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# ENERGY-DEPENDENT ACCUMULATION OF IRON BY ISOLATED RAT LIVER MITOCHONDRIA

## I. GENERAL FEATURES

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SUMMARY

1. Rat liver mitochondria isolated in sucrose–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) medium rapidly accumulate iron from the suspending medium when $^{59}$Fe(III)–sucrose is used as a soluble and stable model complex.

2. The accumulation proceeds by two different mechanisms, *i.e.* by an energy-independent and an energy-dependent (uncoupler and cyanide-sensitive) mechanism which have different pH and temperature dependencies. Both processes require $Mg^{2+}$ and are inhibited by $P_i$ and chelating agents (*e.g.* EDTA), and reveal maximal rates at an iron concentration of 0.2–0.3 mM. At pH 7.2 the energy-dependent accumulation is found to be 10–15 nmoles iron per mg of protein and is completed in less than 45 s. On the other hand, the energy-independent accumulation proceeds at an approximate rate of 25 nmoles iron per mg of protein per min, with a maximal load of 180–240 nmoles iron per mg of protein.

3. Together with our previous studies (Romslo, I. and Flatmark, T. (1972) *Abstr. Commun. Meet. Fed. Eur. Biochem. Soc.* 8, No. 639) these results strongly suggest that the energy-dependent mechanism of accumulation is responsible for a movement of iron across the mitochondrial inner membrane.

## INTRODUCTION

The biosynthesis of heme in mammalian cells depends on a multienzyme system in which the component enzymes are partitioned between the cytosol and the mitochondria. Thus, it is well established that mitochondria contain enzymes catalyzing (1) the formation of δ-aminolevulinic acid from glycine and succinyl-CoA[1,2]; (2) the formation of protoporphyrin IX from coproporphyrinogen[2,3]; and (3) the incorporation of ferrous iron into the porphyrin ring[4]. The final insertion of ferrous iron into protoporphyrin IX is catalyzed by ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1), an enzyme reported to be firmly bound to the matrix side of the mito-

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; R.C., respiratory control.

chondrial inner membrane[5]. To reach this mitochondrial compartment, the iron ions have to penetrate the outer as well as the inner mitochondrial membrane. The observations by Goodman and Hall[6] of an accumulation of large amounts of iron in the mitochondria of erythroblasts in patients developing mild transitory anemia while on chloramphenicol therapy have suggested that the mitochondrial inner membrane is permeable to iron ions. Similar abnormal accumulations have been reported in patients with porphyria cutanea tarda[6], lead poisoning[7], refractory sideroachrestic anemia[8] and pyridoxine responsive anemia[9]; *i.e.* diseases with deranged heme biosynthesis[6]. Furthermore, an energy-independent accumulation of iron has been demonstrated when isolated mammalian mitochondria are incubated with ferric adenine nucleotide complexes[10] or ferric–ferrous 8-hydroxyquinoline complexes[11]. Finally, ferric iron has been found to stimulate State 4 as well as State 3 respiration in isolated rat liver mitochondria (Flatmark, T., unpublished).

Thus, several lines of evidence point to the presence of a mitochondrial transport system of iron. It is the purpose of this article to show that isolated rat liver mitochondria reveal an energy-dependent in addition to an energy-independent accumulation of iron, using a stable and soluble $^{59}$Fe(III)–sucrose complex as a model compound. A preliminary account of this work has already appeared[12].

## MATERIALS AND METHODS

### *Animals and preparation of mitochondria*

Male albino rats of 250–350 g body weight (Wistar, Möll) were fasted overnight, stunned and decapitated. Liver mitochondria were prepared essentially as described[13] in a medium consisting of 0.25 M sucrose and 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.2 at 20 °C). A 10% (w/v) homogenate was centrifuged ($\int_0^t$ (rev./min)$^2$d$t = 6.6 \cdot 10^7$ min$^{-1}$) and the nuclear fraction was washed three times. The combined supernatants were centrifuged ($\int_0^t$ (rev./min)$^2$d$t = 70 \cdot 10^7$ min$^{-1}$), and the mitochondrial pellet thus obtained was washed twice using the same centrifugation integral. The centrifugation integrals chosen were based on determinations of $s$ values of rat liver mitochondria providing maximal recovery of mitochondria with minimal contamination of other subcellular particles (Slinde, E., unpublished). The swinging-bucket HB-4 rotor of Sorvall RC 2-B refrigerated centrifuge ($R_{min} = 6.2$ cm and $R_{max} = 14.4$ cm) was used at a temperature of 2 °C. The final mitochondrial pellet was resuspended in the homogenization medium at a mitochondrial concentration of 15–20 mg of protein per ml. Nupercain was added at a concentration of approx. 7 nmoles/mg of protein in order to depress the activity of phospholipase $A_2$[14]. All preparative work was conducted at 0–4 °C.

