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# The Role of $Mg^{2+}$ in the Regulation of the Structural and Functional Steady-States in Rat Liver Mitochondria

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## Abstract

A possible relationship between mitochondrial $Mg^{2+}$ levels, structural configurations, and functional steady states has been studied in rat liver mitochondria. The results show that the concentration of mitochondrial $Mg^{2+}$ in respiratory state 4 is definitely higher than in respiratory state 3. The metabolic transition from state 3 to state 4 and vice-versa is associated with reversible influx–efflux of about 10 nmol of $Mg^{2+}$ per mg protein. The net uptake of this aliquot of $Mg^{2+}$ is a necessary condition in order for the metabolic transition to state 4, both structurally and functionally, to occur. This process requires a threshold concentration of external $Mg^{2+}$ greater than 5 mM. The phosphorylative mechanism does not appear to depend on the presence or absence of external $Mg^{2+}$. The role of $Mg^{2+}$ on the attainment and maintenance of the structural and functional steady state 4 seems to be correlated with its regulatory effect on the concentration of the mitochondrial $P_i$.

**Key Words:** $Mg^{2+}$ levels; respiratory states; phosphorylative efficiency; rat liver mitochondria.

## Introduction

Evidence has recently been presented that the metabolically linked configurational changes are primarily involved in the mechanism controlling the mitochondrial respiration (Guarriero-Bobyleva *et al.*, 1982), but do not interfere with the processes of energy conservation and transformation (Blair, 1977; Lang and Bronk, 1978; Webster and Bronk, 1978). As to the mechanism underlying these structural changes, it has been reported that they are related to ion movements (Munn, 1974; Fonyo *et al.*, 1977; Webster and

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Bronk, 1978). In particular, a regulatory role of $Mg^{2+}$ in these processes has been suggested (Brierley, 1976; Blair, 1977). As to this ion, it was previously suggested by Johnson and Pressman (1969) that the energy state of mitochondria might play an important role in the regulation of the $Mg^{2+}$ level within mitochondria. In fact, an aliquot of $Mg^{2+}$ is alternatively bound to, or released from, the membranes of ox heart submitochondrial particles, depending on the energetic state of the membrane (Shuster and Olson, 1973). Little experimental evidence, exist, however, as to this possible relationship between mitochondrial $Mg^{2+}$ levels and the structural-functional steady states in mitochondria. In order to get a better insight into this problem, it appears important to establish which are the levels of $Mg^{2+}$ present inside the mitochondria in different metabolic steady states and to assess whether the transition from one metabolic steady state to another is strictly associated with variations in endogenous $Mg^{2+}$ concentration. The data obtained indicate a regulatory role of $Mg^{2+}$ in controlling the structural and functional metabolic transitions. Preliminary reports of this work have been presented previously (Masini *et al.*, 1981a, 1981b, 1982).

## Materials and Methods

Rat liver mitochondria were prepared according to a standard procedure (Muscatello *et al.*, 1972a). The outer membrane was removed by a gentle physical procedure consisting essentially of inducing a large hypotonic swelling of mitochondria (Muscatello *et al.*, 1978). Aliquots of mitochondria, suspended in 0.25 M sucrose, were made to swell by lowering the osmolarity of the suspending medium down to 0.13 M at 4°C. The suspension was then made isotonic again by adding appropriate amounts of 1.0 M sucrose. Careful analysis by the electron microscope revealed that most of the mitochondria were depleted by the outer membrane, and the inner membrane–matrix complex did not show any appreciable damage. Furthermore, the inability of these preparations to oxidize externally added NADH indicated that the inner membrane was structurally and functionally unaltered (Lehninger, 1951).

The incubation medium for assaying the metabolic and structural parameters had the following composition: 100 mM NaCl; 10 mM $MgCl_2$; 10 mM Tris-HCl buffer, pH 7.4; 10 mM Na, K-phosphate buffer, pH 7.4; and 1.6 mM Na-pyruvate plus 0.4 mM Na-malate as the substrate. The metabolic parameters were assayed with a polarographic Clark-type oxygen electrode at 25°C in a final volume of 3.0 ml. The concentration of mitochondria was 3–4 mg protein per ml. The phosphorylative capacity was measured from the polarographic traces according to Chance and Williams (1956). The respiratory states studied were those defined by Chance and Williams (1956).

The structural parameters were estimated by means of the electron microscope and angular light-scattering techniques. For electron-microscopy examination, the sampling procedure and preparative method especially devised for correlating metabolic and structural states of mitochondria were used (Muscatello *et al.*, 1972a, 1972b). Light-scattering measurements were performed at 25°C with an angular light scattering C.N. Wood Monophotometer model 5000 at 546 nm in the same incubation medium used for the metabolic tests (Guarriero-Bobyleva *et al.*, 1982). The mitochondrial concentration ranged from $5 \times 10^{-3}$ to $5 \times 10^{-4}$ mg protein per ml.

The mitochondrial content of $Mg^{2+}$ was determined in rat liver mitochondria (3–4 mg protein per ml) in the same polarographic chamber used for the metabolic tests. At timed intervals, 0.2-ml samples of the mitochondrial suspension were collected and immediately centrifuged at 130,000 *g* for 15 sec in a Beckman Airfuge. The mitochondrial pellet was washed with cold saline medium without $Mg^{2+}$ and immediately dissolved in 1 ml of 4% (w/v) Na-cholate. The $Mg^{2+}$ content was then determined by means of a Perkin-Elmer atomic absorption spectrophotometer.

