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# The effect of ferric iron complex on isolated rat liver mitochondria. II. Ion movements

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**It has been found that addition of iron(III)-gluconate complex to rat liver mitochondria disturbed the mitochondrial $Ca^{2+}$ transport. Indirect evidence when the changes in the membrane potential during the transport of $Ca^{2+}$ were followed, as well as direct evidence, when the fluxes of $Ca^{2+}$ were monitored by a $Ca^{2+}$-selective electrode, indicated that this iron complex induced an efflux of $Ca^{2+}$ from liver mitochondria. The mechanisms by which iron induced $Ca^{2+}$ release appeared to be linked to the induction of lipoperoxidation of mitochondrial membrane. The mitochondrial membrane, however, did not become irreversibly damaged under these conditions, as indicated by its complete repolarization. It was also shown that the induction by iron of lipoperoxidation brought about an efflux of $K^+$ from mitochondria.**

## Introduction

It has been previously reported [1] that addition of iron(III)-gluconate complex to rat liver mitochondria brings about the induction of lipid peroxidation and a drop in membrane potential. This lipid peroxidation process appeared to be responsible for the induction of energy-consuming processes involving ion translocation which caused the drop in membrane potential. Specifically the enhancement of endogenous $Ca^{2+}$ cycling across the membrane was suggested to contribute to a large extent to the dissipation of energy [1].

It is well established that peroxidation of the mitochondrial membrane can modify the fluidity of the membrane [2], thus influencing mitochondrial ion transport. In this regard it was recently shown that the process of induction of peroxidation reactions in mitochondrial membranes with $Fe^{2+}$ in the presence of ascorbic acid or with cumene hydroperoxide elicits the induction of a net $K^+$ release from mitochondria and a drop in the membrane potential [3]. Relevant to the same problem are the findings [4–6] that exposure of rat liver mitochondria to hydroperoxide and $Ca^{2+}$ induced the release of $Ca^{2+}$, through the activation of a specific $Ca^{2+}$ release process. A shift of the redox steady-state of mitochondrial pyridine nucleotide induced by hydroperoxide was suggested to be involved in regulating the $Ca^{2+}$ balance between mitochondria and the medium. On the other hand, induction of mitochondrial lipid peroxidation by hydroperoxide [7] may also conceivably be a factor in the causing a $Ca^{2+}$ release.

In the present research the effect of iron(III)-gluconate on the mitochondrial transport of some ions, specifically $K^+$ and $Ca^{2+}$, was studied. The

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside.

contribution of $K^+$ and $Ca^{2+}$ movements to the energy-dissipating processes responsible for the membrane potential drop as well as the possible controlling mechanisms were also investigated.

## Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure [8]. Protein was determined by a biuret method, with bovine serum albumin as a standard.

The endogenous content of mitochondrial $K^+$ was determined as follows: liver mitochondria (3–3.5 mg protein/ml) were incubated in a standard medium plus substrate at 25°C. At timed intervals 0.5 ml of mitochondrial suspension was removed and centrifuged for 1 min in an Eppendorf bench centrifuge (Model 5414S). The pellet was washed twice with cold standard medium without $K^+$ or $Mg^{2+}$, then dispersed by extensive vortexing in 1% sodium cholate containing 1 mM EDTA. The $K^+$ was determined by atomic absorption. The standard medium had the following composition: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.4), 10 mM sodium potassium phosphate buffer (pH 7.4) and 1.6 mM sodium pyruvate plus 0.4 mM L-malate as the substrate.

The transmembrane potential ($\Delta\psi$) was measured at 25°C in a final volume of 1.5 ml by monitoring with a tetraphenylphosphonium-selective electrode the movements of tetraphenylphosphonium across the mitochondrial membrane as in Ref. 6. The standard medium for assaying the electrochemical parameters was as follows: 210 mM mannitol, 70 mM sucrose, 10 mM Hepes (pH 7.4).

