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# The effect of ferric iron complex on isolated rat liver mitochondria. I. Respiratory and electrochemical responses

Alberto Masini [a,*], Tommaso Trenti [b], Daniela Ceccarelli-Stanzani [c] and Ezio Ventura [b]

*Istituti di [a] Chimica Biologica, [b] Clinica Medica III e [c] Patologia Generale, Via Campi 287, Università di Modena, 41100 Modena (Italy)*

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**Addition of iron(III)-gluconate complex to isolated rat liver mitochondria resulted in an increased iron content of mitochondria. Iron was accumulated through a relatively fast process (maximal uptake in less than 2 min incubation) by an energy-independent mechanism. The in vitro iron overload of mitochondria was associated with enhancement in the oxygen consumption, which was due to the induction of lipoperoxidative processes catalyzed by iron. It was found that a concentration of iron as low as 0.1 mM elicits a consistent production of malondialdehyde in mitochondria. Concomitant with the induction of lipoperoxidation a progressive fall in the mitochondrial membrane potential was observed. The occurrence of energy-consuming processes as a consequence of iron addition, and particularly the enhancement of endogenous $Ca^{2+}$ cycling across the membrane, was suggested as the cause of the membrane potential drop.**

## Introduction

It is a generally accepted wiew that excess iron deposited in hepatic cells is associated with liver injury. However, the role that iron plays in the pathogenesis of biochemical damage to the liver cells has not yet been clearly established [1]. The mechanisms currently proposed to explain the iron toxicity are the peroxidation of unsatured lipids in cell membranes [2–4] and/or lysosomal disruption with the release of cell-damaging hydrolytic enzymes [5]. Since the mitochondria are essential in the handling of iron within the cell, many studies were performed on the effects of iron overload on the structural and functional properties of mitochondria in vivo [6–13] as well as in vitro [14–17]. In the experimental model of iron overload either the mechanism of intramitochondrial transport of iron, or the localization or the consequence of excess iron on mitochondrial functions are still open problems [7,8,18,19]. In this regard, recent studies from this laboratory [20,21] have shown that liver mitochondria from rats made siderotic by injecting intraperitoneally iron as iron(III)-gluconate complex exhibit an anomalous membrane potential, a lack of antioxidant capability and a low content of endogenous potassium ions when the hepatic iron content was above a threshold value.

The aim of the present research is to investigate the in vitro effects of iron(III)-gluconate complex on the iron content, metabolic performances and membrane stability of isolated rat liver mitochondria.

* To whom correspondence should be addressed.

Abbreviation: $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside.

## Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure [22].

The oxygen uptake was assayed with a Clark oxygen electrode.

The transmembrane potential ($\Delta\psi$) was measured by monitoring with a tetraphenylphosphonium-selective electrode the movements of tetraphenylphosphonium across the mitochondrial membrane as in Ref. 23. An inner mitochondrial volume of 1.1 $\mu$l/mg protein was assumed.

The metabolic medium for assaying both the respiratory and the electrochemical parameters 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. Incubation temperature was 25°C. The mitochondrial concentration as determined by the biuret method, ranged between 3 and 3.5 mg protein/ml.

The amount of malondialdehyde formed in vitro in mitochondria was measured by the thiobarbituric acid method [24] in the presence of 0.1% butylated hydroxytoluene and 0.5 mM $FeCl_3$ in the thiobarbituric acid reagent as in Ref. 8. Mitochondria (1 mg protein/ml) were incubated at 37°C under State 4 conditions. Samples of 1 ml were taken at a specified time and the reaction was stopped by rapid mixing with 2 ml thiobarbituric acid mixture. The amount of malondialdehyde was determined in the supernatant, after centrifugation spectrophotometrically.

The iron accumulation by mitochondria was determined as follows: the mitochondria (3 mg protein/ml) were incubated in the metabolic medium at 25°C. At timed intervals aliquots of 0.5 ml were withdrawn and the iron accumulation was stopped by rapid mixing with 1 ml of ice-cold 0.25 M sucrose containing 20 mM EDTA. The sample was immediately centrifuged for 1 min in an Eppendorf bench centrifuge (Model 5414S). The pellet was washed twice with 1 ml of stopping medium and then dispersed by extensive vortexing in 1 ml 0.1% sodium cholate. The iron content of the pellet was determined by atomic absorption.

