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## Protective action of magnesium ions on mitochondrial ion flux

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**Biochimica.** — *Protective action of magnesium ions on mitochondrial ion flux.* Nota di DAGMAR SILIPRANDI, ANTONIO TONINELLO, MICHELA RUGOLO, FRANCO ZOCCARATO e STEFANO DELLA MEA, presentata (*) dal Corrisp. N. SILIPRANDI.

RIASSUNTO. — Viene descritta l'azione protettiva degli ioni magnesio sulla integrità strutturale dei mitocondri di fegato e sulla permeabilità di alcuni ioni (calcio, potassio e fosfato) rispetto alla membrana mitocondriale interna. Sia la diamide, un blando ossidante dei gruppi tiolici, che il fosfato inorganico inducono alterazioni della membrana mitocondriale interna che si estrinsecano in un efflusso di calcio, potassio e fosfato ioni ed in una accelerazione della respirazione in stato 4. Solo in presenza di fosfato la diamide induce un rigonfiamento dei mitocondri « dipendente da energia ». L'aggiunta al mezzo di incubazione di 2 mM $Mg^{2+}$, una concentrazione che non si discosta di molto da quella fisiologicamente esistente nella cellula, previene o sopprime le alterazioni indotte sia dalla diamide come dal fosfato inorganico. Si è tuttavia osservato che l'azione degli ioni magnesio sull'efflusso dei cationi dai mitocondri richiede anche la presenza di ioni fosfato, mentre questi non sono necessari per la prevenzione dell'efflusso del fosfato endogeno. Gli ioni magnesio prevengono anche il rigonfiamento indotto da diamide in presenza di fosfati. Il possibile meccanismo dell'azione protettiva dei magnesio ioni sui mitocondri isolati di fegato viene ampiamente discusso anche in rapporto all'antagonismo magnesio–calcio ed al possibile ruolo del rapporto citoplasmatico $Mg^{2+}$/Pi nella regolazione della permeabilità dei mitocondri agli ioni inorganici.

There is increasing evidence that $Mg^{2+}$ might control ion flux across inner mitochondrial membrane (Ref. [1] for review). Removal of endogenous $Mg^{2+}$ more or less severely affects the permeability and transport properties of mitochondria [2–6], whereas addition of $Mg^{2+}$ to mitochondria preparations "stabilizes" membrane structure [6, 7] and restricts the permeability of inner membrane for cations [8, 9]. External $Mg^{2+}$ also inhibits the mitochondrial flux of $K^+$ induced by valinomycin [10].

We have recently found that both inorganic phosphate (Pi) and diamide, a mild oxidizing agent of thiol groups, induce alterations of mitochondrial permeability implying, among other consequences, a respiratory efflux of endogenous ions ($Mg^{2+}$, $K^+$ and Pi) [11] and in some instances, an energy dependent swelling [12]. Some of these effects could be prevented or reversed by added $Mg^{2+}$.

In the present paper we describe the multiple aspects of $Mg^{2+}$ action in preserving the structural integrity of rat liver mitochondria and the normal permeability and transport properties of the inner mitochondrial membrane.

*Abbreviations*: Diamide = diazenedicarboxylic acid bis-dimethylamide; FCCP = *p*-trifluoromethoxyphenylhydrazone; EGTA = ethylene glycol-bis-(2-amino ethyl ether)-N,N'-tetracetic acid; Ruthenium red = $Ru_2(OH)_2Cl_4 \cdot 7\ NH_3 \cdot 3\ H_2O$; NEM = N-ethyl maleimide.

(*) Nella seduta del 13 maggio 1978.

## Experimental

Rat liver mitochondria were isolated according to Schneider [13]. Protein concentration was determined by the biuret method [14]. Oxygen uptake was measured with a Clark oxygen electrode. $Ca^{2+}$ and $K^+$ movements were estimated by atomic absorption spectroscopy on the supernatant [15] and total cation amount by acid extraction of the pellet [16].