$Ca^{2+}$ movements were followed by a $Ca^{2+}$-selective electrode as described in detail in Refs. 9 and 10. The incubation medium (1.5 ml, maintained at 25°C) contained 120 mM KCl, 10 mM Hepes (pH 7.4), 4.5 mg mitochondrial protein, 5 $\mu$g of rotenone and 50 $\mu$M $CaCl_2$. After a preincubation period of 5 min, the initial rates of calcium influx were measured following addition of 5 mM succinate ($K^+$ salt) as respiratory substrate.

Ferlixit (iron(III)-gluconate complex) was purchased from Natterman and Cie, Köln (F.R.G.), Desferal (desferrioxamine) was obtained from Ciba-Geigy SA, Basel (Switzerland), and Trolox-C was a gift from Roche-Italia, Milano (Italy).

## Results

The effect of addition of iron(III)-gluconate complex on the endogenous $K^+$ content of liver mitochondria respiring under State 4 conditions is presented in Fig. 1. It is seen that after an initial lag period iron induces a net efflux of $K^+$ from mitochondria. An incubation period of 7.5 min in the presence of iron causes a net release of approx. 70% intramitochondrial $K^+$. It is noteworthy that if an antioxidant agent such as butylated hydroxytoluene is present from the start of incubation the release of $K^+$ is almost completely prevented. The addition of either oligomycin, a specific inhibitor of ATP-synthetase, or ruthenium red, a specific inhibitor of electrophoretic $Ca^{2+}$ uptake, markedly reduces the extent of net $K^+$ release.

Fig. 2 shows the effect of the external concentration of $K^+$ on the net fluxes of mitochondrial $K^+$ induced by iron. It appears that when the external $K^+$ concentration is raised from 2 mM to

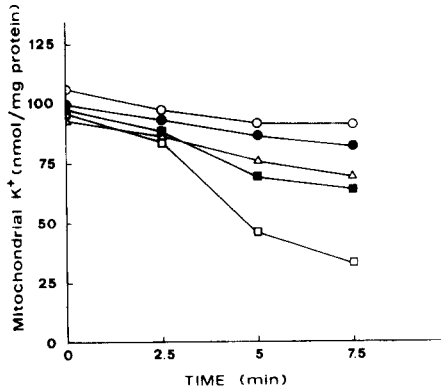


Fig. 1. Effect of iron(III)-gluconate on the mitochondrial content of $K^+$. The mitochondria were incubated in the standard medium as described in the Methods. The intramitochondrial $K^+$ concentration was determined by atomic absorption. The medium also contained at the beginning of the incubation the following additions: ○, none; □, 0.6 mM iron(III)-gluconate; ■, 0.6 mM iron(III)-gluconate + 2 $\mu$g/mg oligomycin; △, 0.6 mM iron(III)-gluconate + 2 $\mu$M ruthenium red; ●, 0.6 mM iron(III)-gluconate + 30 $\mu$M butylated hydroxytoluene. The data represent mean values of five separate experiments.

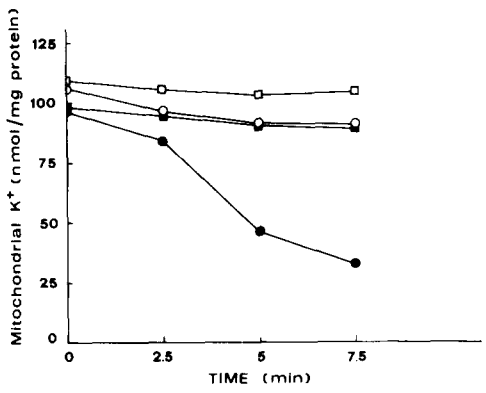


Fig. 2. Effect of iron(III)-gluconate on the mitochondrial content of $K^+$ at two concentrations of external $K^+$. The mitochondria were incubated in the standard medium containing at the beginning of the incubation 1.9 mM $K^+$ (○) and 30 mM $K^+$ (□) in the absence (○, □) and in the presence (●, ■) of 0.6 mM iron(III)-gluconate, respectively. All other conditions were as in Fig. 1.

30 mM the intramitochondrial $K^+$ concentration remains nearly at a steady-state value during the incubation period tested in the presence of iron.

