# INTERACTION OF A CYTOPLASMIC FACTOR WITH ELECTRON AND ION TRANSFER COUPLED FUNCTIONS OF MITOCHONDRIA

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SUMMARY: Intramitochondrial $Mg^{2+}$ is labilized by uncouplers of oxidative phosphorylation or by valinomycin. This effect of uncouplers and of valinomycin exhibits ADP dependence, while the $Mg^{2+}$ labilizing action of valinomycin requires $K^+$ also. A trace amount ($\sim 10^{-6}$ M) of a purified cytoplasmic factor (CMF) prevents the induction of mitochondrial $Mg^{2+}$ ejection by both uncouplers and valinomycin and simultaneously activates mitochondrial metabolism. Oxidizable substrates protect mitochondrial $Mg^{2+}$ in a transient manner against labilization by uncouplers but not against valinomycin. It is concluded that the CMF-sensitive bound $Mg^{2+}$ containing system of mitochondria regulates energy coupled electron and $K^+$ transfer as an energy transducer.

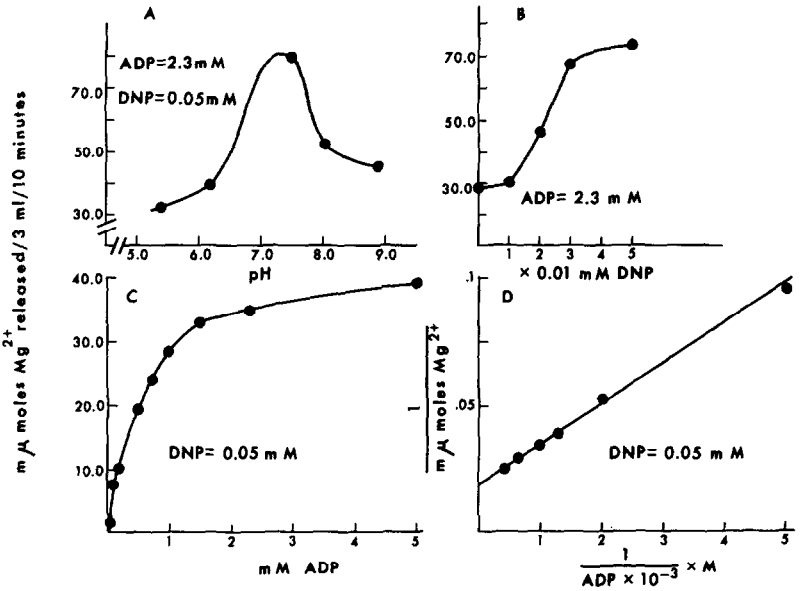
Incubation of liver mitochondria with ADP and DNP causes a rapid ejection of mitochondrial bound $Mg^{2+}$ into the suspending medium (1). The ability of mitochondria to oxidize glutamate at maximal rates in the presence of DNP was lost simultaneously with the depletion of mitochondrial $Mg^{2+}$. Both effects of preincubation with ADP + DNP were prevented by a partially purified cytoplasmic factor (CMF, cf. 1) in the presence of approximately 0.1 mM $Mg^{2+}$.

Since the ejection of mitochondrial $Mg^{2+}$ depended both on ADP and an uncoupler of oxidative phosphorylation, it was assumed that induced $Mg^{2+}$ loss by these agents represents an artificially modified function of a $Mg^{2+}$ containing system which is related to energy transduction. It follows that more detailed studies of rate limiting factors which influence $Mg^{2+}$ extrusion from mitochondria could lead to a better understanding of the chemical mechanism of energy transduction. Not only electron transfer along the respiratory chain, but ion movements in mitochondria are also energy linked (2); therefore, it was of importance to determine whether or not reagents which specifically induce $K^+$ movements can affect the mobilization of bound mitochondrial $Mg^{2+}$. It seemed possible that the two types of energy dependent processes (i.e. electron and $K^+$ transfer) may be separable in terms of their relationship to the mitochondrial $Mg^{2+}$ system. Although apparent regulation of $H^+$ and $K^+$ permeability of mitochondria has been the subject of numerous experiments (3) it is not known what mechanism couples these processes to the oxidative phosphorylative system. The metabolic significance of induced $Mg^{2+}$ loss of mitochondria was

