 1963). In these studies the provision of energy has led to an influx of the cations, and no case has been described in which respiration or ATP hydrolysis induces an efflux of a bivalent cation from mitochondria. Schuster & Olson (1973) observed that respiration caused a small loss of Mg^{2+} from ox heart submitochondrial particles; in this case, the membranes of the particles were inside-out with respect to the inner membrane of intact mitochondria, and thus the direction of the flux of Mg^{2+} was consistent with other studies using intact mitochondria.

The present study reports the effect of energy from respiration and from the hydrolysis of ATP on the movement of Mg^{2+} between heart mitochondria and the external medium. It reveals that the nature of the permeation of Mg^{2+} in heart mitochondria differs in several respects from that established for Ca^{2+} and other bivalent cations; among these, it is shown that energy-linked respiration can elicit an efflux of Mg^{2+} from heart mitochondria by a process that may require phosphate.

Experimental

Isolation of mitochondria

Rat heart mitochondria were prepared by using the proteolytic enzyme Nagarse as described by Pande & Blanchaer (1971), in an extraction medium containing 0.21 M-mannitol, 0.07 M-sucrose, 10 mM-Tris/HCl and 0.1 mM-EDTA, final pH 7.4. The mitochondrial pellet

was washed twice in extraction medium without EDTA. The respiratory-control ratio (Lardy & Copenhaver, 1954) of the mitochondria prepared in this way varied between 4 and 5 when succinate was used as a substrate.

Determination of mitochondrial protein

Mitochondrial protein was determined by a modification of the biuret procedure (Kröger & Klingenberg, 1966), with bovine plasma albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

Measurement of the efflux of Mg^{2+} from mitochondria

Mitochondria (containing about 20 mg of protein) were added to 10 ml of medium containing 120 mM-NaCl, 20 mM-Tris/HCl (pH 7.4) and 15 μ g of rotenone at 25°C; further additions to the medium are stated in the Figure legends. The suspension was stirred continuously and, at intervals of time, 1 ml portions of the suspension were removed and centrifuged for 1 min in an Eppendorf bench centrifuge (model 3200) operating at 15000 rev./min; this centrifugation was sufficient to sediment at least 98% of the mitochondria. The supernatant fluids were removed and their Mg^{2+} , Ca^{2+} and K^{+} contents determined by using a Perkin-Elmer atomic absorption spectrophotometer (model 503).

Measurement of the influx of Mg^{2+} into mitochondria

Incubations were carried out at 25°C as described above (under 'Measurement of the efflux of Mg^{2+} from mitochondria'), except that [U - ^{14}C]sucrose (5 mM, containing approx. 1 μ Ci) and $MgCl_2$ were added to the incubation medium. After certain periods of time, 0.2 ml portions of the suspension were withdrawn and introduced into Beckman centrifuge tubes (capacity 0.4 ml) that already contained a lower

layer of 12.3% (w/v) sucrose (100 μ l) and an upper layer (50 μ l) of silicone (AR50; Wacker-Chemie, Munich, Germany). The tubes were centrifuged for 1 min in a Beckman bench centrifuge (model 152) operating at 12000 rev./min, so that the mitochondria sedimented through the silicone into the sucrose layer. The mitochondria were extracted and their radioactivity content was determined as described previously (Crompton & Chappell, 1973). The Mg^{2+} content of the mitochondrial extract was determined as described above. The intramitochondrial contents of Mg^{2+} were calculated by correcting for the Mg^{2+} present in the extramitochondrial space (determined from the radioactivity content of the mitochondria).

The true extramitochondrial concentrations of free Mg^{2+} in the incubations in Fig. 7 were determined with Eriochrome Blue (Scarpa, 1974). The determinations were done on complete incubation media (see Fig. 7) plus antimycin A to inhibit the uptake and release of Mg^{2+} by the mitochondria. The actual concentrations of free Mg^{2+} were about 10% less than the concentration of Mg^{2+} added.

