A CYTOPLASMIC MOLECULE ACTIVE ON MEMBRANAR Mg^{2+} MOVEMENTS. II – ITS ROLE AS A FACTOR OF THE MEMBRANE INTEGRITY IN MITOCHONDRIA, CHLOROPLASTS AND BACTERIA*

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1. Introduction

It has been reported by Loh et al. in 1968 [1] that a small molecular factor (CMF), isolated from cytosol of animal tissues, activated glutamate mitochondrial metabolism under specific conditions (preincubation with ADP and high concentrations of dinitrophenol in a KCl-Tris medium). At a critical concentration of DNP, metabolic rates reached a maximum, but at higher concentration of DNP, inhibition of metabolism was observed. This inhibitory effect has been explained by Hemker [2] who assumed that the uncoupler formed a complex with one of the factors involved in oxidative phosphorylation, preventing thereby optimal operation of the phosphorylation pathway.

For Van Dam [3] and Kraayenhof and Van Dam [4], the inhibition occurs at a very early stage of the interaction between the substrate and the mitochondrion. Kun et al. [5] explained this inhibition by a selective labilization of the mitochondrial Mg^{2+}. Lee et al. [6] postulated that this Mg^{2+} would be necessary in the reactions leading from α-ketoglutarate to malate and that, bound to mitochondrial membrane, would reveal itself as an obligatory component of the mitochondrial energy transducing apparatus. CMF wholly prevents the Mg^{2+} ejection provoked by DNP.

As evidence of the role of CMF had been so far obtained only under pharmacological conditions (presence of an uncoupler), this work was undertaken to investigate the possible physiological role of CMF and its significance. CMF suppresses the spontaneous ejection of Mg^{2+} which occurs in mitochondria and chloroplasts during aging. It prevents also the lethal effect provoked by a drug levallorphan on bacterial growth. A general role of CMF as a control factor of Mg^{2+} movements and its implication in the maintenance process of the biological membrane integrity are postulated.

Abbreviations:
CMF : cytoplasmic metabolic factor
DNP : 2,4-dinitrophenol
TMPD: *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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2. Experimental procedures

CMF was prepared from pig liver according to Binet et al. [7]. CMF was assayed at the approximative concentration of 10^{-9} M. Rat liver mitochondria were obtained from Long Evans rats and prepared according to Kun et al. [5]. For the estimation of the membrane Mg^{2+} ejection, aerobic incubations were performed in a Dubnoff Shaker at 30° and stopped at various times by centrifugation at 18,000 *g* for 2 min in an Eppendorf microcentrifuge. Mg^{2+} concentration in the supernatant was determined by atomic absorption (Perkin Elmer 290). Pig Heart sarcosomes were prepared according to the technique of Leblanc et al. [8]. Radioactivity determinations were performed with a Nuclear Chicago gas flow de-

tector. Class I chloroplasts were isolated from *Zea mays* leaves in accordance with the method of Johnson and Bruff [9]. *E. coli* CR 341 was grown in the medium described by Boquet et al. [10]. Growth rate was followed by measuring the absorbance at 546 nm in an Eppendorf photometer. For survival experiments, exponentially-growing bacteria were harvested when their concentration reached approximately 5×10^8/ml. They were diluted a thousand times in a saline medium in the presence or in the absence of Levallorphan and CMF. The mixture was incubated at 37°. Samples were withdrawn, diluted at room temperature, and the number of viable cells were determined according to the time by plating on complete agar medium.

3. Results

3.1. *Interaction between CMF and Mg^{2+} movements in various cellular organelles*