Only mitochondrial preparations showing a respiratory control index above 4 were used.

Ferlixit (iron(III)-gluconate complex) was purchased from Nattermann 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

When isolated rat liver mitochondria, respiring on endogenous substrates, were incubated in the presence of 0.6 mM Fe(III)-gluconate complex, iron was rapidly accumulated. The time dependency of the accumulation process and its relation to the concentration of protein in the medium are presented in Fig. 1. It is seen that the uptake of iron-gluconate is a rapid process (maximal uptake within 2 min). It is also seen that the maximal accumulation of iron varies with the concentration of mitochondria. Indeed, the maximal accumulation of iron decreased progressively with increasing protein concentration and reached a steady-state level at approx. 3 mg protein per ml.

The effect of iron concentration on the iron accumulation in rat liver mitochondria is presented in Fig. 2. A saturation level of 100–120 nmol/mg protein was reached at 0.6–0.8 mM of iron. This accumulation was insensitive both to the

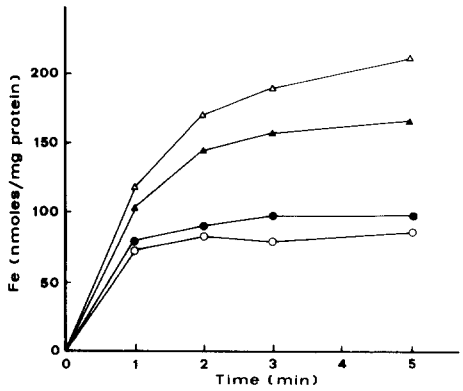


Fig. 1. Time course of iron accumulation by liver mitochondria as a function of mitochondrial protein concentration. The mitochondria were suspended in the standard incubation medium in the presence of 0.6 mM iron(III)-gluconate. The mitochondrial protein concentration was: △, 0.5 mg/ml; ▲, 1 mg/ml; ●, 3 mg/ml; ○, 5 mg/ml. Iron was determined by atomic absorption. Each point indicates the average value obtained for four different mitochondrial preparations.

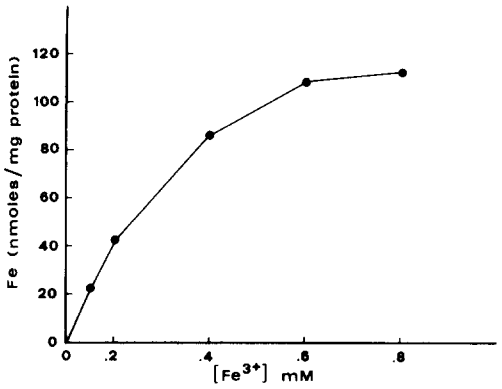


Fig. 2. Effect of iron(III)-gluconate concentration on the iron accumulation in rat liver mitochondria. The mitochondria were suspended in the standard incubation medium as described in the Methods. The incubation period with iron was 5 min. All other conditions were as in Fig. 1.

uncoupler dinitrophenol (20 $\mu$M) or to respiratory chain inhibitors such as KCN (2 mM) and rotenone (5 $\mu$M) when added to mitochondria 10 min before the iron.

The effect of iron on the mitochondrial respiration under different respiratory states is presented in Fig. 3. It is seen that the addition of iron under

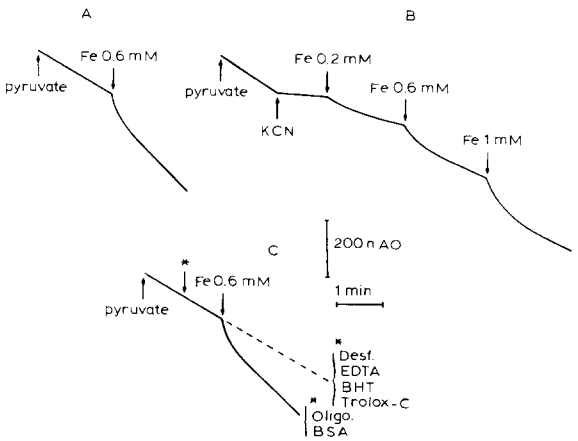


Fig. 3. Effect of iron(III)-gluconate addition on the oxygen uptake of rat liver mitochondria. The mitochondria were incubated for 1 min in the standard metabolic medium as described in the Methods. Arrows indicate the following additions: 1.6 mM pyruvate plus 0.4 mM L-malate (pyruvate); iron(III)-gluconate (Fe); 2 mM KCN; 2 mg/mg protein desferrioxamine (Desf.); 2 mM EDTA; 30 $\mu$M butylated hydroxytoluene (BHT); 200 $\mu$M Trolox-C; 2 $\mu$g/mg oligomycin (Oligo.); 0.1% bovine serum albumin (BSA).