The morphology of the mitochondrial preparations was controlled by electron microscopy. Glutaraldehyde–$OsO_4$-fixed preparations were embedded in Epon, sliced and stained essentially as described[15] and examined in a Siemens Elmiskope I electron microscope. The percentage distribution of mitochondria and other particles was counted in 4 different electron micrographs at a magnification of 40000. In each micrograph a minimum of 200 particles was classified. 92% of the particles were mitochondria with well preserved cristae.

The functional integrity of the preparations was tested by measuring the

respiratory control ratio with ADP ($R.C._{ADP}$)[16] using succinate as the substrate. Only mitochondria with $R.C._{ADP}$ values > 2.5 were used.

*Preparation of $^{59}Fe$(III)–sucrose*

Soluble and stable complexes of ferric iron and sucrose were prepared essentially as described by Charley *et al.*[17]. To a solution of ferric nitrate and sucrose (pH approx. 1.9) was added $^{59}FeCl_3$ (specific activity 5.1–7.7 Ci/g of iron) to give about 100000 cpm/$\mu$mole of iron. The solution was titrated to pH 7.2 and adjusted with distilled water to a final concentration of 5 mM iron and 1 M sucrose. The product thus obtained has been resolved into four Fe(III)–sucrose polymers with different isoelectric points (Romslo, I., unpublished). Approx. 50% consists of a polymer with $pI_{4°C}$ around 5.7.

*Enzymic assays and other analytical methods*

Acid phosphatase (EC 3.1.3.2) was assayed as described by Richterich *et al.*[18]. Glucose-6-phosphatase (EC 3.1.3.9) was assayed as described by de Duve *et al.*[19]; the $P_i$ liberated was determined by the method of Rathbun and Betlach[20]. Urate oxidase (EC 1.7.3.3) was assayed as described by Beaufay *et al.*[21], cytochrome oxidase (EC 1.9.3.1) as described by Yonetani and Ray[22], monoamine oxidase (EC 1.4.3.4) as described by Aas[23], and glutamate dehydrogenase (NAD) (EC 1.4.1.3) as described by Beaufay *et al.*[21]. The particle-bound activity (*i.e.* latent activity) of acid phosphatase and urate oxidase was calculated from the difference in activity in the presence and absence of 0.1% Triton X-100 (see legend to Table II). Protein was determined as described by Eggstein and Kreutz[24], using bovine serum albumin from Sigma as a standard[25]. The total iron content of the mitochondria was determined after wet ashing[26] as described by Ballentine and Burford[27].

*Spectrophotometry*

The measurement of oxidation–reduction level of *b*-type cytochromes was performed as described[13].

*Iron accumulation*

The mitochondria were incubated in a medium containing in a volume of 3.3 ml: 225 mM sucrose, 5 mM $MgCl_2$ and 5 mM HEPES buffer, pH 7.2. Alterations and additions are indicated in the legends to figures and tables. Incubations were carried out in thermostated and magnetically stirred chambers ($\pm$0.1 °C). At different time intervals aliquots of 1 ml were withdrawn, and the iron accumulation was instantaneously terminated by rapid mixing with 4 ml of chilled (0 °C) 225 mM sucrose containing 20 mM EDTA and 5 mM $MgCl_2$ (see below). The iron accumulation was determined by measurement of radioactivity in the mitochondria following density gradient centrifugation (see below).

*Density gradient centrifugation*

In order to separate mitochondria from the Fe(III)–sucrose complex left in the incubation medium, the mitochondrial suspension was subjected to density gradient centrifugation. Discontinuous density gradients were prepared by layering precooled (0–2 °C) sucrose solutions of decreasing molarity one above the other

in a centrifuge tube (Fig. 1a). The gradient was composed of 2.5 ml of 1.62 M sucrose and 5% (w/v) Ficoll (Solution A), 5.0 ml of 1.32 M sucrose containing 5 mM $MgCl_2$ (Solution B), and 4 ml of 225 mM sucrose containing 20 mM EDTA and 5 mM $MgCl_2$ (Solution C). All these solutions contained 2 mM HEPES buffer, pH 7.2. 1 ml of the mitochondrial incubation mixture was initially mixed with the upper sucrose solution (C), and the gradient centrifuged at a centrifugation integral $\int_0^t (\text{rev./min})^2 dt = 215 \cdot 10^7$ $\text{min}^{-1}$ in the swinging-bucket HB-4 rotor of Sorvall RC 2-B refrigerated centrifuge ($R_{min} = 6.1$ cm and $R_{max} = 13.9$ cm) at 2 °C. Four fractions were collected (see Fig. 1b) and analyzed for radioactivity, protein and various enzymic activities (see Table II).