The membrane potential of mitochondria during the transport of a fixed amount of external $Ca^{2+}$ is presented in Fig. 3. Energization of mitochondria with pyruvate results in the development of a $\Delta\psi$ (negative inside) of about 180 mV. The addition of 100 $\mu$M $Ca^{2+}$, after the potential has reached the steady-state, induces a sudden decrease of $\Delta\psi$, which reflects the dissipation of energy during the transport of $Ca^{2+}$ into mitochondria. After a lag which corresponds to the time necessary to accumulate this pulse of $Ca^{2+}$, the potential trace reverses itself spontaneously and a new steady-state almost identical to the pre-$Ca^{2+}$ level is reached. Addition of 0.6 mM iron(III)-gluconate complex in this new steady-state elicits the drop in the membrane potential. The pattern of the $\Delta\psi$ trace seems to reflect the re-uptake of $Ca^{2+}$ released from mitochondria in the presence of iron, i.e., an enhancement of the '$Ca^{2+}$ cycle'. This suggestion is supported by the experiment shown in Fig. 3B, in which the re-uptake of $Ca^{2+}$ is blocked by the ruthenium red added before iron. The same figure shows that when the induction by iron of the peroxidative process is suppressed by butylated hydroxytoluene the $Ca^{2+}$ release is also prevented. Oligomycin under these experimental conditions, i.e., a metabolic medium lacking $Mg^{2+}$ and $P_i$ and without ATP, did not appreciably prevent the drop in membrane potential induced by iron (not shown).

Fig. 4 shows the effects of a higher $Ca^{2+}$ pulse

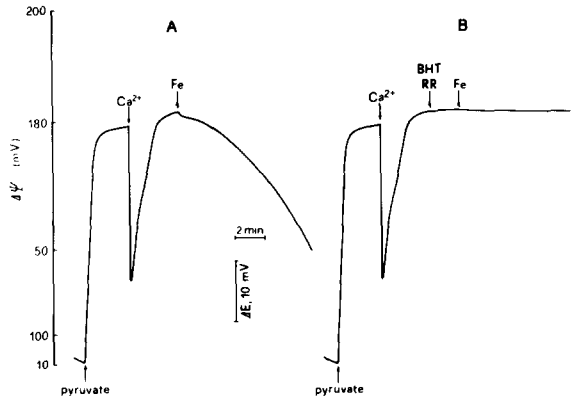


Fig. 3. Effect of iron(III)-gluconate on the transmembrane potential of liver mitochondria pre-loaded with a low $Ca^{2+}$ pulse. The mitochondria in standard incubation medium, described in the Methods, were energized by addition of 1.6 mM pyruvate plus 0.4 mM L-malate (pyruvate). Arrows indicate the following additions: 100 $\mu$M $Ca^{2+}$; 0.6 mM iron(III)-gluconate (Fe); 30 $\mu$M butylated hydroxytoluene (BHT); 2 $\mu$M ruthenium red (RR). The transmembrane potential ($\Delta\psi$) was measured, as described in the Methods, in the presence of 20 $\mu$M tetraphenylphosphonium chloride. $\Delta E$, electrode potential.

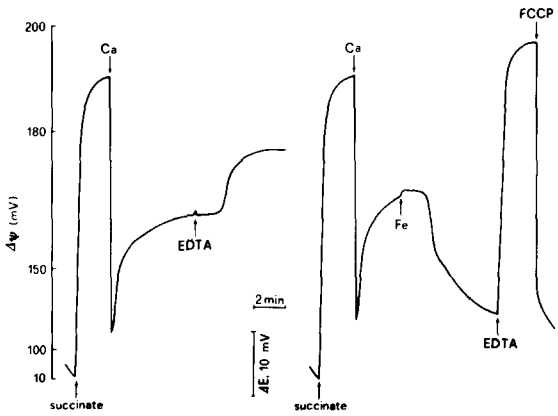


Fig. 4. Effect of iron(III)-gluconate on the transmembrane potential of liver mitochondria preloaded with a high $Ca^{2+}$ pulse. The mitochondria in standard reaction medium, described in the Methods, to which was added 5 $\mu$M rotenone, were energized by 2.5 mM succinate. Additions were: 200 $\mu$M $Ca^{2+}$; 1 mM EDTA; 0.6 mM iron(III)-gluconate (Fe); 1 $\mu$M FCCP. All other conditions were as in Fig. 3.