Measurement of the phosphate content of the mitochondria

Portions (1 ml) of mitochondrial incubations (the compositions of which are stated in the text) were centrifuged in an Eppendorf bench centrifuge (model 3200) operating for 1 min at 15000 rev./min. The mitochondrial pellet was extracted immediately with 1 ml of 0.3 M-HClO₄ for 10 min at 0–2°C. The extract was centrifuged for 30 s in the Eppendorf bench

centrifuge and the phosphate content of the supernatant fluid was determined as described by Stewart (1974).

Results

Efflux of Mg^{2+} from heart mitochondria induced by respiration

Rat heart mitochondria isolated as described in the Experimental section contain 17–24 nmol of Mg^{2+} /mg of mitochondrial protein (from determinations on 21 different mitochondrial preparations); this value agrees approximately with the data of Bogucka & Wojtczak (1971). When the mitochondria are suspended in a medium containing rotenone, to inhibit endogenous respiration, a small fraction of the Mg^{2+} (about 2 nmol/mg of protein) is released immediately into the medium (Fig. 1a); the major part of the endogenous Mg^{2+} is lost extremely slowly, however (about 5% is lost after incubation for 12 min). Thus in the absence of respiration, heart mitochondria retain 84–87% of their endogenous Mg^{2+} after incubation for 12 min. Fig. 1(a) shows that the introduction of succinate induces a release of about 7 nmol of Mg^{2+} /mg of protein. There is no release of Mg^{2+} , however, when succinate oxidation is either inhibited (by antimycin A) or uncoupled (by carbonyl cyanide *m*-chlorophenylhydrazone), which indicates that the capacity of succinate to induce the release of Mg^{2+} is due to its providing a source of energy. A similar release of Mg^{2+} is observed when ascorbate plus tetramethylethylenediamine or β -hydroxybuty-

Fig. 1. Efflux of Mg^{2+} (a) and Ca^{2+} (b) from mitochondria

Mitochondria (containing 21 mg of protein) were incubated in 10 ml of medium containing rotenone as described in the Experimental section. Additions (at the arrow) were made after incubation for 5 min as follows: ●, none; ▲, 2 mM-succinate; □, 2 mM-succinate+20 μ M-Ruthenium Red; ○, 2 mM-succinate+4 μ M of antimycin A; △, 2 mM-succinate+0.5 μ M-carbonyl cyanide *m*-chlorophenylhydrazone.

rate (in the absence of rotenone) are used as respiratory substrates instead of succinate (experiments not shown). From a series of 12 experiments it is concluded that energy-linked succinate oxidation causes the release of 31–43% of the total Mg^{2+} retained by the mitochondria in the absence of energy. The composition of the suspending medium is not critical, since identical results are obtained, with respect to both the amount of Mg^{2+} retained by the mitochondria in the absence of respiration and the amount liberated during coupled respiration, if the NaCl of the incubation medium is replaced by either 120mM-KCl or 240mM-sucrose.

It is noteworthy that if 10mM-ATP is used instead of a respiratory substrate, the release of Mg^{2+} is only about 12% of that otherwise observed, although ATP does not inhibit the efflux of Mg^{2+} induced by respiratory substrates (experiments not shown). Thus externally added ATP seems to be a poor source of energy for the release of Mg^{2+} .

The changes described above may be compared with those occurring in the fluxes of endogenous Ca^{2+} under the same conditions (Fig. 1*b*). There is an efflux of Ca^{2+} in the absence of respiration, but the addition of succinate causes a prompt re-uptake of Ca^{2+} , and the re-uptake is inhibited by Ruthenium Red (Moore, 1971; Vasington *et al.*, 1972). Under these experimental conditions therefore the net fluxes of endogenous Mg^{2+} and Ca^{2+} respond in an opposite manner to energization of the mitochondria by respiration. The distinction between the movements of Mg^{2+} and Ca^{2+} is further emphasized by the observation that the movement of Mg^{2+} is insensitive to Ruthenium Red (Fig. 1*a*).