The mitochondrial $P_i$ content was determined as follows: rat liver mitochondria (3–4 mg protein per ml) were incubated in 3 ml of the standard medium, except that the $P_i$ was labeled with $^{32}P$. At timed intervals, 0.2 ml of mitochondrial suspension were withdrawn and rapidly vacuum filtered through Millipore filters of pore size 0.45 $\mu$m. The filters were rapidly washed with 0.150 M sucrose containing 50 mM Na,K-phosphate, pH 7.4, in order to remove the background binding of $^{32}P$ to the filters. The radioactivity of the filters was then determined in vials containing 10 ml Intagel (Packard) as scintillation liquid. The concentration of mitochondrial proteins was determined by the biuret method.

**Table I.** Mitochondrial content of $Mg^{2+}$ under Various Metabolic Steady-States[a]

| Metabolic steady state | Endogenous $Mg^{2+}$ (nmol/mg protein) |
|---|---|
| 0.25 M Sucrose | 21.2 ± 1.2 |
| State 3 | 20.8 ± 1.6 |
| State 4 | 29.9 ± 1.4 |

[a]The intramitochondrial $Mg^{2+}$ concentration was determined as described in the experimental section on samples incubated 1 min in the various metabolic steady states at 25°C. The effect of endogenous substrates on the $Mg^{2+}$ content of mitochondria in sucrose has not been analyzed. It should be noted, however, that the content of $Mg^{2+}$ in mitochondria incubated in 0.25 M sucrose at 0°C is slightly lower than at 25°C.

## Results

Table I shows the concentration of $Mg^{2+}$ in rat liver mitochondria under different conditions of incubation. It appears that the amount of $Mg^{2+}$ in freshly prepared mitochondria suspended in 0.25 M sucrose is 21.2 ± 1.2 nmol per mg protein, a value of the same order as that found by other authors (Judah *et al.*, 1965; Johnson and Pressman, 1969; Bogucka and Wojtczak, 1971; Kun, 1976; Siliprandi *et al.*, 1978; Hayashi and Tagashira, 1980). It also appears from the Table that the mitochondrial concentration of $Mg^{2+}$ is significantly higher in respiratory state 4 than in respiratory state 3, the difference being of the order of about 10 nmol of $Mg^{2+}$ per mg protein.

Figure 1 illustrates that rat liver mitochondria are able to maintain their steady-state concentration of $Mg^{2+}$, both in state 4 and in state 3, over a period of at least 10 min, provided that an adequate concentration of $Mg^{2+}$ is present in the incubation medium.

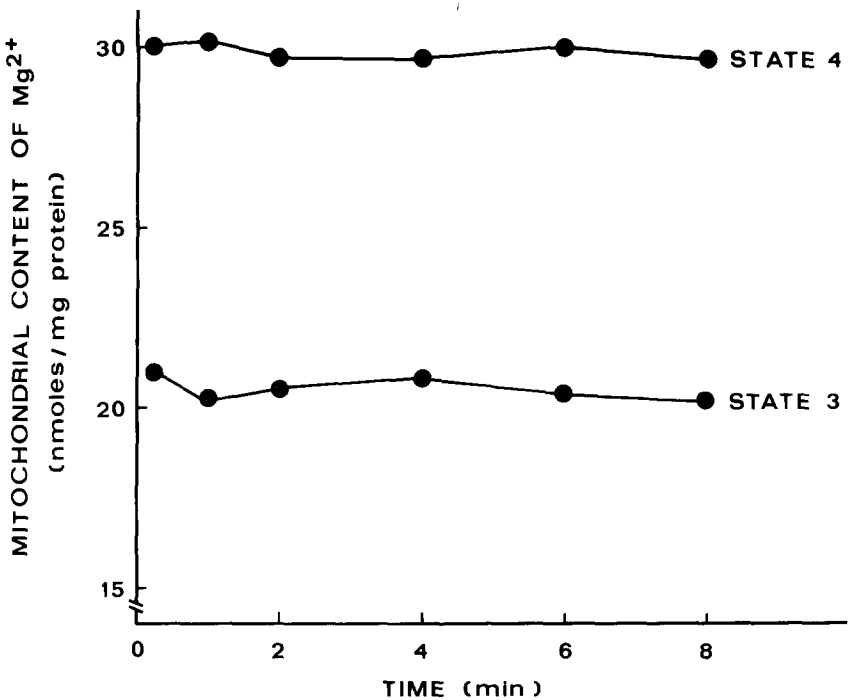


**Fig. 1.** Time course of mitochondrial content of $Mg^{2+}$ in respiratory states 4 and 3. Mitochondria (3.3 mg protein/ml) were incubated at 25°C in states 4 and in 3 as described in the Methods. The mitochondrial content of $Mg^{2+}$ was determined as reported in the experimental section. All the sampling procedures were performed in the same polarographic chamber used to test the respiratory states. Under the conditions used, the mitochondria maintained a linear respiration in state 4 for more than 8 min.

