# PREVENTION OF $Ca^{2+}$ INDUCED CATION EFFLUX FROM LIVER MITOCHONDRIA BY A CYTOPLASMIC FACTOR AND BY OLIGOMYCIN

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## 1. Introduction

It was shown in preceding studies that trace amounts of a cytoplasmic metabolic factor (CMF) prevented certain deleterious effects of uncouplers on isolated mitochondria [1–6]. The possibility was considered that CMF may be a naturally occurring cytoplasmic stabilizing agent of mitochondrial bioenergetic functions [7]. Since demonstration of the effect of CMF on mitochondria required artificial uncouplers, or potentially toxic cellular metabolites (e.g. oleic acid or bilirubin, cf. [7]), the apparent cellular role of CMF seemed to be confined to protection against mitochondrial damage. More recent work revealed that artificial uncouplers could be replaced by $Ca^{2+}$ in concentrations found in the cytosol [8]. Since, in the presence of $Ca^{2+}$, energy coupled cation movements were influenced by CMF in the absence of any artificial uncoupler, the $Ca^{2+}$-CMF antagonism assumed cell physiological interest.

The present report is concerned with the time course of $Ca^{2+}$-induced cation movements. As an extention of a previous observation [cf. 8], it was confirmed that oligomycin inhibits $Ca^{2+}$-induced cation flux, and this effect of oligomycin is similar to that of CMF.

*Abbreviation*

CMF = cytoplasmic metabolic factor

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## 2. Experimental procedures

Because the published methods for the isolation of partially purified CMF [2–4] underwent some modification, a brief description of a procedure for partial purification of CMF used in these experiments will be given. Pig liver (1.8 kg) kept in cracked ice during transportation from the slaughterhouse was homogenized in 300 g portions for 45 sec (at 4°) in a Sorvall Omni-Mixer with 1.8 litre distilled $H_2O$ and crude sediments were removed by centrifugation at 16,300 *g* for 1 hr at 4°. The bulk of protein of the supernatant fluid was removed by heat coagulation (submersion of 70 ml batches in Erlenmeyer flasks for 2.5 to 3 min in boiling $H_2O$ baths), and the volume of the supernatant of the heat-treated extract obtained by a second centrifugation (40 min at 34,800 *g* at 4°) was reduced to 250 ml by freeze-drying. This concentrated extract, which contained all CMF present in the liver, was fractionated on a large Sephadex G-25 column. equilibrated and developed with $H_2O$ at 4°. The size of the column was 9.5 × 50 cm, bed volume = 3.5 l, void volume = 1.2 l. The flow rate was 10 ml/min and 15 ml fractions were collected. Analysis for CMF activity was performed by the metabolic assay [cf. 3]. After the void volume, residual proteins were eluted in 500 to 600 ml. This was followed by the CMF containing peak (800 to 1000 ml) and finally by a yellow colored peak (500 to 600 ml) which had no activity. The volume of the CMF containing eluate was reduced to 100 ml by freeze-drying. This extract was passed through a Chelex (BioRad) column (3.5 × 4.5 cm in $Na^+$ form, containing 35 g resin, equilibrated at neutral pH), then through a Dowex-1-bicarbonate

column (3 × 1 cm, 6 g resin), both developed with $H_2O$. The eluate (about 100 ml) was freeze-dried until a solid material was formed (about 3 g). This material contained 70 to 80% of CMF present in the original extract, comprising on a weight basis a 600-fold purification, although this may be greater because the material was hygroscopic. It contained no $Ca^{2+}$, $Mg^{2+}$ or nucleotides, and no detectable mitochondrial subunits, but was contaminated with variable quantities of ninhydrin reactive and carbohydrate material. Based on further purification, when these contaminants were removed*, the purity of this extract with respect to the active component of CMF was estimated to be 1 to 3%. When activity was related to total organic matter, (dichromate reducing material with glucose as primary standard, cf. [9]), maximal CMF activity was obtained at 0.4 to 0.6 mg/ml concentrations. Partially purified CMF in this form is stable for several months in the frozen state. Various characteristics of the active small molecular (less than 1000 daltons) component have been reported [cf. 5].

Isolation of rat liver mitochondria was carried out by the Tris–sucrose–mannitol–BSA procedure [cf. 10]. The method of determination of cation flux, based on atomic absorption analyses of cations both in mitochondria and in the suspending medium at various time intervals, was the same as reported earlier [3, 5, 8].

## 3. Results

In the experiments shown (fig. 1), rat liver mitochondria were incubated in sucrose–Tris medium at 30° and cation movements determined at 2 to 3 min intervals. In the absence of externally added $Ca^{2+}$, only traces of $Mg^{2+}$ and $Ca^{2+}$ were ejected into the suspending medium. There was also a steady, relatively slow outward flux of $K^+$. In the presence of externally added $Ca^{2+}$ (60 or 37 nmoles $Ca^{2+}$/mg mitochondrial protein), almost all $Ca^{2+}$ was rapidly taken up. For a period of 2 to 3 min, only a small increase of $Mg^{2+}$ and $K^+$ efflux was detectable. After this period of latency, $Mg^{2+}$ and $K^+$ efflux greatly accelerated, while $Ca^{2+}$ was still retained for an additional 2–3 min. When virtually all mitochondrial $Mg^{2+}$ and $K^+$ were ejected, mitochondrial $Ca^{2+}$ was also released and even some of the originally bound $Ca^{2+}$ appeared in the suspending medium. Cation ejection was prevented, $Ca^{2+}$ uptake increased, and $Ca^{2+}$ retention sustained when CMF or oligomycin were present during incubation, or were