Inorganic phosphate was determined according to the method of Sumner [17] on 10 % ClCCOOH extracts from the pellet obtained by rapidly centrifuging portions of the incubated suspension containing 10.5 mg mitochondrial protein (Fig. 3).

The Pi efflux from mitochondria was followed by the swelling method (Fig. 5) based on the generation of Pi in the mitochondrial matrix by FCCP stimulated ATP hydrolysis as described by Klingenberg *et al.* [18].

Swelling was monitored by absorption at 520 nm using an Aminco Chance spectrophotometer. ATPase activity was estimated from the pH records [19].

## Results

A) *Effect of* $Mg^{2+}$ *on the efflux of some ions* ($Ca^{2+}$, $K^+$ *and* Pi) *from rat liver mitochondria.*

*Calcium.* In the presence of 400 nmoles diamide/mg mitochondrial protein, practically all $Ca^{2+}$ initially present in the incubation medium as contaminant was taken up rapidly, but unlike in untreated mitochondria, after about 8 minutes a progressively increasing $Ca^{2+}$ efflux ensued (Fig. 1) Addition of both $Mg^{2+}$ and Pi completely prevented $Ca^{2+}$ efflux, whereas neither of the two ions, when added separately, was effective. Pi requirement was also confirmed by the observation that mersalyl, an inhibitor of Pi transport, prevented the combined action of $Mg^{2+}$ plus Pi. No other tested anion (see acetate in Fig. 1) could replace phosphate.

*Potassium.* It was previously shown that both 2 mM Pi and 0.15 mM diamide induce a respiration dependent efflux of $K^+$ sensitive to $Ca^{2+}$ and Pi transport inhibitors [11]. As shown in Fig. 2, addition of $Mg^{2+}$ and, in the case of diamide, of $Mg^{2+}$ plus Pi fully prevented $K^+$ efflux. Again the presence of Pi in the incubation medium was a necessary condition for the observed effect.

*Phosphate.* Fig. 3 shows that diamide promoted a progressive efflux of mitochondrial Pi, which was prevented by $Mg^{2+}$ *also in the absence of added* Pi. It is quite peculiar that exogenous $Mg^{2+}$ prevents the efflux of cations only in the presence of added Pi, whereas it is capable of preventing Pi efflux in the absence of this anion. This would indicate that cations and