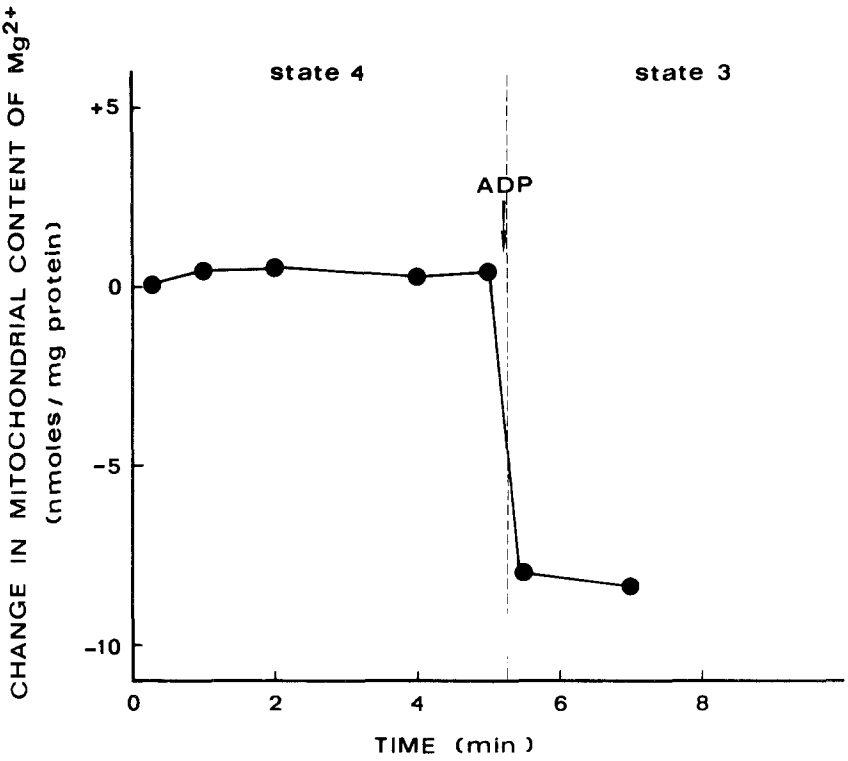


**Fig. 2.** Change in mitochondrial content of $Mg^{2+}$ associated with the metabolic transition from state 4 to state 3. Mitochondria (3.2 mg protein/ml) were incubated under state 4 conditions. The transition to state 3, as indicated by the dotted line, was obtained by the addition of 0.33 mM ADP. All other conditions as in Fig. 1.

Figure 2 demonstrates that the transition from state 4 to state 3 is associated with a rapid and parallel efflux of about 8–10 nmol of $Mg^{2+}$ per mg protein.

The reverse transition from state 3 to state 4 is associated with a parallel influx of the same amount of $Mg^{2+}$. The cycle of efflux–influx may be repeated several times by inducing cyclical transitions from state 4 to state 3 and vice versa. This is illustrated in Fig. 3.

Table II shows that the steady-state concentration of $Mg^{2+}$ characteristic of state 4 is reached only when the supplied energy can be utilized. In fact, in the presence of either KCN or the uncoupler DNP (2,4-dinitrophenol), the mitochondrial concentration of $Mg^{2+}$ equals that observed when mitochondria are suspended in 0.25 M sucrose, i.e., in the absence of energy supply and $P_i$. It appears from Table II that the $Mg^{2+}$ steady-state concentration of state 3 does not seem to require an energy supply.

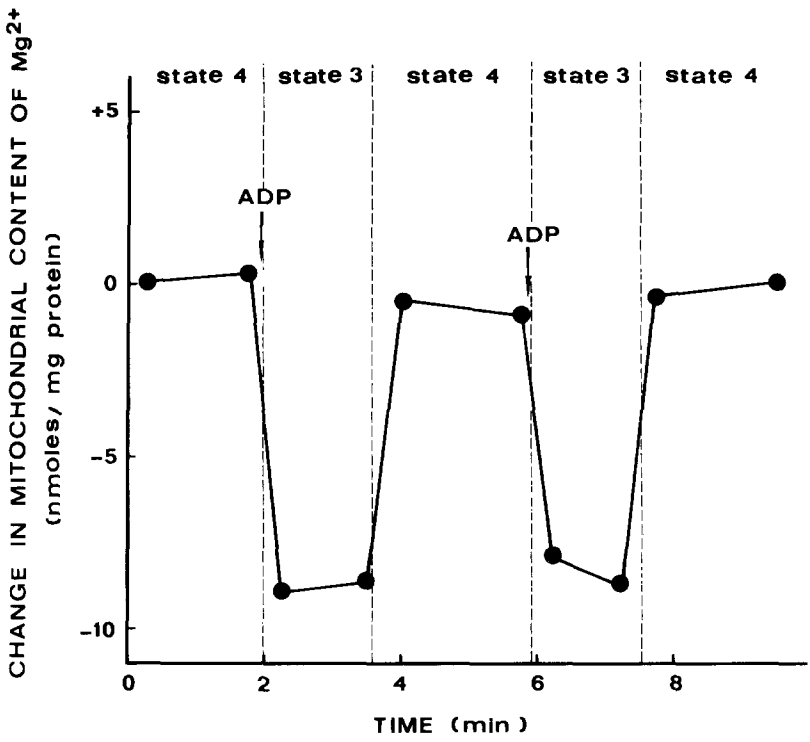


**Fig. 3.** Constant oscillation in mitochondrial content of $Mg^{2+}$ concomitant with a cycle of metabolic transitions. Mitochondria (3.6 mg protein/ml) were incubated under state 4 conditions. The cycle of metabolic transitions from state 4 to state 3 and vice versa was obtained by repeated additions of 0.33 mM ADP. All other conditions as in Fig. 1.

**Table II.** Mitochondrial Content of $Mg^{2+}$ under Various Conditions of Energy Supply[a]

| Metabolic steady state | Endogenous $Mg^{2+}$ (nmol/mg protein) |
|---|---|
| State 4 | 30.6 ± 2.1 |
| Plus 1.33 mM KCN | 20.8 ± 1.6 |
| Plus 25 μM DNP | 21.4 ± 1.8 |
| State 3 | 21.3 ± 1.2 |

[a]The concentration of endogenous $Mg^{2+}$ in respiratory state 4 was determined after 1 min of incubation. The concentration of $Mg^{2+}$ in state 3 was determined 1 min after the addition of 0.33 mM ADP. When 25 μM DNP or 1.33 mM KCN were present, the concentration of endogenous $Mg^{2+}$ was determined 1 min after the addition, respectively, of the uncoupler and the inhibitor. The results are the mean of five separate experiments ± S.D.