Effects of phosphate and oligomycin on the efflux of Mg^{2+}

Fig. 2 reports the effects of phosphate and oligomycin on the efflux of Mg^{2+} induced by respiration. Both phosphate and oligomycin increase the rate and the final amount of Mg^{2+} released. Phosphate and oligomycin cause no loss of Mg^{2+} from the mitochondria in the absence of respiration (Fig. 2; plus antimycin A). The amount of Mg^{2+} released by respiration with either phosphate or oligomycin present was about 59% of the total Mg^{2+} retained by the mitochondria in the absence of respiration.

Maintenance of mitochondrial integrity during efflux of Mg^{2+}

It is important to establish that the release of Mg^{2+} reported above is not due to a loss of the structural integrity of the mitochondria. In this regard, other experiments have shown that the endogenous K^+ content of rat heart mitochondria (75–95nmol/mg of mitochondrial protein) decreases by less than 1nmol/mg of protein during incubation for 8min under experimental conditions identical with those

Fig. 2. Effect of P_i and oligomycin on the efflux of Mg^{2+} from mitochondria

Mitochondria (containing 19.4mg of protein) were incubated in 10ml of medium containing rotenone as described in the Experimental section. The media also contained at the beginning of the incubations: ▲, 2mM-succinate; ◻, 2mM-succinate+1mM- P_i ; ○, 2mM-succinate+5µg of oligomycin; △, 2mM-succinate, 1mM- P_i +4µg of antimycin A; ●, 2mM-succinate, 5µg of oligomycin+4µg of antimycin A.

of Fig. 2 (plus succinate and either phosphate or oligomycin, i.e. when the efflux of Mg^{2+} is maximum). In addition, negligible swelling of the mitochondria occurs during the efflux of Mg^{2+} , as judged by the absorbance changes of the mitochondrial suspension. The initial absorbance of the incubation (of Fig. 2; plus succinate and either phosphate or oligomycin) decreased by only about 1% during incubation for 8min [this may be compared with a decrease in absorbance of 20–30% after 1min when mitochondria are suspended in iso-osmotic $(NH_4)_2HPO_4$, for example, which results in large-amplitude swelling (Chappell & Crofts, 1966; Crompton *et al.*, 1974)]. Finally, rat heart mitochondria exhibit good respiratory control after incubation for 8min in the presence of succinate and phosphate (the respiratory-control ratio was 3–4 with succinate as substrate). These observations indicate that the specific permeability properties of heart mitochondria are preserved during the efflux of Mg^{2+} .

Effect of ADP on the efflux of Mg^{2+}

Fig. 3(*a*) shows that the efflux of Mg^{2+} induced by succinate oxidation is strongly inhibited by ADP;

other experiments revealed that about $100\mu\text{M}$ -ADP is required for maximum inhibition. The inhibition by ADP is abolished completely by the inclusion of phosphate in the incubation medium (Fig. 3*a*, uppermost curve). In this case, more Mg^{2+} is released than with succinate alone because of the presence of phosphate; this point is evident from Fig. 3(*a*), since the release of Mg^{2+} in the presence of succinate, phosphate and ADP is the same as in the presence of succinate and phosphate, i.e. ADP does not inhibit when phosphate is present. The inhibition of the efflux of Mg^{2+} by ADP is not prevented by acetate.

Fig. 3(*a*) also shows that the inhibition maintained by ADP for a period of time (3 min) is promptly abolished when phosphate is added. This experiment was accompanied by a parallel one, under identical conditions, in which the consumption of O_2 by the mitochondrial suspension was monitored and used to establish the time at which the ADP was completely phosphorylated; this was marked by a sharp decrease in the respiratory rate [transition from state 3 to state 4 respiration (Chance & Williams, 1955)] and occurred 4 min after the addition of phosphate. Since the inhibition by ADP is nullified immediately after the addition of phosphate it may be concluded that phosphate acts by counteracting the effect of ADP and not by removing ADP (by phosphorylation).

The inhibition of the efflux of Mg^{2+} by ADP is also prevented when mitochondria are added to a medium containing oligomycin in addition to ADP (Fig. 3*b*); under these conditions oxidative phosphorylation is prevented. However, Fig. 3(*b*) also reveals that once the inhibition by ADP is established then the addition of oligomycin does not restore the capacity for respiration-dependent release of Mg^{2+} . Other experiments showed that atractyloside, which blocks the access of extramitochondrial ADP to the mitochondrial adenosine triphosphatase (Klingenberg & Pfaff, 1966; Vignais & Duee, 1966), also prevents the inhibition by ADP when added to the mitochondria at the same time as ADP, but it does not release the inhibition when added 1 min after ADP. Thus oligomycin and atractyloside prevent, but do not reverse, the inhibition of the respiration-dependent efflux of Mg^{2+} by ADP.