on the transmembrane potential. Addition of 200 $\mu$M $Ca^{2+}$ to energized mitochondria results in a rapid drop in $\Delta\psi$. When $Ca^{2+}$ added has been accumulated the $\Delta\psi$ trace reverses to a new steady-state which is considerably lower than the pre-$Ca^{2+}$ level. Addition of EDTA to limit the operation of the $Ca^{2+}$ cycle by lowering the extramitochondrial $Ca^{2+}$, and thus inhibiting its uptake, results in a gradual shift of the potential trace towards a higher value (Fig. 4A). However, this new level is considerably lower than the pre-$Ca^{2+}$ level. Fig. 4B indicates that the addition of iron completely releases all the $Ca^{2+}$ accumulated. Indeed in this case, the addition of EDTA after $Ca^{2+}$ has been released induces a rapid and almost complete reversal of the potential trace to the pre-$Ca^{2+}$ steady-state level.

Direct experimental evidence of the effect of iron(III)-gluconate complex on the mitochondrial $Ca^{2+}$ transport is presented in Fig. 5. Indeed it is seen that addition of iron to the $Ca^{2+}$-loaded mitochondria leads to the release of mitochondrial $Ca^{2+}$ (Fig. 5A). It appears from Fig. 5B that when either desferrioxamine, a specific chelator of $Fe^{3+}$, or Trolox-C, an antioxidant agent, is added after $Ca^{2+}$ has been accumulated by mitochondria, they markedly prevent the $Ca^{2+}$-releasing action of iron.

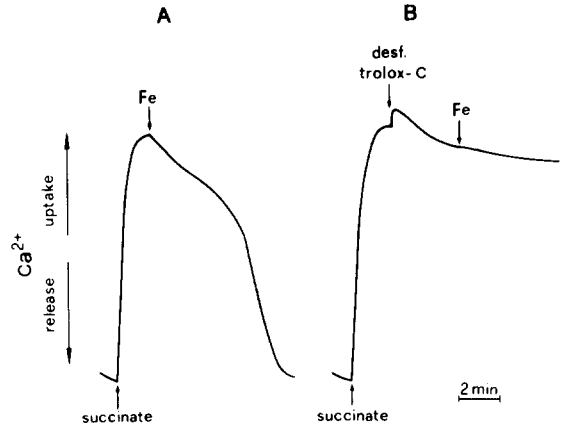


Fig. 5. Effect of iron(III)-gluconate on the $Ca^{2+}$ fluxes of liver mitochondria. The mitochondria were incubated for 5 min in the standard reaction medium described in the Methods, plus 50 $\mu$M $Ca^{2+}$. Respiration-dependent uptake of $Ca^{2+}$ was started by the addition of 5 mM succinate. Further additions were: 0.6 mM iron(III)-gluconate (Fe); 2 mg/mg desferrioxamine (desf.); 200 $\mu$M Trolox-C. $Ca^{2+}$ movements were measured with a $Ca^{2+}$-selective electrode as described in the Methods.

## Discussion

The results of the present paper lead to the conclusion that the addition of iron(III)-gluconate complex to rat liver mitochondria induces a release of mitochondrial $Ca^{2+}$, thus supporting a previous suggestion [1]. Two lines of experimental evidence strengthen this conclusion. Firstly, the mitochondrial membrane potential after a pulse of $Ca^{2+}$ has been accumulated is rapidly and markedly decreased by the addition of iron. The drop in the $\Delta\psi$ trace may be accounted for by an increased $Ca^{2+}$ cycling, as a result of the release of mitochondrial $Ca^{2+}$. Indeed, the addition of EDTA, which complexes $Ca^{2+}$ outside the mitochondria, in this case immediately reverses the potential trace to control levels (see Fig. 4B). Furthermore, the fall of the membrane potential induced by iron in mitochondria pre-loaded with $Ca^{2+}$ is prevented by ruthenium red (see Fig. 3B), which blocks the cycling of $Ca^{2+}$ by inhibiting the electrophoretic $Ca^{2+}$-uptake route [11]. Secondly, direct measurements of $Ca^{2+}$ fluxes with a $Ca^{2+}$-selective electrode show that iron causes a rapid efflux of $Ca^{2+}$ from mitochondria (see Fig. 5A).