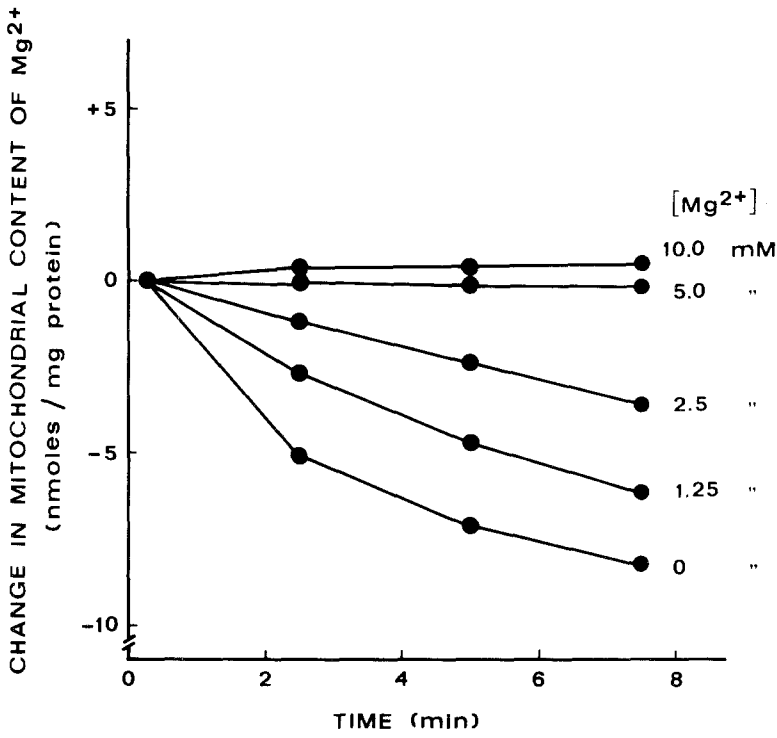


**Fig. 4.** Effect of varied concentrations of external $Mg^{2+}$ on the mitochondrial content of $Mg^{2+}$ in respiratory state 4. Mitochondria (3.3 mg protein/ml) were incubated in state 4 in the presence of various concentrations of external $Mg^{2+}$ as indicated in the figure. All other conditions as in Fig. 1.

Figure 4 illustrates that the steady-state concentration of $Mg^{2+}$ of state 4 requires not only the availability of energy, but also a suitable concentration of $Mg^{2+}$ in the incubation medium. In fact, rat liver mitochondria are able to maintain their steady-state concentration of $Mg^{2+}$ under the condition of state 4 only when the external concentration of $Mg^{2+}$ is greater than 5 mM. At lower concentrations there is progressive loss of $Mg^{2+}$ from the mitochondria, the extent of which is inversely related to the external concentration of $Mg^{2+}$. In the absence of $Mg^{2+}$ in the incubation medium, there is a net efflux of about 10 nmol of $Mg^{2+}$ per mg protein from mitochondria during the incubation in state 4. As a consequence, in the absence of external $Mg^{2+}$ the mitochondrial concentration of $Mg^{2+}$ in state 4 equals that observed in state 3.

Figure 5 shows that upon addition of external $Mg^{2+}$ to the incubation medium, the $Mg^{2+}$ steady-state concentration characteristic of state 4 is restored.

Figure 6 shows that, in contrast to state 4, the mitochondrial concentra-

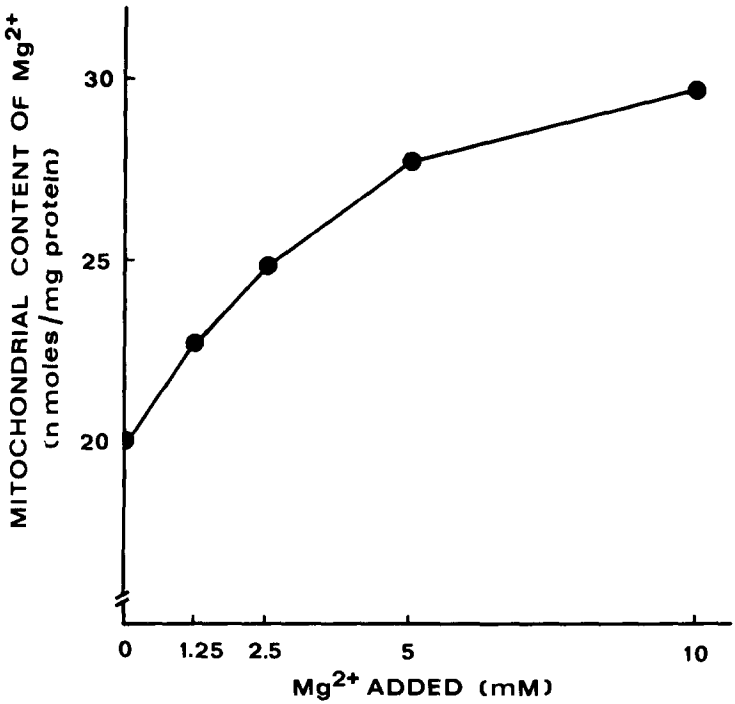


**Fig. 5.** Restoration of mitochondrial content of $Mg^{2+}$, characteristic of state 4, by the addition of external $Mg^{2+}$. Mitochondria (3.2 mg protein/ml) were incubated in state 4 for 6 min in the absence of external $Mg^{2+}$. Varied concentrations of exogenous $Mg^{2+}$ were then added and the mitochondrial content of $Mg^{2+}$ was determined after 1 additional minute. All other conditions as in Fig. 1.

tion of $Mg^{2+}$ in state 3 is not dependent on the external concentration of $Mg^{2+}$ over a period of incubation of at least 10 min.

It appears from Fig. 7 that the influx of 10 nmol of $Mg^{2+}$ per mg protein is an essential requirement for mitochondria to modify their metabolic steady state from state 3 to state 4. In fact, under incubation conditions that reduce or abolish the influx of 10 nmol of $Mg^{2+}$ (i.e., in the absence of external $Mg^{2+}$), during the transition from state 3 to state 4 the respiratory rate remains high and consequently the R.C.I. (the respiratory control index) appears to be reduced.