The data of Fig. 3 suggest a working hypothesis, i.e. that phosphate may be necessary for the efflux of Mg^{2+} induced by respiration. According to this hypothesis, ADP would inhibit by removing endogenous phosphate by oxidative phosphorylation, and both oligomycin and atractyloside would prevent (but not reverse) the inhibition by ADP, since they prevent phosphorylation of ADP. In addition, the inhibition by ADP would be reversed by phosphate

Fig. 3. Inhibition of the efflux of Mg^{2+} by ADP

Mitochondria (containing 21 mg of protein) were incubated in 10 ml of medium containing rotenone as described in the Experimental section. The media also contained at the beginning of the incubations the following additions. Fig. 3(*a*): ●, 2 mM-succinate; ○, 2 mM-succinate + 1 mM-ADP; ■, 2 mM-succinate, 1 mM-ADP + 10 mM-potassium acetate; □, 2 mM-succinate, 1 mM-ADP + 2 mM- P_i ; △, 2 mM-succinate + 2 mM- P_i ; ▲, 2 mM-succinate + 1.5 mM-ADP, and a further addition of 2 mM- P_i was made after incubation for 3 min (marked by arrow). Fig. 3(*b*): ●, 2 mM-succinate, 1 mM-ADP + 5 μg of oligomycin; ○, 2 mM-succinate + 1.5 mM-ADP, and a further addition of 5 μg of oligomycin was made after incubation for 3 min (marked by arrow).

itself. Measurements of the phosphate content of the mitochondria under these conditions gave the following values (Table 1). The endogenous content of phosphate in rat heart mitochondria in the presence of succinate is about 5.5 nmol/mg of mitochondrial protein and is decreased to a minimum value of about 2.1 nmol/mg of protein after incubation with ADP and succinate for 1 min; the content of phosphate is not decreased further by incubation with ADP and succinate for longer periods. There is no detectable decrease in the content of phosphate when ADP is added in the presence of oligomycin.

The effects of α -oxoglutarate and Ca^{2+} reported below were designed to test the above hypothesis further.

Effect of α -oxoglutarate on the efflux of Mg^{2+}

Intramitochondrial phosphate may be consumed in the presence of oligomycin by the substrate-level phosphorylation reaction catalysed by succinic thio-kinase (EC 6.2.1.4). This forms the basis of the experiment reported in Fig. 4, in which α -oxoglutarate was added to generate intramitochondrial succinyl-CoA. Succinate and oligomycin were present in every incubation.

Fig. 4. Inhibition of the efflux of Mg^{2+} by α -oxoglutarate

Mitochondria (20.6 mg of protein) were incubated in 10 ml of medium as described in the Experimental section, except that rotenone was omitted and both 2 mM-succinate and 5 μ g of oligomycin were added. Other additions to the media at the beginning of the incubations were: Δ , 1 mM-ADP; $\bullet$, 1 mM-ADP+2 mM- α -oxoglutarate; $\circ$, 2 mM- α -oxoglutarate; $\blacktriangle$, 1 mM-ADP, 2 mM- α -oxoglutarate+2 mM- P_i ; $\square$, 1 mM-ADP+2 mM- α -oxoglutarate+2 mM-arsenite.

Table 1. Intramitochondrial content of phosphate after different treatments

Heart mitochondria (containing 9.0 mg of protein) were added to 5 ml of medium containing 120 mM-NaCl, 20 mM-Tris/HCl, 0.1 mM-EDTA and 2 mM-succinate. Further additions to the incubations are stated in the Table. Portions (1 ml) of the suspension were withdrawn after incubation for 1 min and the phosphate content of the mitochondria was determined as described in the Experimental section. Values obtained with two different preparations of mitochondria are reported (Expts. a and b).