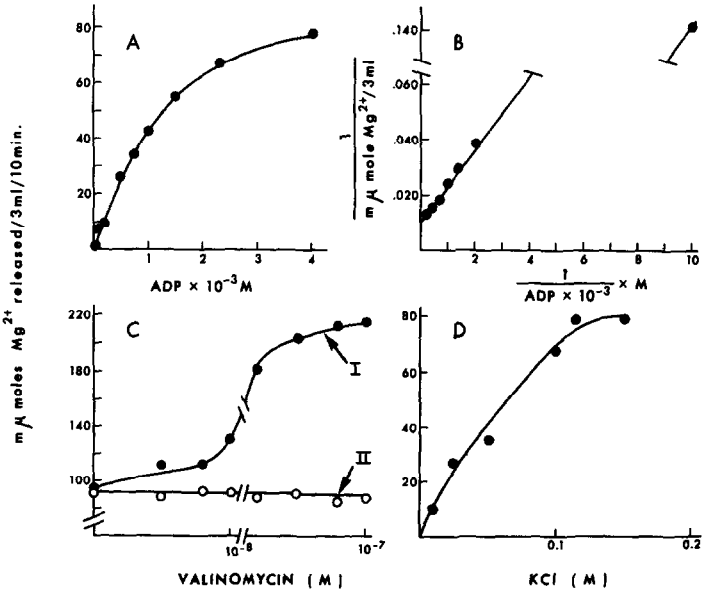
determined by correlating rates of oxidation of NAD linked substrates (e.g. glutamate) and those of succinate with the kinetics of $Mg^{2+}$ ejection in the presence of reagents which act selectively either on electron transfer or on $K^+$ movements.

MATERIALS AND METHODS. Analytical and preparative procedures were the same as described earlier (1). It was found recently that mitochondria prepared in EDTA free sucrose-mannitol (4) required no subsequent washing in 0.15 M KCl (in contrast to mitochondria isolated in sucrose-EDTA), are more stable, and tend to be more specific in their metabolic response to CMF. For this reason, EDTA free mannitol-sucrose was used for isolation of liver mitochondria. Initial rates of $Mg^{2+}$ ejection were determined after 10 minutes incubation at $30^0$, as described earlier (1). Rabbit liver CMF preparations were identical with those employed previously (1). In some instances, purified CMF was re-chromatographed on Bio-Gel P-2, developed with 0.03 M KCl, which separated $Mg^{2+}$ and the bulk of contaminating ninhydrin positive substances from the active component of CMF. Adjacent biochemically active (as measured by both the metabolic test and $Mg^{2+}$ ejection, cf. 1) and inactive fractions were hydrolyzed in 6 N HCl (at $115^0$ for 16 hours) and amino acid analyses performed on both samples. The active fraction contained 0.51 μmoles/ml acid labile $NH_3$, which could not be accounted for as glutamine or asparagine, and 0.32 μmoles/ml cystine. Trace amounts of other amino acids were nearly the same in both inactive and active fractions, therefore were considered to be contaminants. Since 0.04 ml of the active fraction was maximally effective in 3 ml test systems, the concentration of CMF in terms of cystine residues was 0.004 μmoles/ml, and, in terms of $NH_3$, 0.007 μmoles/ml. It is probable that CMF contains components which are undetectable by ninhydrin. Since cystine and $NH_3$ obtained by acid hydrolysis are likely to be in molar proportions to other components in the unhydrolyzed active constituent of CMF, it is estimated that CMF is active in the micromolar range, while $Mg^{2+}$ required for maximal activity is 0.1 mM. Rotenone and antimycin were gifts received from Dr. T.P. Singer and valinomycin from Dr. H.A. Lardy; 4,5,6,7-tetrachlorotrifluoromethylbenzimidazole was a gift of Dr. B. Chance, atractyloside was a gift of Dr. I. Edelman. All other compounds were of analytical grade.

RESULTS. Induction of $Mg^{2+}$ release from liver mitochondria requires ADP and either an uncoupler of oxidative phosphorylation (Fig. 1), or valinomycin and $K^+$ (Fig. 2). The saturation type ADP concentration dependence is restricted to a specific range of uncoupler or valinomycin levels. All kinetic studies were carried out in this range. ADP could not be replaced by ATP, AMP (1), GDP or IDP. Inhibitors of electron transfer (rotenone, antimycin) or atractyloside, which specifically inhibits adenine nucleotide translocation (5) do not replace uncouplers or valinomycin + $K^+$. Kinetic properties of the ADP + DNP dependent $Mg^{2+}$ release are illustrated in Fig. 1. A pH optimum around 7.5 (Fig. 1A), sigmoidal relationship between rates and DNP concentration (B) and hyperbolic relationship between rates and ADP concentration (C and D)




LEGEND TO FIGURE 1. pH dependence and kinetics of $Mg^{2+}$ release from liver mitochondria induced by DNP + ADP. The rate of $Mg^{2+}$ ejection during 10 minutes of aerobic incubation at 30° of mitochondria (5 mg protein) in 3 ml KCl-Tris was determined as described in Ref. 1. In A & B, endogenous $Mg^{2+}$ release (30 mμ moles/10 min) was not subtracted, while C & D were corrected for this background value.