Subsequent additions of $Mg^{2+}$ to the incubation medium cause the respiration to be controlled again, and the R.C.I. is restored to normal values. This is illustrated in Fig. 8. Similar results are obtained when 10 mM $P_i$ is replaced by 1 mM $P_i$ in the metabolic medium (results not shown).

Table III shows that, if the influx of $Mg^{2+}$ during transition from state 3 to state 4 is reduced or even abolished, the structural modification from

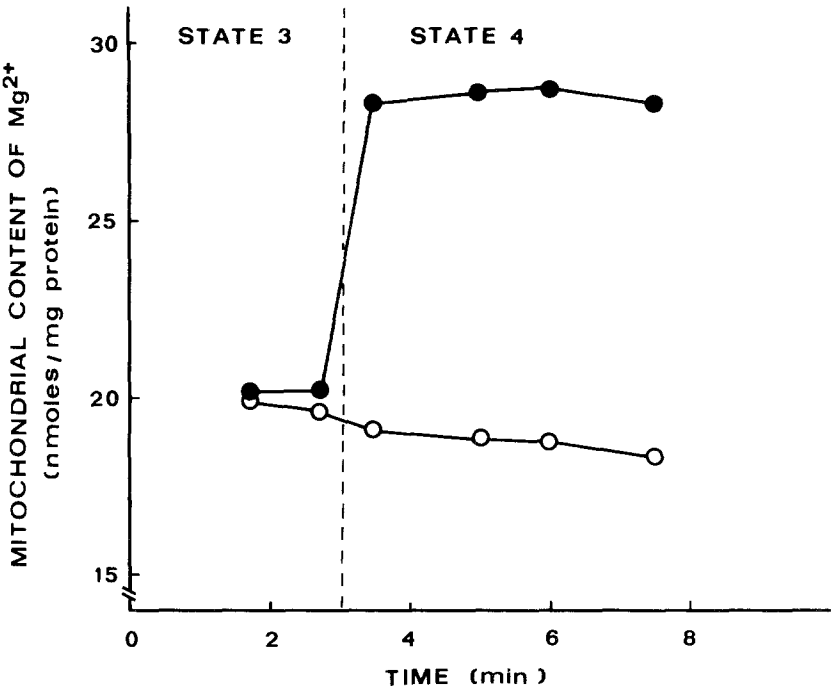


**Fig. 6.** Effect of external $Mg^{2+}$ on the mitochondrial content of $Mg^{2+}$ in respiratory states 3 and 4. Mitochondria (3.1 mg protein/ml) were incubated under state 3 conditions in the presence of 10 mM $Mg^{2+}$ (●) and in the absence of $Mg^{2+}$ (○). All other conditions as in Fig. 1.

condensed to expanded configuration that characteristically parallels this metabolic transition does not occur. The mitochondrial configuration remains condensed as in state 3. It appears from Table III that the phosphorylative efficiency remains unaltered, in contrast to the respiratory control index which progressively tends to unity.

The effects, both structural and functional, observed during transition from state 3 to state 4 in the absence of $Mg^{2+}$ are not due to parallel movements of endogenous $Ca^{2+}$ consequent to the removal of $Mg^{2+}$ from the medium. In fact, similar results are obtained either by removing $Ca^{2+}$ residues by 0.1 mM EGTA, a chelating substance with a higher affinity constant for $Ca^{2+}$, or by addition to the incubation medium of 1 $\mu$M Ruthenium Red, a specific inhibitor of $Ca^{2+}$ transport (results not shown).

It appears from Table IV that the effect of the absence of external $Mg^{2+}$ from the metabolic medium in the respiratory state 4 on the inner membrane–matrix complex is of order comparable to that observed in the whole mitochondria, both at the functional and the structural level. It must be noted, however, that the rate at which the configurational and functional

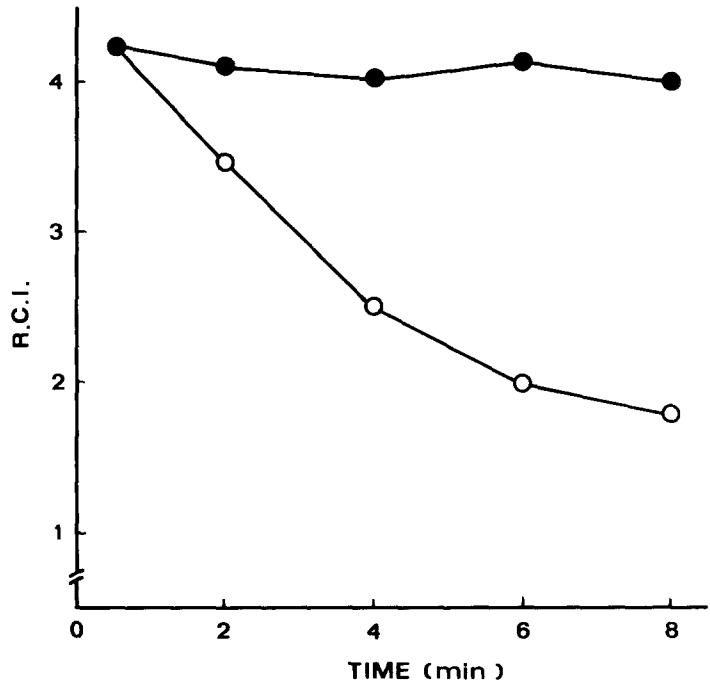


**Fig. 7.** Effect of varied concentrations of external $Mg^{2+}$ on the respiratory control index. Mitochondria (3.4 mg protein/ml) were incubated for different intervals of time in state 4 in the presence of 10 mM $Mg^{2+}$ (●) and in the absence of external $Mg^{2+}$ (○). 0.33 mM ADP was then added to obtain state 3 conditions. Results of a typical experiment.

modifications occur is quite different under the same conditions. It can be seen, in fact, that rat liver mitoplasts respiring in state 4 without added $Mg^{2+}$ undergo a volumetric reduction of about 30% in 2 min, while a change of the same extent requires 6 min in whole mitochondria. The changes in the respiratory rates show a similar behavior. The phosphorylative mechanism of the inner membrane–matrix complex does not appear to be modified, as in whole mitochondria.