State 4 conditions (Fig. 3A) immediately induces a transient jump in the oxygen consumption and then the oxygen uptake remains markedly stimulated. The pattern of the increase in the $O_2$ consumption is very similar under all the metabolic conditions tested, i.e., State 1 and State 3 respiratory states (not shown). The same figure (Fig. 3B) shows that, when the respiratory chain is inhibited by KCN, the addition of iron elicits a stimulation in the $O_2$ consumption very similar to that observed in functionally competent mitochondria. It also appears that addition of increasing concentrations of iron induces a proportional stimulation in the $O_2$ consumption. It is noteworthy that the enhancement in the $O_2$ consumption was of the very same order under all the metabolic conditions tested, i.e., approx. 7–9 ng atoms O/min per mg protein. Both the initial jump and the following stimulation in the $O_2$ uptake are prevented by iron-chelating agents such as desferrioxamine and EDTA, as well as by antioxidant compounds such as buthylated hydroxytoluene and Trolox-C (Fig. 3C). In contrast, neither oligomycin, a specific inhibitor of tightly coupled respiration, nor albumin, a scavenger of uncoupling agents, appreciably modify the enhancement of $O_2$ consumption. Polarographic measurements of the ferric complex in the absence of mitochondria excluded the possibility that this stimulation of $O_2$ consumption was due to impurities of ferrous iron (not shown).

Fig. 4 presents the time course of malondialdehyde formation induced by various concentrations of iron. It appears that concentrations of iron as low as 0.1 mM induce a significant enhancement of the lipid peroxidation process. Fig. 5 shows that this process was suppressed by antioxidant agents such as butylated hydroxytoluene or Trolox-C. Oligomycin revealed itself to be a weak antioxidant, its inhibition being of the order of approx 30%. Desferrioxamine and EDTA largely prevented malondialdehyde formation.

The effect of iron on the transmembrane potential of liver mitochondria is presented in Fig. 6. Rat liver mitochondria on addition of substrate develop a membrane potential of about 180 mV. Addition of ADP, which causes transition to State 3, induces a sudden decrease of $\Delta\psi$. After the phosphorylation of ADP has been completed, $\Delta\psi$

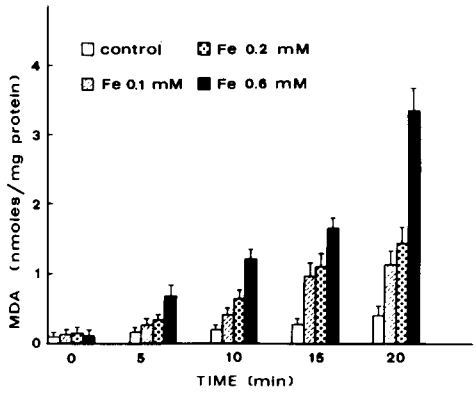


Fig. 4. Malondialdehyde (MDA) formation in rat liver mitochondria induced by various concentrations of iron(III)-gluconate. The mitochondria were incubated under State 4 conditions at 37°C and malondialdehyde was determined as described in the Methods. The reaction was started by the addition of the mitochondria. Fe, iron(III)-gluconate. The results represent the means ± S.D. of five separate experiments.

returns to nearly its original value. Addition of 0.6 mM iron(III)-gluconate at this point does not appreciably modify the $\Delta\psi$ trace for the first 2–3 min. The $\Delta\psi$ starts progressively decreasing (Fig. 6A). When either desferrioxamine, EDTA, butylated hydroxytoluene, Trolox-C, oligomycin or ruthenium red is present before the addition of