Addition	Content of P_i (nmol/mg of mitochondrial protein)	
	Expt. (a)	Expt. (b)
(1) None	5.1	5.9
(2) 1 mM-ADP	2.1	2.2
(3) 1 mM-ADP and 3 μ g of oligomycin	4.9	6.0
(4) 1 mM-ADP, 3 μ g of oligomycin and 2 mM- α -oxoglutarate	2.4	2.3

The release of Mg^{2+} that occurs when mitochondria are added to a medium containing succinate, oligomycin and ADP (i.e. as in Fig. 3b) is severely inhibited if α -oxoglutarate is also included (i.e. plus succinate, oligomycin, ADP and oxoglutarate). Under these conditions the mitochondrial content of phosphate decreased to a minimum value of about 2.3 nmol/mg of protein within 1 min (Table 1). The addition of α -oxoglutarate also causes some inhibition of the efflux of Mg^{2+} in the absence of added ADP (i.e. plus succinate, oligomycin and α -oxoglutarate), but this inhibition is much less than when ADP is present (i.e. plus succinate, oligomycin, oxoglutarate and ADP). Thus, in summary, although ADP does not inhibit the efflux of Mg^{2+} when it is added to a medium containing oligomycin (Fig. 3a) and does not decrease the mitochondrial content of phosphate (Table 1), ADP does inhibit in the presence of oligomycin if the medium also contains α -oxoglutarate (Fig. 4), and in this case the intramitochondrial phosphate content is decreased (Table 1).

The inhibition of the efflux of Mg^{2+} in the presence of oligomycin, oxoglutarate and ADP is abolished when further additions are made of either arsenite, which inhibits oxoglutarate dehydrogenase and hence substrate-linked phosphorylation, or phosphate.

Effect of Ca^{2+} on the efflux of Mg^{2+}

Another treatment that might be predicted to lower the intramitochondrial concentration of free phosphate is that after uptake of Ca^{2+} by the mitochondria, since the Ca^{2+} that is accumulated would

Fig. 5. Inhibition of the efflux of Mg^{2+} by Ca^{2+}

Mitochondria (17.2 mg of protein) were incubated in 10 ml of medium containing rotenone (see the Experimental section). The media also initially included: ●, 2 mM-succinate; ○, 2 mM-succinate + 80 μ M- $CaCl_2$; △, 2 mM-succinate + 80 μ M- $CaCl_2$ + 1 mM- P_i ; ▲, 20 μ M-Ruthenium Red and a further addition of 2 mM-succinate + 80 μ M- $CaCl_2$ was made after incubation for 10 s.

be available to complex with the endogenous phosphate. Fig. 5 shows that when Ca^{2+} is included in the incubation medium, the efflux of Mg^{2+} produced by respiration is prevented almost completely. Ruthenium Red, which inhibits the uptake of Ca^{2+} by mitochondria, prevents the effect of Ca^{2+} ; this indicates that Ca^{2+} needs to permeate to inhibit the release of Mg^{2+} . Moreover, the inhibition of the efflux of Mg^{2+} by Ca^{2+} is also prevented when phosphate is added.

Influx of Mg^{2+} into heart mitochondria induced by respiration

Brierley *et al.* (1963) and Schuster & Olson (1974) have reported that heart mitochondria take up added Mg^{2+} in the presence of phosphate and energy from respiration. Both groups of workers used high external concentrations of Mg^{2+} (5–17 mM). We have investigated further the conditions necessary for the uptake of Mg^{2+} in order to compare this process with the efflux of Mg^{2+} reported here.

Fig. 6 shows that there is no uptake of Mg^{2+} in the absence of respiration (i.e. in the presence of antimycin A) and little uptake when phosphate is omitted. In the absence of phosphate, the uptake that occurs is complete within 1 min and is not changed when acetate is included. The inability of acetate to

stimulate the uptake of Mg^{2+} contrasts with its ability to augment the energy-dependent uptake of Ca^{2+} by mitochondria and thereby provide a substitute for phosphate in this respect (Rasmussen *et al.*, 1965; see Lehninger *et al.*, 1967). It is evident that phosphate is required for prolonged uptake of Mg^{2+} and that this process is not influenced by the addition of Ruthenium Red, which completely inhibits uptake of Ca^{2+} by mitochondria from heart tissue and other sources (Moore, 1971; Vasington *et al.*, 1972).