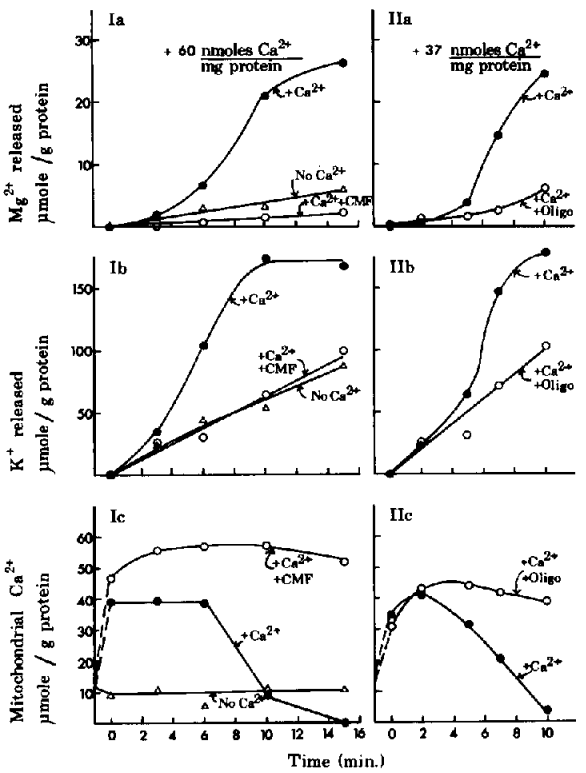


Fig. 1. Liver mitochondria (2.8 mg/ml in I and 2.4 mg in II) were incubated at 30° in a Dubnoff shaker in 50 ml beakers. The volume of incubation mixture was 6 ml, consisting of 250 mM sucrose + 30 mM Tris-HCl (pH 7.4). Cation content of mitochondria and the suspending medium were determined simultaneously at 2 to 3 min intervals after rapid centrifugal sedimentation of mitochondria at 0 to 4° [cf. 3]. Time is shown on the abscissa. Mitochondrial $Mg^{2+}$ and $K^+$ release is calculated as µmoles of cations extruded from 1 g mitochondrial protein as recorded on the ordinate (a and b). The movement of $Ca^{2+}$ is calculated in the same manner by expressing the variation of mitochondrial $Ca^{2+}$ content. Original $Ca^{2+}$ content of mitochondria was 10 µmoles $Ca^{2+}$/g mitochondrial protein. In Ic and IIc, $Ca^{2+}$ uptake is shown by the increase in mitochondrial $Ca^{2+}$ above and $Ca^{2+}$ loss by the decrease below this value (ordinate). The amounts of externally added $Ca^{2+}$ are shown on the top of the figure; CMF = 0.46 mg organic matter/ml; oligomycin = 2.8 µg/mg mitochondrial protein.

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added within 3 min after the exposure of mitochondria to $Ca^{2+}$. This relatively short period of reversibility of the effects of $Ca^{2+}$ by CMF may be of cellular physiological importance, since it allows short cycles of oscillations of energy linked processes of the inner membrane [cf. 7]. Endogenous respiration is essential for $Ca^{2+}$-induced cation flux; rotenone ($10^{-7}$ M) prevented $Mg^{2+}$ and $K^+$ efflux, and inhibited the effect of CMF. Oligomycin did not inhibit endogenous respiration.

## 4. Discussion

Similarity in action between the cellular substance CMF and oligomycin suggests that the mitochondrial site for oligomycin is a part of a cellular regulatory system which, under physiological conditions, responds to CMF. It is known [11] that mitochondrial ATP synthesis is inhibited during energy coupled $Ca^{2+}$ uptake, presumably because metabolic energy is used for $Ca^{2+}$ translocation in preference to the phosphorylation of ADP. Our experimental conditions correspond to this energetic pattern during the first 3 to 5 min after exposure of mitochondria to $Ca^{2+}$. Thereafter, energy coupling to cation retention fails unless CMF or oligomycin is added, indicating that both agents stabilize a component of the energy transfer system which can couple respiration to cation retention. It is known from the work of Lardy et al. [12–15], that oligomycin blocks energy transfer reactions leading to ATP synthesis, but not to other processes (e.g. cation translocation); in fact, oligomycin was shown to promote energy transfer under non-phosphorylating conditions [16–19]. We conclude that the common site of CMF and oligomycin is this – as yet unknown portion of the energy transducing system, preceding ATP synthesis. Previous experiments [3–8] indicated that this site may be a ligand system of $Mg^{2+}$, thus $\sim X{-}Mg^{2+}$ could represent a non-phosphorylated energized intermediate of energy transfer. The significant difference between CMF and oligomycin is that the former sustains oxidative phosphorylation [cf. 7], while the latter inhibits it. This difference indicates that the toxic antibiotic simulates only one mode of action of CMF. The common site of CMF and oligomycin is not shared by aurovertin [cf. 8].

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