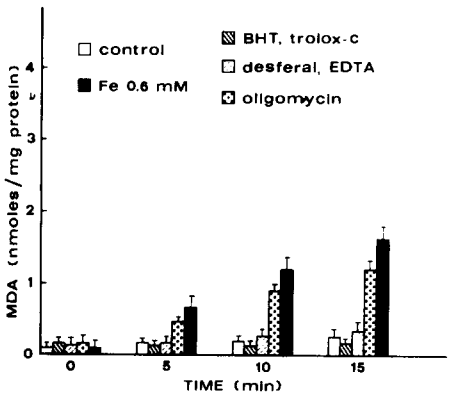


Fig. 5. Effect of antioxidants, iron chelators and oligomycin on the malondialdehyde (MDA) formation in rat liver mitochondria induced by iron(III)-gluconate. When present, 30 $\mu$M butylated hydroxytoluene (BHT), 200 $\mu$M Trolox-C, 2 mg/ml desferrioxamine (desferal), 2 mM EDTA and 4 $\mu$g/ml oligomycin were added to the mitochondrial incubation before the addition of 0.6 mM iron(III)-gluconate (Fe). All other conditions were as in Fig. 4

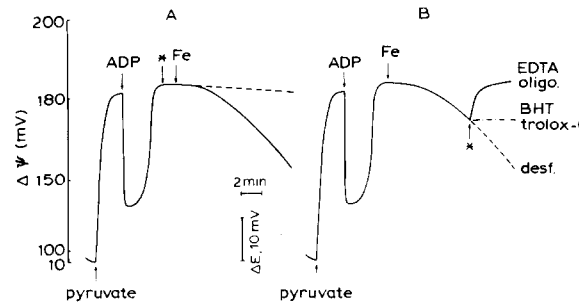


Fig. 6. Effect of iron(III)-gluconate on the transmembrane potential of rat liver mitochondria. The mitochondria were incubated in the standard medium as described in Methods. Arrows indicate the following additions: 1.6 mM pyruvate plus 0.4 mM L-malate (pyruvate); 0.33 mM ADP; 0.6 mM iron(III)-gluconate (Fe). The dashed line in A indicates the membrane potential trace when either 30 $\mu$M butylated hydroxytoluene (BHT), 200 $\mu$M Trolox-C, 2 mM EDTA, 2 mg/mg desferrioxamine (desf.), 1 $\mu$M ruthenium red (RR) or 2 $\mu$g/mg oligomycin (oligo.) was added 1 min before the addition of iron at the point indicated by the marked arrow. 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.

iron they all prevent the $\Delta\psi$ drop (Fig. 6A). In contrast, when these compounds are added after iron, i.e., when the decrease of $\Delta\psi$ is occurring, oligomycin and EDTA restore the normal membrane potential, and butylated hydroxytoluene and Trolox-C arrest the decrease of $\Delta\psi$ but do not restore the original value; desferrioxamine does not appreciably modify the $\Delta\psi$ trace (Fig. 6B). When iron was added at a concentration of 0.2 mM, the $\Delta\psi$ trace remained unchanged for a longer period than in the case of 0.6 mM iron. The following decrease in the $\Delta\psi$ trace was also lower than that seen in mitochondria in the presence of a higher concentration of iron (not shown).

Similar effects on $\Delta\psi$ were obtained when succinate was used as a substrate and when iron was added in State 4(a), i.e., in the absence of endogenously formed ATP (not shown).

## Discussion

The results of the present work lead to the conclusion that iron(III)-gluconate complex is accumulated in vitro by rat liver mitochondria by a fast process, the maximal uptake being reached in

less than 2 min incubation. This iron complex is accumulated essentially by an energy-independent process. The following experimental lines of evidence support this conclusion: (a) the accumulation of iron is not affected by uncouplers or by respiratory-chain inhibitors such as KCN and rotenone; (b) the membrane potential trace does not decrease in the phase when iron is accumulated, thus indicating that no energy-utilizing process is occurring. These findings are in agreement with the conclusions of Strickland and Davis [25] and Cederbaum and Wainio [26,27] obtained by incubating liver mitochondria in the presence of iron(III)-ADP and iron (III)-8-hydroxyquinoline complex, respectively. They do not agree with the results of Romslo and Flatmark [28–31] and Ulvik et al. [32], which indicated the occurrence of both a high-affinity energy-dependent and a low-affinity energy-independent process when liver mitochondria were incubated in the presence of iron(III)-sucrose and iron(III)-transferrin complex, respectively. The discrepancy between the present data and those previously reported [28–32] might be due to the fact that different iron complexes have been used. It could be that in some iron complexes the iron charges are well screened so that the complex can easily penetrate the membrane, whereas other complexes might behave more like lipophilic cations and penetrate faster in response to a negative inside membrane potential. Also relevant to this point are the observations by Barnes et al. [3] that the extent of the influx of iron into the matrix space was almost completely dependent on the permeability of the inner mitochondrial membrane to the various iron complexes.