The suggestion that $Mg^{2+}$ is an essential requirement, in addition to oxygen and a suitable substrate, for $P_i$ uptake by mitochondria (Brierley *et al.*, 1962; Abou-Khalil and Hanson, 1977), that is, under respiratory state 4 conditions (as defined by Chance and Williams, 1956), has been confirmed and extended by the experiments presented in Fig. 9. These results in fact show that an appropriate concentration of external $Mg^{2+}$ is required to maintain a steady state in the mitochondrial $P_i$ concentration during state 4 incubation. The value of mitochondrial $P_i$ content measured under these conditions is in accordance with that found for rat liver mitochondria (Reed

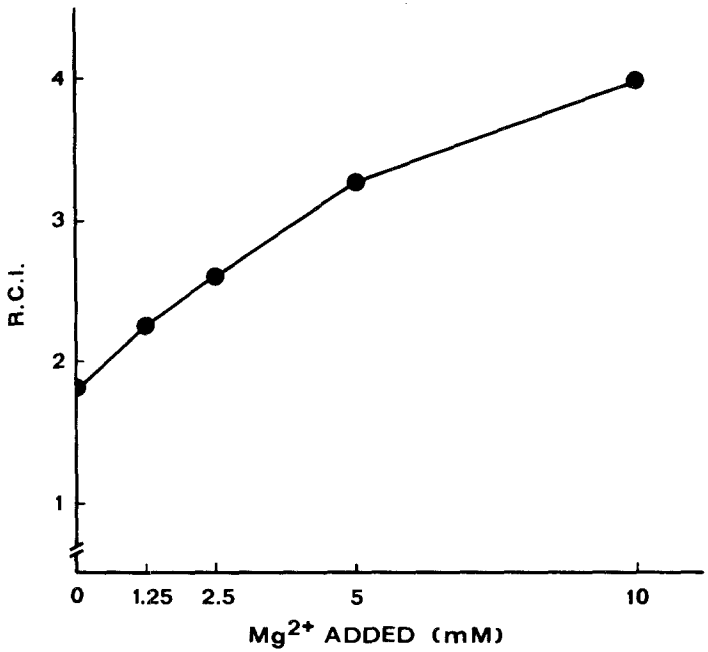


**Fig. 8.** Restoration of the normal respiratory control index by the addition of external $Mg^{2+}$. Mitochondria (3.5 mg protein/ml) were incubated for 6 min in state 4 in the absence of external $Mg^{2+}$. Exogenous $Mg^{2+}$ at increasing concentrations was then added. 0.33 mM ADP was added after 1 additional minute. The respiratory control index is the ratio of the respiratory rate in state 3 to the respiratory rate in state 4, as measured after the addition of $Mg^{2+}$. Results of a typical experiment.

and Lardy, 1972; Azzone *et al.*, 1976; Fonyo *et al.*, 1977; Fiskum and Lehninger, 1979; Roos *et al.*, 1980; Zoccarato and Nicholls, 1981). However, if $Mg^{2+}$ is absent from the metabolic medium, a net efflux of about 20–22 nmol of $P_i$ per mg protein takes place in the first 4 min of incubation. A dampening of the same extent in the endogenous $P_i$ level of rat liver mitochondria respiring in state 4, in the absence of added $Mg^{2+}$, has been shown by Roos *et al.* (1980). Figure 9 also shows that the addition of 10 mM $Mg^{2+}$ rapidly restores the level of $P_i$ characteristic of state 4.

## Discussion

The present data show that isolated rat liver mitochondria, under definite metabolic conditions (i.e., in the presence of energy supply, $P_i$, and $Mg^{2+}$), are able to maintain a constant concentration of $Mg^{2+}$, whose value is signifi-

**Table III.** Effect of Different External $Mg^{2+}$ Concentrations on the Structural and Functional Steady States in Rat Liver Mitochondria[a]

| | Structural parameters | | | Functional parameters | | | |
|---|---|---|---|---|---|---|---|
| | Specific turbidity $\tau/c$ ($cm^2$/mg protein) | | | Respiration rate $QO_2$($\mu$l $O_2$/mg protein/h) | | | |
| External $Mg^{2+}$ | State 3 | State 4 | $(\tau/c)_4/(\tau/c)_3$ | State 3 | State 4 | R.C.I. | ADP/O |
| 10 mM | 0.610 ± 0.022 | 0.891 ± 0.032 | 1.46 | 28.86 ± 1.12 | 6.81 ± 0.38 | 4.24 | 2.63 |
| 2.5 mM | 0.611 ± 0.021 | 0.770 ± 0.028 | 1.26 | 27.82 ± 1.13 | 8.40 ± 0.39 | 3.31 | 2.55 |
| 0 mM | 0.614 ± 0.023 | 0.626 ± 0.023 | 1.02 | 26.77 ± 1.11 | 11.54 ± 0.28 | 2.32 | 2.49 |

[a]Mitochondria were incubated for 6 min in state 4, then 0.33 mM ADP was added. The respiratory control index R.C.I. is the ratio of the respiratory rate in state 3 to the respiratory rate in state 4. The variations in specific turbidity were measured at 546 nm. $(\tau/c)_4/(\tau/c)_3$ is the ratio of the specific turbidity observed in state 4 to that observed in state 3. All values are given as mean ± S.E.M. The differences between the mean values were found to be statistically significant at $P < 0.01$. The differences betweeen the mean values of specific turbidity in state 3 and those of state 4, at an external $Mg^{2+}$ concentration of 0 mM, were not statistically significant. Results of seven separate experiments.