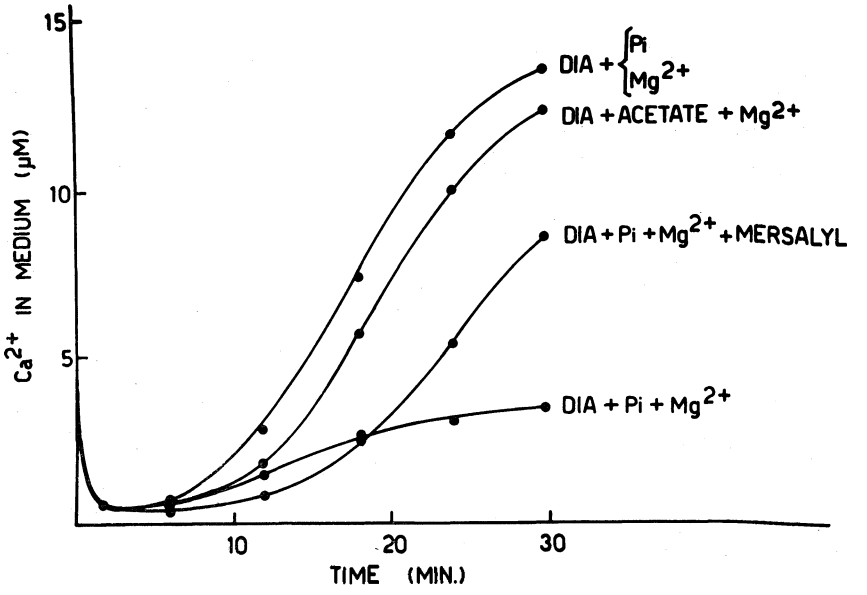


Fig. 1. – Effect of $Mg^{2+}$ on the diamide induced efflux of $Ca^{2+}$ from liver mitochondria. Rat liver mitochondria (RLM) were suspended (1 mg protein/ml) in a medium containing: 170 mM sucrose, 10 mM Tris-Cl ph 7.4, 5 mM Na-succinate, 1.25 μM rotenone, 40 μM diamide; temperature 25 °C. Final volume 20 ml. 2 mM $MgCl_2$, 2 mM Na-phosphate pH 7.4, 2 mM Na-acetate, 40 μM mersalyl (when present), $Ca^{2+}$ content at zero-time: 13 nmoles/mg protein.

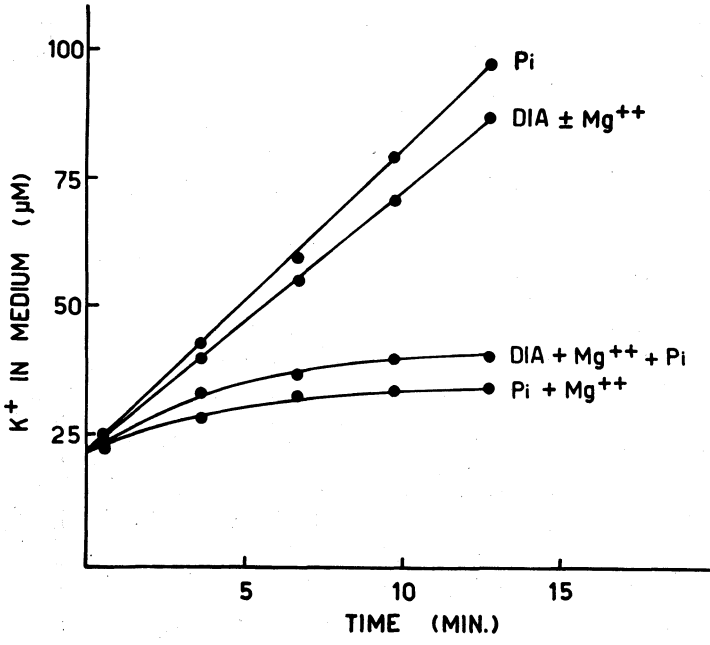


Fig. 2. – Effect of $Mg^{2+}$ on the diamide or phosphate induced efflux of $K^+$ from liver mitochondria.

Experimental conditions as in Fig. 1 except for 10 mM Tris-Cl pH 6.5 in the experiments done in the absence of diamide. 150 μM diamide, 2 mM Na-phosphate pH 6.5, 2 mM $MgCl_2$ (when present). $K^+$ content at zero-time: 125 nmoles/mg protein.

Pi effluxes are not necessarily correlated events. Fig. 3 also shows that NEM, an inhibitor of Pi mitochondrial transport, inhibited, though not completely, the efflux of Pi from mitochondria.