Data of Brierley *et al.* (1962) that are relevant to the present work have been confirmed in this laboratory. First, the influx of Mg^{2+} (10 mM) in the presence of phosphate and respiration is inhibited by about 90% when ADP (Mg^{2+} salt; 5 mM) is also added to the incubation medium. Thus almost no influx of Mg^{2+} occurs during oxidative phosphorylation (whereas efflux of Mg^{2+} does occur; Fig. 3a). When oxidative phosphorylation is inhibited by oligomycin, however, the uptake of Mg^{2+} is completely restored to the extent observed when no ADP is added. Secondly, when the succinate in the incubation medium is replaced by ADP (Mg^{2+} salt; 2 or 10 mM), plus antimycin A to inhibit endogenous respiration, the uptake of Mg^{2+} after 9 min incubation is decreased by 85–90%, i.e. ATP is a poor source of energy for the uptake of Mg^{2+} .

Fig. 6. Uptake of Mg^{2+} by mitochondria

Mitochondria (4 mg of protein) were incubated in 2 ml of medium containing rotenone and 13 mM- $MgCl_2$ as described in the Experimental section. The media included also the following: ●, 2 mM-succinate, 2 mM- P_i + 0.7 μ g of antimycin A; ○, 2 mM-succinate; ▲, 2 mM-succinate + 10 mM-potassium acetate; △, 2 mM-succinate + 2 mM- P_i ; □, 2 mM-succinate + 2 mM- P_i + 20 μ M-Ruthenium Red.

Fig. 7. Changes in the mitochondrial content of Mg^{2+} after incubation in various concentrations of external Mg^{2+}

Mitochondria (1.9 mg of protein) were incubated for 10 min in 1 ml of medium containing rotenone, 2 mM-succinate and 2 mM- P_i as described in the Experimental section (under 'Measurement of the influx of Mg^{2+} into mitochondria'). Different concentrations of $MgCl_2$ also were added to the incubation medium and the actual concentration of free Mg^{2+} in each case was determined by using Eriochrome Blue (see the Experimental section). The change in the mitochondrial content of Mg^{2+} was obtained by subtracting the endogenous content of Mg^{2+} from the content after incubation for 10 min (when the net fluxes of Mg^{2+} were complete).

Dependence of the direction of net flux of Mg^{2+} on the external concentration of Mg^{2+}

It is clear from the data of Fig. 6 and the preceding Figures that the movement of Mg^{2+} across the inner mitochondrial membrane may constitute either a net efflux or a net influx and that the direction of the net flux depends on the presence or absence of external Mg^{2+} . The relation between the direction of the net movement of Mg^{2+} and the external concentration of free Mg^{2+} is reported more fully in Fig. 7. In this experiment the net fluxes of Mg^{2+} were allowed to proceed to completion.

The final amount of Mg^{2+} that is released from the mitochondria is progressively decreased as the extramitochondrial concentration of free Mg^{2+} is increased. Net efflux of Mg^{2+} occurs when the external concentration of free Mg^{2+} is less than about 2.5 mM, whereas net influx takes place when the external concentration is higher than this. No net movement of Mg^{2+} occurs if about 2.5 mM free Mg^{2+} is added to the external medium.

Discussion

The present study shows that energy-linked respiration induces a net efflux of Mg^{2+} from rat heart mitochondria. Further, on the basis of our data it seems possible that phosphate may be required for this process; the evidence for this is summarized as follows. The respiration-dependent release of Mg^{2+} is stimulated by phosphate (Fig. 2) and is inhibited by those treatments that decrease the endogenous content of free phosphate in the mitochondria; these treatments involved the use of ADP (Fig. 3), α -oxoglutarate (Fig. 4) and Ca^{2+} (Fig. 5). After all of these treatments, the addition of phosphate restores the capacity for respiration-induced efflux of Mg^{2+} . Finally the inhibition of release of Mg^{2+} caused by treatment with ADP or α -oxoglutarate is prevented when inhibitors (e.g. oligomycin, atractyloside or arsenite) are included to stop the phosphate-depleting reactions; these inhibitors do not reverse the inhibition once it has been established; only the addition of phosphate achieves this.