Fig. 1 shows the effects of DNP on the Mg^{2+} ejection in liver mitochondria. For all DNP concentrations used (0.2 to 1.0^{-4} M), the totality of the mitochondrial Mg^{2+} (around 30 nmole Mg^{2+}/mg protein) is recovered within 10 min in the incubation medium. CMF suppresses completely this Mg^{2+} ejection. These results are in good agreement with the assertions of Kun et al. [5]. We observed that it is possible to follow a Mg^{2+} ejection, in the absence of an uncoupler, when liver mitochondria are incubated in KCl-Tris medium and that CMF prevents this Mg^{2+} efflux. Fig. 2 indicates that 60% of the mitochondrial Mg^{2+} is already ejected when liver mitochondria are incubated for 60 min in KCl-Tris and that CMF stops this spontaneous ejection. Adding 10 mM glutamate to the incubation medium, we only obtain an ejection of 30% of the mitochondrial Mg^{2+}. When pig heart sacrosomes are incubated in the presence of ADP, an important energy dependent Ca^{2+} uptake may be observed. This uptake decreases strikingly in aged sarcosomes. Fig. 3 indicates the Ca^{2+} uptake obtained from fresh pig heart sarcosomes incubated with ascorbate-TMPD as substrates. The Ca^{2+} uptake, with 8 day old sarcosomes, decreases considerably (−75%). But, if the effect of CMF on the Ca^{2+} uptake in fresh sarcosomes is moderate (+30%), it becomes very important in 8 day old sarcosomes (+200%). In this latter case, CMF brings back the Ca^{2+} uptake level to the one observed normally in fresh sarcosomes.

It has been reported by Dilley and Vernon [11] that important movements of cations (K^+ and Mg^{2+}) occur when spinach chloroplasts are illuminated in presence of an appropriate electron acceptor. Fig. 4 points out clearly that we observed a significative Mg^{2+} ejection when *Zea mays* leaf chloroplasts are illuminated and incubated in a KCl-Tris medium at 30°, and that this ejection is totally suppressed by CMF.

3.2. *Interaction between CMF and Mg^{2+} movements in E. coli*

Boquet et al. [10] have shown that the lethal effect of Levallorphan (1,3-dihydroxy-N-allyl morphinane) on bacterial cultures is due to an important Mg^{2+} ejection from the bacteria, causing irreversible damages to their structure. Results reported in fig. 5 indicate that CMF reverses the levallorphan effect. This CMF action is demonstrated with two series of experiments: fig. 5a reports results obtained with levallorphan and CMF on the exponential growth rate of *E. coli* and fig. 5b shows their effects on the survival of these bacteria when transferred in a saline medium.

Preliminary experiments suggest that a molecule, identical to CMF with respect to its activity on rat liver mitochondria and its chromatographic behaviour, exists in the supernatant of sonicated *E. coli*. Demonstration of this identity is under way.

4. Discussion

The results reported here indicate that the cytoplasmic factor (CMF) is equally active on mammalian and plant organelles as well as on bacteria. This factor seems to act specifically on Mg^{2+} movements in various biological membranes. It is commonly accepted that liver mitochondria incubated during periods exceeding 2 to 3 hr lose partially or totally their ability to oxodize various substrates or the integrity of certain transport systems of their membranes. It was tempting to correlate these damages with the Mg^{2+} ejection which occurs over the same period of time, and to assume that Mg^{2+} plays an important part in the conservation of the mitochondrial membrane structure. Similar conclusions have been reached by Dilley and