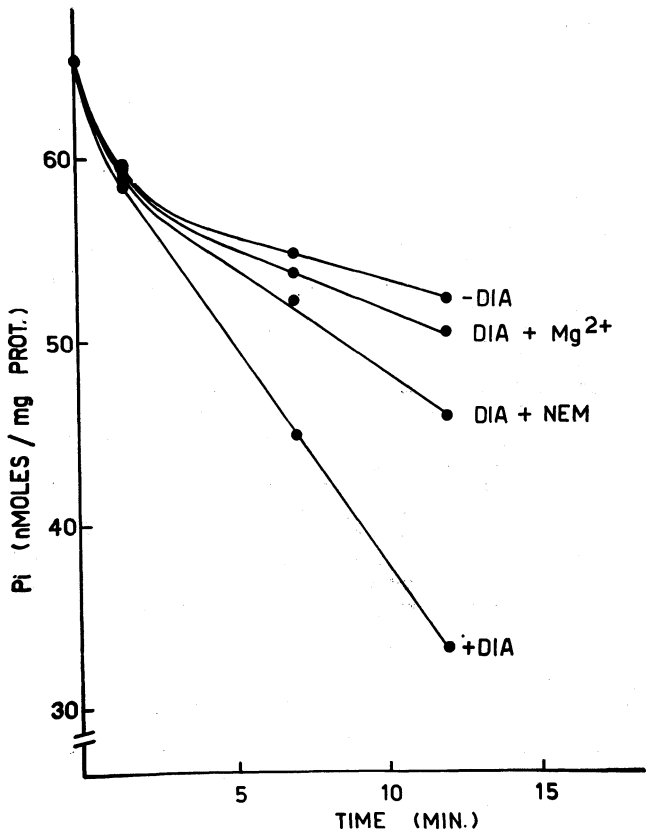


Fig. 3. – Effect of diamide on the efflux of inorganic phosphate from liver mitochondria.
Experimental conditions as in Fig. 1; final volume 30 ml. 310 μM diamide (DIA); 2 mM $MgCl_2$ and 30 μM NEM, (when present).

B) *Inhibition of the release of state 4 respiration.*

A common feature of ion efflux mediated by Pi or diamide is its complete prevention when calcium transport is inhibited by ruthenium red or $La^{3+}$ and when external $Ca^{2+}$ is sequestered outside the mitochondria by EGTA. The circumstance that inhibition of calcium flux reduces the rate of respiration in state 4 makes it reasonable to assume that a cyclic, energy dissipating flux of $Ca^{2+}$ is involved in the respiration dependent release of calcium ions from mitochondria [11]. Fig. 4 shows that also $Mg^{2+}$ was capable of dampening the accelerated rate of state 4 respiration, even in the absence of added Pi.

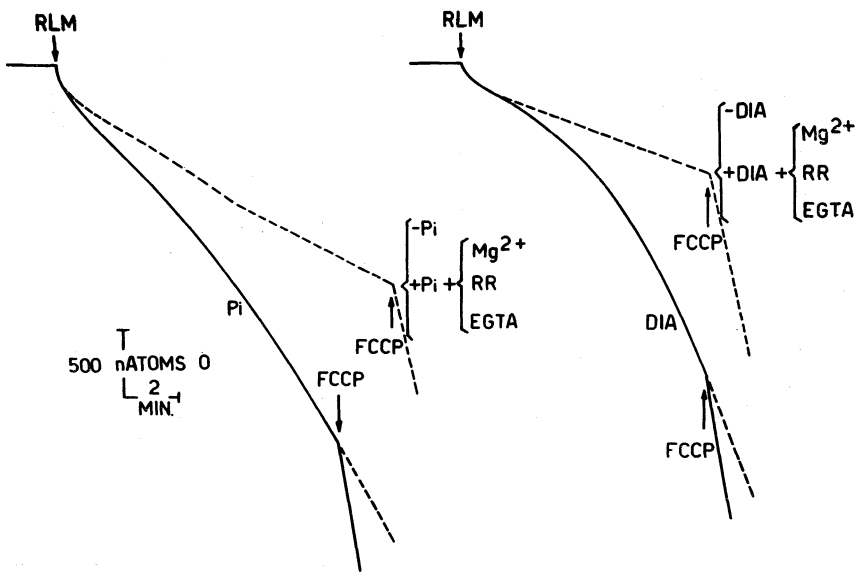


Fig. 4. – Inhibition by $Mg^{2+}$ of the release of state 4 respiration induced by diamide or phosphate.
Experimental conditions as in Fig. 1, except for 10 mM Tris-Cl pH 6.5 for the experiment in which state 4 respiration is released by Pi. 2 mM Na-phosphate (pH 6.5), 150 μM diamide, 2 mM $MgCl_2$, 5 μM ruthenium red (RR), 1 mM EGTA and 0.8 μM FCCP, (when present).