The postulate that phosphate is required for the efflux of Mg^{2+} is in accordance with all of these data. However, since the treatments used for depleting the mitochondria of phosphate removed only about 60% of the total endogenous P_i , the above postulate can only be a tentative one. Nevertheless, it does seem possible that the non-removable fraction of the P_i may be sequestered in an inactive form (perhaps precipitated with bivalent cations), so that the decrease in the amount of free, available P_i obtained by the treatments was greater than 60%. In this regard, McGivan & Klingenberg (1971) found that about 50% (or 3.8 nmol/mg of mitochondrial protein) of the P_i contained in liver mitochondria does not exchange with externally added phosphate.

Phosphate is transported electroneutrally across the inner mitochondrial membrane by co-transport with protons [i.e. a net movement of H_2PO_4 occurs (Chappell & Crofts, 1966; Klingenberg *et al.*, 1974)]. The net process of the permeation of acetate is similar [the undissociated acid being the permeant species (Chappell & Crofts, 1966)]. However, acetate does not share the ability of phosphate to elicit efflux of Mg^{2+} , which indicates that any role exerted by phosphate in the movement of Mg^{2+} cannot be due to its ability to transport protons across the inner membrane.

The uptake of Mg^{2+} by heart mitochondria is not supported by ATP and is not inhibited by Ruthenium Red. These results suggest that the mechanisms of the uptake of Mg^{2+} and Ca^{2+} may be distinct, although it is premature to infer that the influxes occur by completely independent processes. Certainly Mg^{2+} ions do bind to the transport system for Ca^{2+} in heart mitochondria (Sordahl, 1974; Jacobus *et al.*, 1975). However, work in our laboratory has shown that the

affinity for Mg^{2+} of the transport system for Ca^{2+} is very low, about 20mM- Mg^{2+} being required to inhibit by 50% the initial rate of uptake of 50 μ M- Ca^{2+} .

A loss of Mg^{2+} from rat liver mitochondria has been reported by Kun and his co-workers (Kun *et al.*, 1969; Lee *et al.*, 1971) and by Scarpa (1974) when respiration occurs in the presence of an uncoupling agent plus ADP. Intramitochondrial K^+ is lost at the same time (Lee *et al.*, 1971). No efflux of Mg^{2+} occurs in the absence of uncoupling agents and the amount of uncoupler needed to elicit loss of Mg^{2+} exceeds that required for maximum uncoupling of respiration (Kun *et al.*, 1969); this suggests that the uncoupling agents may exert their effect by perturbing the membrane structure rather than by uncoupling. Binet & Volfin (1974) reported that respiration-dependent uptake of Ca^{2+} induces a loss of Mg^{2+} from liver mitochondria. The loss of Mg^{2+} is followed by a loss of the accumulated Ca^{2+} and it has been suggested that the integrity of the inner mitochondrial membrane is impaired by the uptake of Ca^{2+} (Binet & Volfin, 1975).

Since efflux of Mg^{2+} from heart mitochondria occurs during respiration in the presence of ADP and phosphate (Fig. 3a), the intramitochondrial content of Mg^{2+} *in vivo* is presumably maintained by a concentration of Mg^{2+} in the cytosol that counteracts the potential net efflux. Our data (Fig. 7) suggest that about 2.5mM of free Mg^{2+} may be required for no net flux of Mg^{2+} to occur between mitochondria and cytosol. However, this concentration of Mg^{2+} is clearly a highly provisional one, since the fluxes of Mg^{2+} *in vivo* may be subjected to controlling factors, so that extrapolation from data of this type obtained *in vitro* provides only a tentative value. Measurements by Endo (1975) suggest that the concentration of free Mg^{2+} in skeletal muscle of toad is about 0.9mM, a somewhat similar value.

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