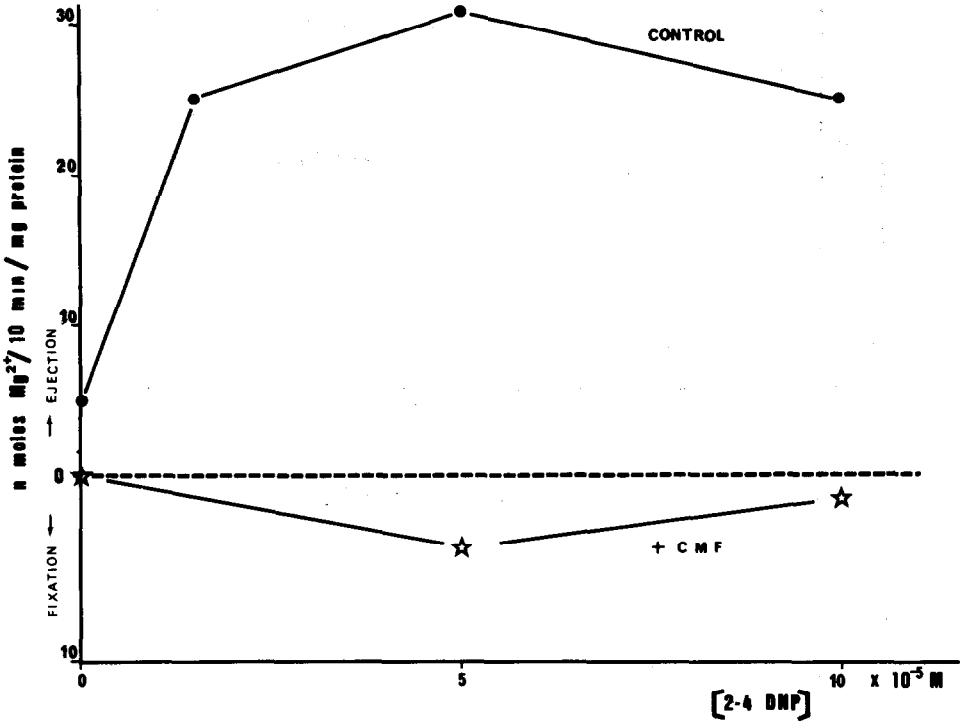

Fig. 1. Effect of CMF on the DNP-induced ejection of the mitochondrial Mg^{2+}. Rat liver mitochondria (6 mg protein) were incubated for 10 min at 30° in a glass vessel in 3 ml of the following medium: KCl 150 mM, Tris-HCl pH 7.4, 30 mM, ADP 2.3 mM. For Mg^{2+} measurement see experimental procedures.

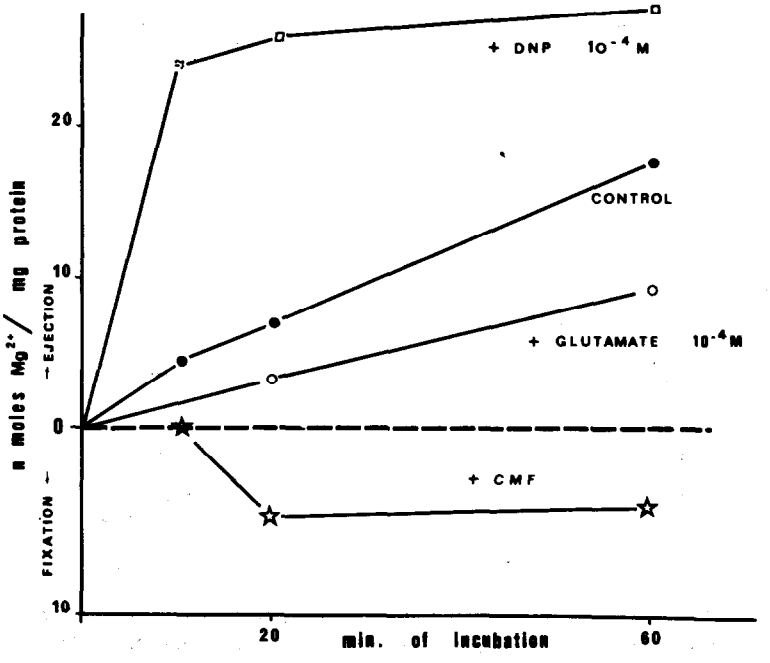

Fig. 2. Effect of CMF, uncoupler and glutamate on the kinetics of the mitochondrial Mg^{2+} ejection. Same conditions than in fig. 1.