C) *Inhibition of the "active mitochondrial swelling induced by diamide or* $Ca^{2+}$.

Fig. 5 shows the close similarity between rat liver mitochondria swelling induced by diamide and $Ca^{2+}$. In both cases addition of 0.5–1 mM Pi was required to induce the swelling, which was prevented by uncouplers and by respiratory chain inhibitors. Addition of $Mg^{2+}$ completely prevented the swelling induced by diamide as well as by $Ca^{2+}$. In the presence of high concentrations of Pi (above 2 mM) or $Ca^{2+}$ (above 0.3 mM) the antagonist effect of $Mg^{2+}$ resulted much less evident.

D) *Effect on the inhibition of mersalyl on* Pi *transport across inner mitochondrial membrane.*

When Pi is generated within the matrix space from added ATP by the action of FCCP stimulated ATPase, addition of mersalyl, inhibiting Pi efflux, induces a progressively increasing mitochondrial swelling due to an accumulation of Pi inside the mitochondria. As Fig. 6 shows, addition of $Mg^{2+}$, restoring Pi efflux, abolished the mersalyl induced swelling. It would then appear that $Mg^{2+}$ prevents or antagonizes the mersalyl block on Pi efflux.

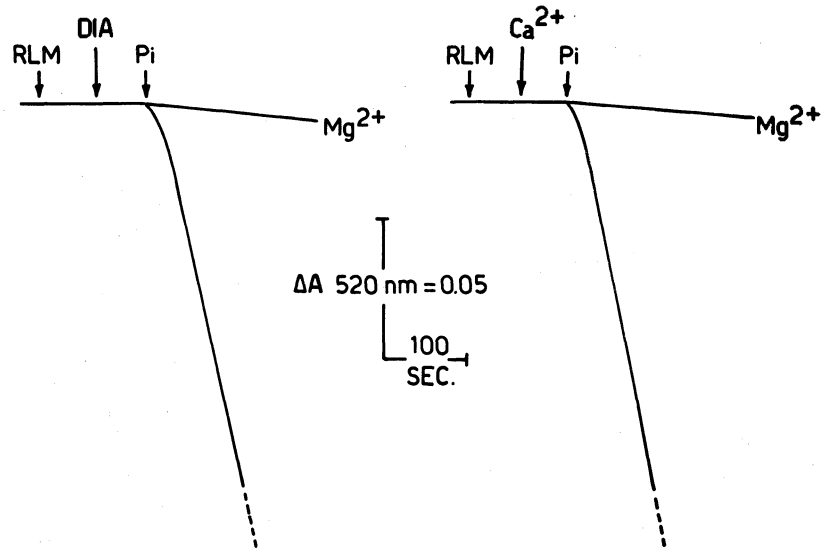


Fig. 5. – Inhibition by $Mg^{2+}$ of the active mitochondrial swelling induced by diamide or $Ca^{2+}$.

Rat liver mitochondria (RLM) were suspended in a medium (final volume 2 ml, temperature 25 °C) containing: 250 mM sucrose, 10 mM Tris-Cl pH 7.4, 5 mM K-succinate, 2.5 μM rotenone, 500 μM was added. 100 μM diamide, 100 μM $CaCl_2$, 500 μM $MgCl_2$, (when present).