The stimulation of $O_2$ consumption by iron, as can be evaluated by the pattern of the polarographic traces, is prevented by EDTA, a chelator of ferrous iron, by desferrioxamine, a chelator of ferric iron, and by antioxidants. These findings clearly indicate that this extra oxygen consumption does not represent a stimulation of respiration but is due to lipoperoxidative processes induced by the redox system $Fe^{2+}/Fe^{3+}$. The presence and the functioning of the redox system $Fe^{2+}/Fe^{3+}$ was indicated by Barnes et al. [33] when ferric iron and some of its complexes were added in vitro to liver mitochondria. The occurrence of lipoperoxidative processes under the experimental conditions used here is directly supported by the measurement of the accumulation of a significant amount of malondialdehyde. In this regard it is worthy of note that we have found the occurrence of peroxidative reactions at concentration of iron, e.g. 0.1 mM, where no lipid peroxidation was detected by Romslo and Flatmark [16] with iron(III)-sucrose complex. However, it is well established that the ionic iron ($Fe^{2+}$,$Fe^{3+}$) or iron chelates in vitro can initiate lipid peroxidation in isolated mitochondria [34–38].

It was generally accepted that the development of lipid peroxidation reactions in mitochondrial membranes disturbs mitochondrial functions [34,37,39]. However, lipid peroxidation may have also a pathophysiological importance [40]. In this regard it was recently shown that induction of peroxidation reactions in mitochondrial membranes with $Fe^{2+}$ or with cumene hydroperoxide brings about the drop in membrane potential and the induction of $K^+$ transport [41]. The prevention by oligomycin of these processes, only in energized mitochondria, led to the suggestion of a functional link between lipid peroxidation, cation transport and the ATP-synthetase complex. The present results show that the induction of lipid peroxidation by iron is paralleled by a progressive decrease in the membrane potential. However, the mitochondrial membrane is not irreversibly depolarized, as indicated by the restitutive effect on it of oligomycin and EDTA (see Fig. 6B). These observations, and the finding that antioxidant compounds such as butylated hydroxytoluene and Trolox-C are able to arrest the drop of $\Delta\psi$ induced by iron but not to restore it, support the conclusion that the progressive decrease of $\Delta\psi$ is due to the activation of energy-requiring and/or dissipating processes linked to the ATP-synthetase functioning [42,43]. Furthermore the finding that ruthenium red, as well as EDTA, added to liver mitochondria respiring in State 4 in the presence of endogenously formed ATP, completely prevents the iron effect on $\Delta\psi$ seems to indicate that the enhancement of endogenous $Ca^{2+}$ cycling may contribute to a large extent to the dissipation of energy. The above considerations and the weak antioxidant capability presented by oligomycin in comparison with other antioxidants tested lead

also to the conclusion that this antibiotic does not act on the membrane potential by preventing lipid peroxidation catalyzed by iron but rather by blocking the occurrence of energy-utilizing processes linked to ATP synthesis.

As to the problem of the pathological effects on the liver cell of iron overload in vivo, the present results appear promising for the comprehension of the biochemical mechanism which underlies mitochondrial malfunction. Indeed, here we have shown that iron in vitro catalyzes a lipoperoxidative process whose extent is related to the amount of iron given. These data agree with the findings of Bacon et al. [19], who measured significant mitochondrial lipid peroxidation, as well as with our recent observations [20,21] of a lack of antioxidant capacity in liver mitochondria, when the hepatic iron was above a threshold level. Furthermore, the observed decrease of $\Delta\psi$ induced by iron in vitro and the restitutive effect of oligomycin resemble the anomalous pattern of $\Delta\psi$ exhibited by liver mitochondria from rats with hepatic chronic iron overload [20,21]. It is conceivable that both in vitro and in vivo iron overload elicts energy utilizing processes involving ion movements.

In the light of the present findings it appears of importance to study the types of ions involved in these energy-utilizing processes as well as the possible controlling mechanisms.

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