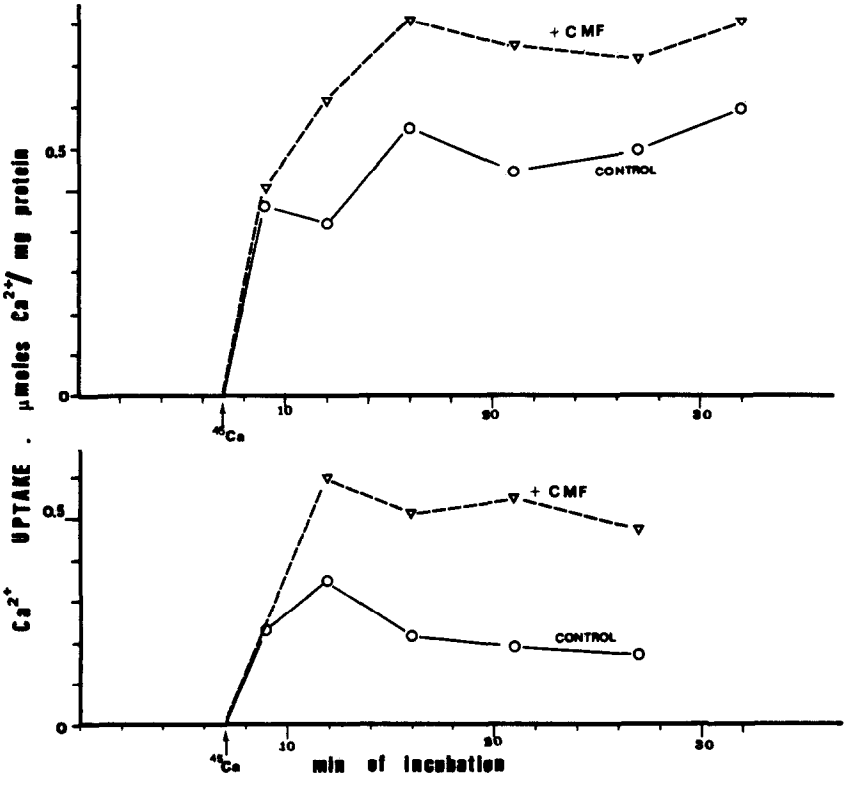

Fig. 3. Effect of CMF on the calcium uptake in fresh and aged sarcosomes. Pig heart sarcosomes (5 mg protein) were incubated at room temperature in 4.2 ml of the following medium: sucrose 60 mM, Tris-HCl pH 7.0 60 mM; $MgCl_2$ 8 mM; KH_2PO_4 10 mM; ascorbate 1 mM; TMPD 0.5 mM, oligomycin 10 µg, rotenone 3 µg. $^{45}Ca^{2+}$ was stipped in after 7 min of preincubation. Reaction was stopped by filtration on millipore – mitochondrial pellet was dissolved in formic acid and radioactivity measured on aliquots. Figure above: results obtained with fresh sarcosomes. Figure below: with 8 days old sarcosomes.

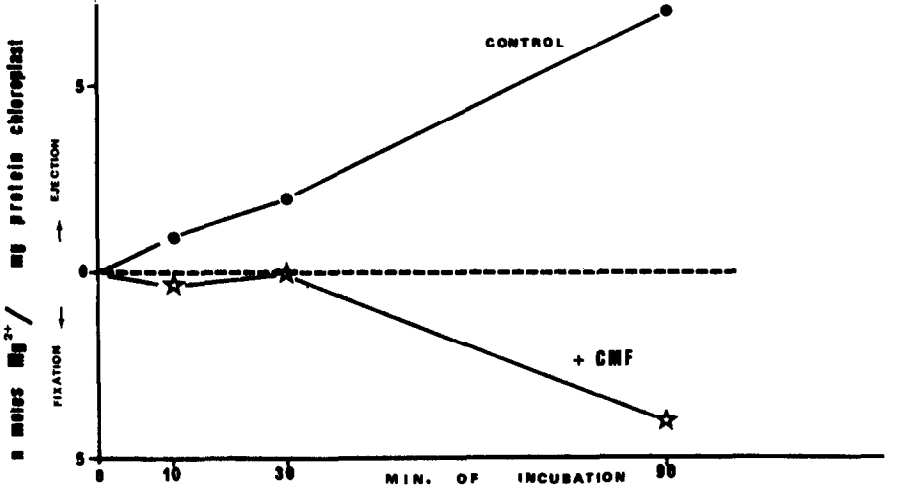

Fig. 4. Effect of CMF on Mg^{2+} movements in chloroplasts. Chloroplasts (72 mg protein) from *Zea mays* leaf were incubated at 30° in a glass vessel in 6 ml of KCl 150 mM, Tris-HCl pH 7.4 30 mM. They were illuminated and Mg^{2+} determined on aliquots taken at various time as indicated in experimental procedures.