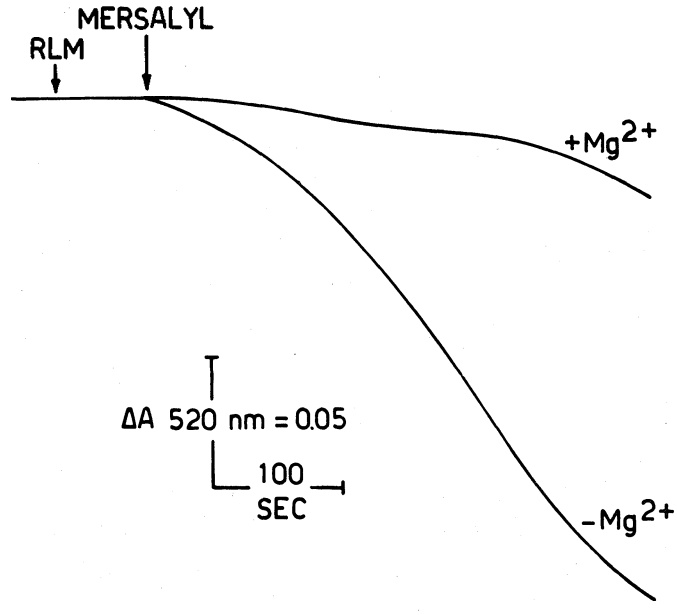


Fig. 6. – Reversal by $Mg^{2+}$ of the inhibition of mitochondrial Pi efflux induced by mersalyl.

Rat liver mitochondria (RLM) (2.5 mg protein/ml) were suspended in a medium containing 250 mM sucrose, 20 mM Tris-Cl pH 7.4, 1 mM ATP, 1 μM FCCP. 200 μM mersalyl was added. $Mg^{2+}$ was 4 mM, (when present).

E) *Inhibition of diamide stimulated* ATPase.

As shown in Fig. 7, diamide stimulated ATPase activity of intact liver mitochondria [12] was completely inhibited by 2 mM $Mg^{2+}$.

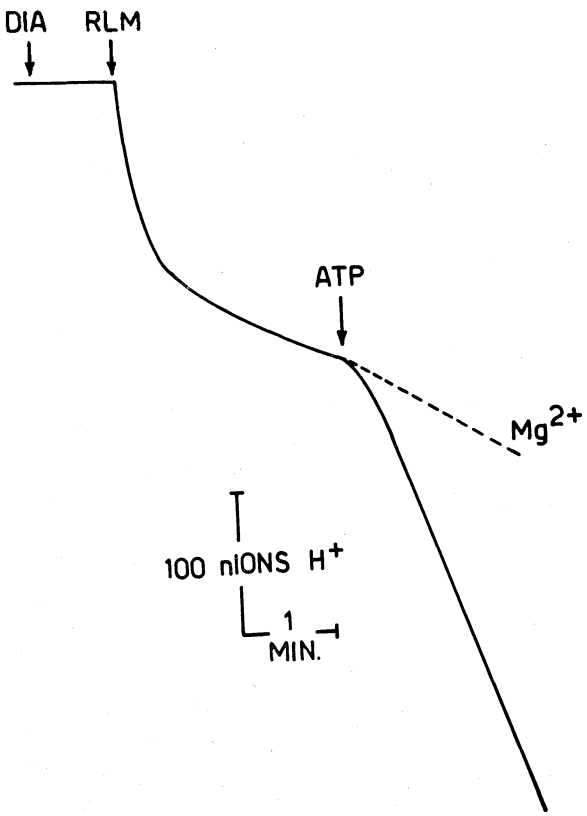


Fig. 7. – Inhibition by $Mg^{2+}$ of diamide stimulated mitochondrial ATPase. 5 mg of mitochondrial protein (RLM) were added to the assay medium (final volume 2 ml, temperature 20 °C) containing 80 mM KCl, 5 mM Tris-Cl pH 7.4, 400 μM diamide. 0.5 mM ATP was added. 2 mM $MgCl_2$, (when present).