LEGEND TO FIGURE 2. Kinetics of $Mg^{2+}$ release induced by valinomycin + $K^+$. In A & B, valinomycin concentration was 1.5 x 10-8 M and the concentration of ADP was varied. In C the effect of variation in valinomycin concentration is shown in 0.15 M KCl (Curve I) and in Tris-sucrose-mannitol (Curve II). In C the amount of mitochondria was increased to 8.3 mg/3 ml in order to improve the assay of endogenous $Mg^{2+}$ release. This value is not subtracted in C and corresponds to the valinomycin and $K^+$ independent rate found in Tris-sucrose-mannitol medium (Curve II). In D, valinomycin level was 1.5 x 10-8 M, and the suspending medium Tris-sucrose-mannitol (cf. 4). In both C & D, ADP was 2.3 mM.

were observed. Uncouplers of oxidative phosphorylation apparently related to DNP by their proton donor properties (6,7) are inducers of $Mg^{2+}$ ejection in the presence of ADP, except the concentration range, where the characteristic ADP-dependent kinetics is observed, varies with the chemical structure of the uncoupler. Half maximal rate of $Mg^{2+}$ ejection (35 x $10^{-6}$ M $Mg^{2+}$ per 10 minutes) was induced by 25 x $10^{-6}$ M DNP, 3 x $10^{-7}$ M pentachlorophenol, or 8 x $10^{-7}$ M tetrachlorotrifluoromethylbenzimidazole, in the presence of 2.3 mM ADP. Uncouplers were equally effective when the suspending medium was 0.15 M KCl or ($K^+$-free) sucrose-mannitol.

Kinetics of $Mg^{2+}$ release by valinomycin showed typical ADP dependence in the presence of 1.5 x $10^{-8}$ M valinomycin (Fig. 2, A & B), and a characteristic requirement for $K^+$ (Compare Fig. 2C, Curves I and II, with 2D). At fixed level of $K^+$ and ADP, a sigmoidal correlation exists between rates of $Mg^{2+}$ release and the concentration of valinomycin (Fig. 2C).

A typical experiment illustrates the temperature dependence of $Mg^{2+}$ ejection in the presence of 2.3 mM ADP + 0.05 mM DNP. Mitochondria (4.8 mg/3 ml KCl-Tris) at 0 to $4^0$, lose no $Mg^{2+}$ in 10 minutes, while at $20^0$ the rate is 20 mμmoles, at $30^0$, 67 mμmoles, and at $37^0$, 97 mμmoles.

As shown previously (1), prevention of $Mg^{2+}$ release by CMF modified the metabolic capacity of mitochondria and permitted maximal rates of glutamate oxidation in the presence of ADP + DNP. Since valinomycin + $K^+$ also labilized $Mg^{2+}$ (Fig. 2), it was of importance to test the metabolic consequences of preincubation of mitochondria with this antibiotic and CMF.

CMF stimulated the rates of oxidation of glutamate (and to a varying degree the oxidation of other NAD linked substrates), but not of succinate in the presence of ADP + DNP. Valinomycin and $K^+$ replaced DNP and under these conditions CMF increased the oxidation of <u>both</u> glutamate and succinate (Table I).

As shown previously (1), CMF without $Mg^{2+}$ did not influence metabolism. On the other hand, metabolic rates in the presence of 0.1 mM $Mg^{2+}$ alone were significantly below the rates obtained with CMF + $Mg^{2+}$ (Compare Nos. 2,4,10,11,13 and 14, Table I).