As to the mechanism by which iron induces the release of mitochondrial calcium it may be concluded that iron does not directly induce $Ca^{2+}$ efflux, but it does so by catalyzing the induction of lipoperoxidative processes [1] which elicit calcium release. Indeed, when lipoperoxidation is blocked with antioxidant agents, no calcium release from mitochondria is seen (see Figs. 3B and 5B). Relevant to this point are the observations that hydroperoxides induce a specific $Ca^{2+}$ release from mitochondria [4–6,13]. The $Ca^{2+}$ release in this case was paralleled by oxidation of intramitochondrial pyridine nucleotides. On the other hand, it is well established that hydroperoxides induce lipoperoxidation in mitochondria [3,7]. It has also to be considered that $Fe^{2+}$ was found to catalyze the oxidation of NAD(P)H in liver mitochondria, which resulted in polyunsaturated fatty acid degradation [14]. Furthermore it was reported [15,16] that bovine heart mitochondria and submitochondrial particles catalyzed the peroxidation of endogenous lipids with NAD(P)H as an electron donor in the presence of ADP-$Fe^{3+}$ chelate. The above considerations and the results here re-

ported may suggest that a conceivable factor in the induction of the $Ca^{2+}$ fluxes in mitochondria loaded in vitro with iron is the fluidity state of the membrane, which can be affected by peroxidation reactions [2].

The induction of lipoperoxidation by iron(III)-gluconate brings about, the release of $K^+$ from mitochondria, in addition to that of $Ca^{2+}$ (see Fig. 1), in agreement with previous findings [3] showing a net efflux of $K^+$ from mitochondria as a result of the induction of peroxidation reactions in mitochondrial membranes. The efflux of $K^+$, however, seems to be secondary to that of $Ca^{2+}$. Indeed we have found that when the fall of the membrane potential is prevented, i.e., when the energy-dissipating process due to enhancement of $Ca^{2+}$ cycling is blocked, with agents which primarily inhibit $Ca^{2+}$ uptake, such as ruthenium red or oligomycin [1], the $K^+$ release is also largely prevented. Preliminary observations at the $K^+$ transport level (unpublished observations) also indicated that preloading of mitochondria with a pulse of $Ca^{2+}$ markedly stimulated $K^+$ efflux induced by iron.

The findings that no net release of $K^+$ from mitochondria occurs when the external $K^+$ concentration is increased (see Fig. 2) may suggest that the permeability of the inner mitochondrial membrane to $K^+$ has been modified. In this regard it has recently been suggested that some $Ca^{2+}$-releasing agents, among them hydroperoxides, operate by a mechanism which can also produce a nonspecific increase in inner membrane permeability [17,18]. The loss of endogenous $K^+$ and $Mg^{2+}$ was reported to accompany the release of $Ca^{2+}$ in the presence of these agents; the release of endogenous $Mg^{2+}$ was thus taken as an indication of the induction of nonspecific alterations in membrane permeability [17,18]. On the other hand, the observation that no loss of endogenous $Mg^{2+}$ occurred under the experimental conditions used here (unpublished observations) seems to indicate that the alterations in membrane permeability induced by iron are rather specific. As to this problem it is noteworthy that the experiments showing the loss of $Mg^{2+}$ induced by $Ca^{2+}$-releasing agents [17,18] were carried out under metabolic conditions, i.e., in the absence of external $Mg^{2+}$, where a respiration-dependent net efflux of endogenous $Mg^{2+}$ occurred per se either in heart [19] or in liver mitochondria [20].

The present results appear of interest for the comprehension of the biochemical mechanisms underlying mitochondrial malfunction induced in vivo by iron overload [21–28]. The in vitro induction by iron, described here, of release of $Ca^{2+}$ may be regarded as one of the possible mechanisms of hepatic cell damage induced by iron overload in vivo, given the major role that mitochondria play in the control of $Ca^{2+}$ compartmentation and regulation of cytosolic $Ca^{2+}$.

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