## DISCUSSION

The reported results concordantly demonstrate that $Mg^{2+}$ acts on liver mitochondria protecting their native permeability properties from perturbations induced by different agents. The agents used in the present work are physiological components of the cell ($Ca^{2+}$ and Pi), or inducers of reversible modifications, which are not far from those possibly occurring as a consequence of variations in the redox state of inner membrane (diamide). Therefore the observed effects of $Mg^{2+}$ may be considered of physiological significance. In particular the inhibition by $Mg^{2+}$ of K efflux induced by added Pi (Fig. 2) makes it reasonable to assume that in the intact cell the

extra mitochondrial Mg/Pi ratio might control potassium transport across mitochondrial membrane. Recently Ligeti and Fonyo [10] have found that $Mg^{2+}$ inhibits valinomycin stimulated transport of $K^+$ across liver mitochondrial membrane. The Authors have interpreted this action as due to a decreased potassium carrier mobility as a consequence of membrane fluidity changes induced by $Mg^{2+}$. Such an interpretation can be maintained also for explaining the action of $Mg^{2+}$ on endogenous potassium permeability described in this paper.

The observation that $Mg^{2+}$ inhibits $K^+$ and $Ca^{2+}$ efflux induced by diamide only in the presence of external Pi (Figs. 1 and 2) while the release of mitochondrial Pi is inhibited by $Mg^{2+}$ alone (Fig. 3), raises a rather intriguing problem. A plausible interpretation may emerge from the following considerations. External Pi is actively taken up by liver mitochondria and accumulated in the matrix space whereas $Mg^{2+}$ does not seem to be accumulated by liver mitochondria [6]. However accumulation of Pi is facilitated by $Mg^{2+}$, which permits a better access of Pi to its carriers in the membrane [20]. Once accumulated Pi contributes with its negative charges in preventing the efflux of $K^+$ and other cations through diamide damaged inner membrane. On the other hand the binding of external $Mg^{2+}$ to the negatively charged phospholipids on the outer face of inner membrane [21] restricts the leakage of endogenous Pi, probably through modifications of membrane fluidity. In other words it is very likely that the prevention of diamide induced cation efflux requires both an increase of negative charges inside and a restoration of membrane tightness; Pi fulfills the former condition, $Mg^{2+}$ the latter. On the contrary, the prevention of Pi efflux only requires the maintenance of the native membrane permeability which is fulfilled by $Mg^{2+}$.

External $Mg^{2+}$ also prevents more severe alterations such a those induced by high diamide concentrations. Indeed the efflux of $Ca^{2+}$ induced by diamide reflects profound alterations in the inner membrane also involving a collapse of electrical transmembrane potential. Nevertheless 2 mM $Mg^{2+}$, a concentration not far from that existing in the liver cell [22], fully prevents such damage (Fig. 1); similarly, added $Mg^{2+}$ prevents the mitochondrial swelling promoted either by diamide or $Ca^{2+}$ (Fig. 5)

As regards $Mg^{2+}$ action in preventing or reversing diamide stimulated ATPase in intact mitochondria, it is likely that $Mg^{2+}$ could induce conformational changes in the inner membrane which render some critical thiol groups inaccessible to diamide action. It has been postulated that a number of vicinal thiol groups are responsible for the tightness of mitochondrial membrane and for the maintenance of a proper transmembrane potential gradient, responsible for the resting state of ATPase in intact mitochondria [23].

In relation to the probable mechanism of the protective action of $Mg^{2+}$ it is relevant to mention that mitochondrial $Mg^{2+}$ are partly sequestered within the matrix space and partly present in the intermembrane space [24, 25]. It is likely that "labilization" of mitochondria by diamide, Pi and $Ca^{2+}$ mainly involves $Mg^{2+}$ bound to phospholipids in the external pool [26],

which could be preserved, or reconstituted on addition of external $Mg^{2+}$. The findings of Scarpa and Azzi [27] that $Mg^{2+}$ and $Ca^{2+}$ compete for their binding sites within mitochondrial membrane are relevant to this point.

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