**Table IV.** Effect of the Presence and the Absence of External $Mg^{2+}$ on the Structural and Functional Steady States in Rat Liver Mitoplasts[a]

| | Structural parameters | | | Functional parameters | | | |
|---|---|---|---|---|---|---|---|
| | Specific turbidity $\tau/c$ ($cm^2$/mg protein) | | | Respiration rate $QO_2$ ($\mu$l $O_2$/mg protein/h) | | | |
| External $Mg^{2+}$ | State 3 | State 4 | $(\tau/c)_4/(\tau/c)_3$ | State 3 | State 4 | R.C.I. | ADP/O |
| 10 mM | 0.458 ± 0.028 | 0.652 ± 0.038 | 1.42 | 25.05 ± 1.22 | 7.66 ± 0.44 | 3.27 | 2.34 |
| 0 mM | 0.464 ± 0.031 | 0.474 ± 0.033 | 1.02 | 21.71 ± 0.88 | 13.40 ± 0.68 | 1.62 | 2.22 |

[a]Mitoplasts, prepared by a gentle osmotic procedure, were incubated for 2 min in state 4, then 0.33 mM ADP was added. All values are given as mean ± S.E.M. Results of five separate experiments. All other conditions as in Table III.

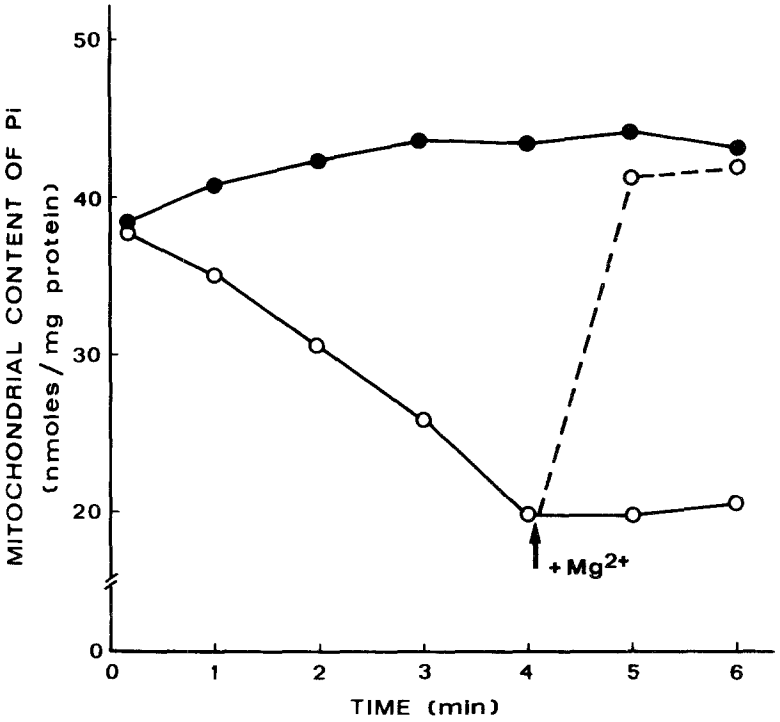


**Fig. 9.** Effect of the external $Mg^{2+}$ on the mitochondrial content of $P_i$ in respiratoty state 4. Mitochondria (3.8 mg protein/ml) were incubated in state 4 in the presence of 10 mM $Mg^{2+}$ (●) and in the absence of $Mg^{2+}$ (○). When 10 mM $Mg^{2+}$ was added, at the point indicated by the arrow, the dotted line shows the subsequent changes in mitochondrial content of $P_i$ where it differs from control incubation in the absence of $Mg^{2+}$. The mitochondrial $P_i$ content was determined as described in the experimental section. Results of a typical experiment.

cantly different in states 4 and 3. The metabolic transition from state 4 to state 3 and vice versa is strictly associated with constant oscillation of $Mg^{2+}$ in the inner membrane–matrix complex. The steady-state concentration of $Mg^{2+}$ characteristic of state 3 represents an amount of $Mg^{2+}$ not easily exchangeable with the external medium. In fact, it is not dependent on the presence of $Mg^{2+}$ in the incubation medium, at least for the incubation time tested, and, further, it equals, in absolute value, that observed when the energy is dissipated or its utilization is made impossible. This leads to the conclusion that a large part of $Mg^{2+}$ in state 3 must be bound, since its concentration is of the same order as that found in mitochondria incubated in 0.25 M sucrose. On the contrary, the steady-state concentration of $Mg^{2+}$ characteristic of state 4 is strictly dependent on the presence of energy supply, inorganic phosphate, and an adequate concentration of $Mg^{2+}$ in the metabolic medium. In fact,

when $Mg^{2+}$ is absent from the incubation medium, a net efflux of $Mg^{2+}$ during the time course of state 4 occurs, and the endogenous $Mg^{2+}$ level rapidly reaches that characteristic of state 3. The results reported here and the above considerations confirm that rat liver mitochondria are able to take up or release $Mg^{2+}$ by a respiration-dependent process (Judah *et al.*, 1965; Johnson and Pressman, 1969; Diwan *et al.*, 1979), although at a definitely lower extent with respect to heart mitochondria (Crompton *et al.*, 1976). They also agree with the finding that, at a definite concentration of external $Mg^{2+}$, the influx and efflux processes tend to be similar, so that no net movement of $Mg^{2+}$ occur (Crompton *et al.*, 1976; Diwan *et al.*, 1979). Further they do not contradict the finding that the addition of ADP to heart mitochondria respiring in state 4 strongly inhibits only the process of $Mg^{2+}$ influx (Crompton *et al.*, 1976) and that varied concentrations of external $Mg^{2+}$ affect only the rate of $Mg^{2+}$ influx in rat liver mitochondria during state 4 (Diwan *et al.*, 1979). The present data are also in accordance with the finding that when the respiration is inhibited or uncoupled, no net movement of $Mg^{2+}$ is observable (Johnson and Pressman, 1969; Crompton *et al.*, 1976; Siliprandi *et al.*, 1978; Diwan *et al.*, 1979). Finally they provide experimental support to the suggestion that the intramitochondrial levels of $Mg^{2+}$ are somewhat related to the energy state (Johnson and Pressman, 1969).