Present results (Table I) and previous studies (1) show that the system which determines the metabolic capacity of mitochondria can be maximally activated by CMF in the presence of agents which, by themselves, labilize mitochondrial $Mg^{2+}$. If a functional relationship exists between the effect of $Mg^{2+}$ labilizing agents, CMF and metabolic rates, it would be expected that substrates and specific metabolic inhibitors should also influence the stability of $Mg^{2+}$ binding in mitochondria in the presence of inducers of $Mg^{2+}$ ejection. When ADP + DNP were the $Mg^{2+}$ labilizing agents, oxidizable substrates (Table II, Nos. 2,6 & 10) inhibited the initial rate of $Mg^{2+}$ ejection, and this effect was counteracted by inhibitors of electron transfer between NADH and $O_2$ (Nos. 3,4,5,7,8 & 9), or by antimycin only when succinate was the substrate (No. 13). Inhibition of ADP + DNP-induced $Mg^{2+}$ ejection by CMF was almost complete (No

TABLE I

RATES OF GLUTAMATE AND SUCCINATE OXIDATION OF LIVER MITOCHONDRIA

| No. | Variable Components | $O_2$* |
|---|---|---|
| 1 | Glu.($10^{-2}$ M) | 4.3 |
| 2 | " + 0.1 mM $Mg^{2+}$ + 5 x $10^{-5}$ M DNP | 10.5 |
| 3 | " + 0 $Mg^{2+}$ + 5 x $10^{-5}$ M DNP | 7.5 |
| 4 | " + 0.1 mM $Mg^{2+}$ + 5 x $10^{-5}$ M DNP + CMF | 21.5 |
| 5 | Succ.($10^{-2}$ M) | 8.7 |
| 6 | " + 0.1 mM $Mg^{2+}$ + 5 x $10^{-5}$ M DNP | 11.8 |
| 7 | " + 0 $Mg^{2+}$ + 5 x $10^{-5}$ M DNP | 11.3 |
| 8 | " + 0.1 mM $Mg^{2+}$ + 5 x $10^{-5}$ M DNP + CMF | 12.2 |
| 9 | Glu. + 5 x $10^{-8}$ M valino. | 10.4 |
| 10 | " + 5 x $10^{-8}$ M valino. + 0.1 mM $Mg^{2+}$ | 18.2 |
| 11 | " + 5 x $10^{-8}$ M valino. 0.1 mM $Mg^{2+}$ + CMF | 24.2 |
| 12 | Succ. + 5 x $10^{-8}$ M valino. | 8.7 |
| 13 | " + 5 x $10^{-8}$ M valino. + 0.1 mM $Mg^{2+}$ | 11.1 |
| 14 | " + 5 x $10^{-8}$ M valino. + 0.1 mM $Mg^{2+}$ + CMF | 15.4 |

*µmoles/10 mg protein per 60 min.

LEGEND TO TABLE I. The effects of preincubation (for 15 minutes, cf.1) with 0.1 mM $MgCl_2$, 0.05 mM DNP and CMF on the rates of glutamate and succinate oxidation by liver mitochondria (Exp. 1-8). The effects of preincubation with valinomycin are shown in Exp. 9-14. Details of the manometric assay are given in Ref. 1.

TABLE II

INFLUENCE OF SUBSTRATES, INHIBITORS AND CMF ON THE INITIAL RATE OF $Mg^{2+}$ EJECTION FROM MITOCHONDRIA

| No. | Inducing Agent | Variable Components | Rate of $Mg^{2+}$ Loss* |
|---|---|---|---|
| 1 | ADP + DNP | - | 103 |
| 2 | " | $10^{-2}$ M glu. | 30 |
| 3 | " | " + 2.4 x $10^{-7}$ M rotenone | 106 |
| 4 | " | " + 5 x $10^{-4}$ M arsenite | 85 |
| 5 | " | " + antimycin** | 100 |
| 6 | " | $10^{-2}$ M α-ktgl. | 56 |
| 7 | " | " + 2.4 x $10^{-7}$ M rotenone | 106 |
| 8 | " | " + 5 x $10^{-4}$ M arsenite | 94 |
| 9 | " | " + antimycin** | 90 |
| 10 | " | $10^{-2}$ M succ. | 42 |
| 11 | " | " + 2.4 x $10^{-7}$ M rotenone | 57 |
| 12 | " | " + 5 x $10^{-4}$ M arsenite | 42 |
| 13 | " | " + antimycin** | 90 |
| 14 | " | CMF | 3 |
| 15 | " | " + 2.4 x $10^{-7}$ M rotenone | 119 |
| 16 | " | " + antimycin** | 154 |
| 17 | ADP + valino + $K^+$ (0.1 M) | - | 98 |
| 18 | " | $10^{-2}$ M glu. | 98 |
| 19 | " | $10^{-2}$ M succ. | 90 |
| 20 | " | CMF | 44 |

*(mµmoles/10 min/10 mg protein at 30°); **0.1 µg antimycin/mg protein.