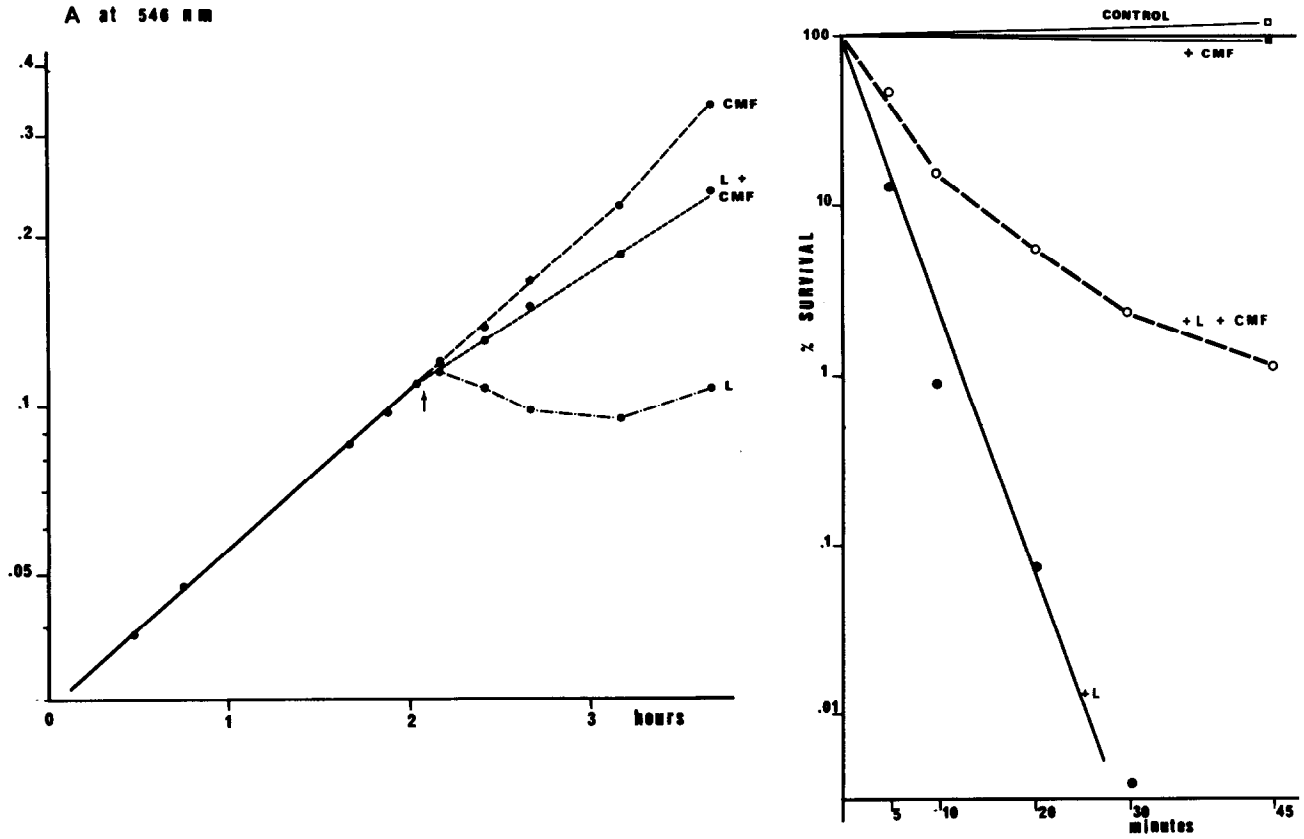

Fig. 5. Antagonistic effect of CMF on the actions of levallorphan on *E. coli*. *E. coli* CR 341 was grown exponentially in the following medium: glucose 0.4% Difco casamino acids 0.4% at pH 8.1. (a) Exponential growth in the absence or the presence of levallorphan ($0.6\ 10^{3}$ M) or CMF: the final Mg^{2+} concentration was 1×10^{-4} M. (b) Effect of levallorphan and CMF on the survival of *E. coli* culture resuspended in a saline medium (pH 8.1).

Vernon [11] for the large scale fluxes of Mg^{2+} and K^{+} observed in illuminated spinach leaf chloroplasts. CMF preventing totally Mg^{2+} efflux which occurs in incubated liver mitochondria or *Zea mays* chloroplasts, may participate in the maintenance of the structural integrity and the physiological activity of various biological membranes. This action and the preliminary indications on the peptidic structure we obtained [7] are different from the properties of an ionophoretic peptide recently discovered by Blondin et al. [12] in beef heart mitochondria. While this molecule is neutral, liposoluble and facilitates the transport of cations across the mitochondrial inner membrane, CMF is negatively charged, hydrosoluble and provokes the retention of the intra membranar Mg^{2+}.

The role of CMF and its cytoplasmic localization prove the importance of the interactions which exist within the cell between organelle membranes and their soluble environment. When mitochondria or chloroplatsts are isolated, they will be withdrawn from the influence of various significant parameters. Owing to its specific action on Mg^{2+} movements, CMF may be noteworthy for the conservation and the protection of the integrity of the biological membranes and thus be part of the tissue specific metabolic control system. Its role in the maintenance of *E. coli* membrane structure seems to indicate a more universal aspect of its mechanism of action.

Acknowledgements

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