Since the transition from state 3 to state 4 is paralleled by a net uptake of about 10 nmol of $Mg^{2+}$ per mg protein, it is reasonable to assume that this uptake is mediated by the inner membrane (Shuster and Olson, 1974) and is dependent on the concentration of available $Mg^{2+}$ in the intermembrane space. The concentration in this space, in fact, equals that in the incubation medium, as the outer membrane is freely permeable. The results presented on the inner membrane–matrix complex support this explanation. From a physiological point of view and consistent with the above, it is important to note that the high concentration of $Mg^{2+}$ binding sites in the liver, providing a large reservoir from which $Mg^{2+}$ may be released or to which it may be bound, ensures a constant free intracellular concentration of $Mg^{2+}$ ranging between 1–5 mM (Veloso *et al.*, 1973; Ligeti and Horvath, 1980).

A similar value of $Mg^{2+}$ concentration in the intermembrane space can also result from a calculation based on the total $Mg^{2+}$ found in this space (Bogucka and Wojtczak, 1971; Heaton and George, 1979) and the volume of the same space (Muscatello *et al.*, 1972a, 1972b). These observations are relevant to the role that $Mg^{2+}$ plays in the maintenance of the integrity of the membrane structure (Binet and Volfin, 1975; Coelho and Vercesi, 1980; Ligeti and Horvath, 1980). The fact that $Mg^{2+}$ uptake into the inner space is conditioned by the availability of a threshold concentration of $Mg^{2+}$ in the incubation medium (i.e., greater than 5 mM) provides a tool for the comprehension of the functional significance and the metabolic implications

of the uptake itself in the transition from state 3 to state 4. On the basis of the present data it can be concluded that this uptake is necessary in order for the transition to state 4 to occur. In fact, in the absence of external $Mg^{2+}$, both the structural and the respiratory modifications that characterize this metabolic transition are largely prevented. On the other hand, the addition of external $Mg^{2+}$ and its consequent uptake completely restores the normal situation. Morphological and functional modifications in rat liver mitochondria associated with a limited decrease in the endogenous $Mg^{2+}$ content have been reported (Binet and Volfin, 1975; Hoser *et al.*, 1976; Blair, 1977). Further, Wherle et al. (1976) showed that heart mitochondria severely depleted of $Mg^{2+}$ by means of the ionophore A23187 restores the respiratory control index and phosphorylative efficiency upon the addition of 2 mM $Mg^{2+}$.

The structural and respiratory modifications observed in the absence of $Mg^{2+}$ cannot be related to concomitant variations in endogenous $Ca^{2+}$ transport. The possibility that, in the absence of external $Mg^{2+}$, the endogenous $Ca^{2+}$ cycling may be enhanced (Siliprandi *et al.*, 1978), with the consequent increase in the $O_2$ uptake and structural contraction, is not supported by the present data. In fact, the use of EGTA or suitable concentrations of Ruthenium Red that do not inhibit *per se* the oxygen uptake (Rigoni *et al.*, 1980) does not appreciably modify the effect of the absence of $Mg^{2+}$ on the respiratory velocity and structural modification in the transition from state 3 to state 4. In this regard, several lines of evidence indicate that movements of $Mg^{2+}$ and $Ca^{2+}$ in mitochondria may occur by independent processes (Binet and Volfin, 1975; Crompton *et al.*, 1976; Wherle *et al.*, 1976; Blair, 1977; Diwan *et al.*, 1979; Åkerman, 1981).

The results of the present study lead to the conclusion that the uptake of a given amount of $Mg^{2+}$ is an essential requirement for the transition from state 3 to state 4 to occur. The control by $Mg^{2+}$ of the attainment and maintenance of the metabolic steady state 4, both structurally and functionally, seems to be correlated with the effect of this ion on the concentration of endogenous $P_i$. This conclusion is further supported by some considerations on the isoosmolality maintenance of the inner mitochondrial compartment (Rottemberg and Solomon, 1969). The variations in the content of the mitochondrial $Mg^{2+}$ are not osmotically significant *per se* to account for the change in both the volume and water content of the inner space. In fact, if we take a value for the water content of the condensed configuration of 0.7 $\mu$l water per mg protein (Harris and Van Dam, 1968; Heaton and Nicholls, 1976) and we consider that a 42% increase in matrix volume is associated with the transition to expanded configuration, the matrix water content would then increase to 1 $\mu$l water per mg protein. In this case, the isoosmolality maintenance of the inner space would require the net uptake of about 80 ng ions per mg protein, a value comparable with the amount of $Mg^{2+}$ and counteranions plus phosphate and

countercations that we have found to be associated with this transition. Consistent with this, Fonyo *et al.* (1977) and Stoner and Sirak (1978) observed that the shrinkage of mitochondria respiring in state 4 after ADP addition is connected with a substantial depletion of internal $P_i$. The possibility that variations in the $P_i$ transport may primarily influence $Mg^{2+}$ movements has recently been excluded by Diwan *et al.* (1980).

The present results provide further evidence for the conclusion that the metabolically linked configurational changes, which are dependent on appropriate ion movements, are closely related to the mechanisms leading to the control of respiration, but they are not involved in the energy transformation and conservation events (Guarriero-Bobyleva *et al.*, 1982).

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