14), and this effect was profoundly reversed by inhibitors of electron transfer (15,16). Since initial rates of $Mg^{2+}$ ejection were measured during the first 10 minutes of aerobic incubation of mitochondria, the contribution of endogenous metabolites under these conditions is highly probable (e.g. in Exp. No. 14). For this reason, it must be concluded that the protective effect of CMF apparently depends on the presence of reducing substrates, either added externally or present as endogenous substrates during short term experiments. The most important difference between CMF and substrates is that dicarboxylic acids alone stabilize mitochondrial $Mg^{2+}$ only for a short time (15-20 minutes) in the presence of ADP + DNP, while CMF + substrates protect mitochondrial $Mg^{2+}$ for two hours and maintain maximal metabolic rates during this period. When the inducing agents for $Mg^{2+}$ release were valinomycin and $K^+$ (in the presence of ADP), oxidizable substrates alone did not stabilize mitochondrial $Mg^{2+}$, only CMF.

DISCUSSION. The kinetic relationship between rates of $Mg^{2+}$ ejection, the concentration of uncouplers and of valinomycin + $K^+$ and ADP, points to an interaction of electron and ion transfer with the $Mg^{2+}$ containing mitochondrial system. As shown in Table II, valinomycin + $K^+$ induce $Mg^{2+}$ ejection as effectively as DNP. There is, however, a significant difference between $Mg^{2+}$ labilization initiated by uncouplers or by valinomycin, which is known to be a specific reagent for the activation of $K^+$ translocation (8). While the action of uncouplers on mitochondrial $Mg^{2+}$ is temporarily counteracted by physiological electron donors and this effect is characteristically prevented by site specific inhibitors of electron transfer, $Mg^{2+}$ ejection by valinomycin + $K^+$ is not altered by reducing mitochondrial substrates. This experiment clearly distinguishes the electron transfer and $K^+$ carrier system. It appears that the succinate system occupies a special place. Succinate oxidation is stimulated by CMF only in the presence of $K^+$ + valinomycin (Table I). A dependence of succinate oxidation on valinomycin-induced $K^+$ uptake has also been observed by Harris, van Dam and Pressman (9).

It is concluded that the bound $Mg^{2+}$ containing mitochondrial system is distinct from both the electron and $K^+$ transfer apparatus, yet it is functionally connected to both. Although the exact nature of the bound $Mg^{2+}$ containing system needs to be further elucidated, its intramitochondrial localization is less hypothetical. We found that 50 to 60% of mitochondrial $Mg^{2+}$, which corresponds to the quantity labilized by uncouplers or valinomycin, is present in the inner mitochondrial membrane which is the well known site of energy coupled functions.

A probable relationship between the bound $Mg^{2+}$ containing system ($x \sim Mg^{2+}$) and the electron and $K^+$ transfer systems is shown in the following schematic diagram:

$$(e^-\text{-system}) \rightleftharpoons (x \sim Mg^{2+}) \rightleftharpoons (K^+\text{-system})$$

The arrows indicate the directions of reversible energy transfer. According to this interpretation ($x \sim Mg^{2+}$) can be energized by or dissipate energy to the electron and $K^+$ transfer systems. Since it is known that both transfer systems are coupled to ATP synthesis, ($x \sim Mg^{2+}$) may function as an energy transducer capable of utilizing energy

derived from either transfer systems for ATP synthesis. Intermediates of ATP synthesis may be unstable $Mg^{2+}$ chelates, localized in hydrophobic membrane sites. In isolated mitochondria the rate limiting role of ($x \sim Mg^{2+}$) escapes detection because the transfer systems and the energy transducer seem to operate as a single apparatus, especially when its function is monitored by transfer process linked ATP synthesis. Recognition of ($x \sim Mg^{2+}$) required both reagents which act on either of the two transfer systems and CMF. In cellular systems, CMF may exert a control function on mitochondrial metabolism by way of ($x \sim Mg^{2+}$) and may regulate cation and indirectly electron flux, provided the effects of uncouplers or valinomycin are simulated by physiological variations of energy (cytoplasmic substrates) or cation supply. It is probable that other cytoplasmic constituents (e.g. $Ca^{2+}$, hormones) may also interact with ($x \sim Mg^{2+}$), thus partially imitate the extreme effect of toxic reagents employed in our model experiments.

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