*Measurement of radioactivity*

The mitochondria were sucked off the sucrose density gradient by a J-shaped Pasteur pipette and diluted to 10 ml with distilled water. After hypoosmotic lysis overnight, 4 ml of the mitochondrial suspension was transferred to scintillation vials containing 5 ml of Unisolve and counted in a Mark I Liquid Scintillation Counter (Nuclear Chicago Corp.). The counting efficiency was approx. 70%. The values given for the mitochondrial fractions were the average of duplicate determinations. The precision of the measurement was calculated from 30 replicate determinations on the same mitochondrial preparation; at the level tested (18.8 nmoles of iron per mg of protein) the precision was found to be approx. 7.4%.

*Chemicals*

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). HEPES (A grade) was purchased from Calbiochem (Luzern, Switzerland) and $^{59}FeCl_3$ from Institutt for Atomenergi, Kjeller, Norway. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr P. G. Heytler of Du Pont. Unisolve was obtained from Koch-Light Laboratories. Other chemicals were of highest purity commercially available. Deionized water was used in making up all solutions. All glasswares were soaked in concentrated HCl and thoroughly rinsed in deionized distilled water.

## RESULTS

Generally, studies on ion movements in mitochondria are carried out in structureless, metabolically inactive media which, with the exception of osmolarity has limited similarity to the cytoplasmic environment. In view of lack of knowledge of how iron is bound in the cytoplasm[28–32], as well as the extreme insolubility of $Fe(OH)_3$, which keeps the free $Fe^{3+}$ concentration below $10^{-15}$ M at pH 7.2 (ref. 33), a rational approach is to use a ferric iron chelate complex which is soluble at pH 7.2 and impermeable to the mitochondrial inner membrane. Since it is well known that reducing sugars and polyols form stable complexes with many metal ions[17] (including Fe(III)) and sucrose is used in the isolation and incubation medium, an Fe(III)–sucrose complex was selected. With this reservation in mind, the partition of $^{59}Fe^{3+}$ between incubation medium and isolated mitochondria will be described in the present study.

*Studies on the stability of the Fe(III)–sucrose complex*

A concentrated solution of Fe(III)–sucrose complex with a molar ratio iron: sucrose of 1:200 was stable for months when stored at −20 °C. As expected[17], however, the stability decreased upon dilution. Thus, the stability depends not only on the iron:sucrose ratio, but also on the absolute concentrations of the two components (Table I). Furthermore, the stability was highly influenced by pH and temperature. Whereas no change in recovery was found at pH $\gtrsim$ 7.2 by varying the temperature between 0 and 55 °C, at pH < 7 all the iron precipitated at temperatures above 37 °C during an incubation period of 5 min. This precipitation was reversible upon addition of 20 mM EDTA (Solution C of the density gradient, Fig. 1).

TABLE I

THE STABILITY OF THE IRON–SUCROSE COMPLEXES AS A FUNCTION OF THE MOLAR RATIO OF IRON TO SUCROSE

The solution of iron(III)–sucrose were prepared as described in the Methods section. Aliquots of freshly prepared solutions were centrifuged ($\int_0^t$ (rev./min)$^2$ $dt = 270 \cdot 10^7$ min$^{-1}$, see Methods) and the per cent recovery of radioactivity in the supernatant was calculated.

| | *Per cent recovery in soluble form* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Sucrose concn (M):* | | | | | *Iron concn (mM):* | | | | | |
| | *1.0* | *0.5* | *0.25* | *0.1* | *0.05* | *5* | *10* | *25* | *50* | *75* | *100* |
| 5 mM $Fe^{3+}$ | 99.0 | 95.7 | 92.0 | 89.0 | 74.2 | | | | | | |
| 1 M sucrose | | | | | | 99.0 | 101.9 | 97.1 | 96.5 | 46.9 | 1.0 |

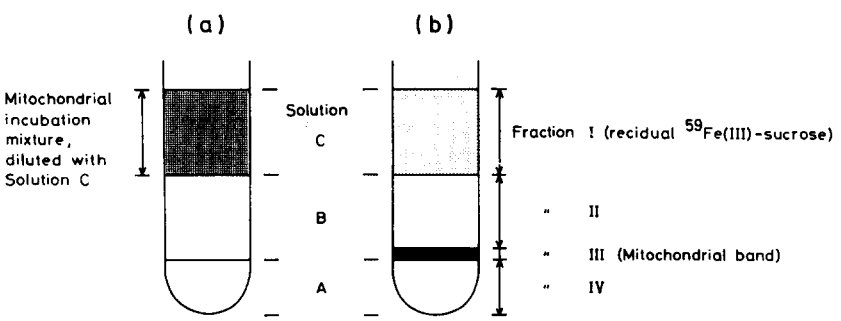



Fig. 1. Outline of the procedure used to fractionate iron-loaded mitochondria on a density gradient of sucrose–Ficoll (for experimental details, see Methods). (a) Initial state. (b) After centrifugation at a time integral of (rev./min)$^2$ equal to $215 \cdot 10^7$ min$^{-1}$ ($t = 15$ min).

*Density gradient centrifugation*

When the mitochondrial incubation mixture was subjected to density gradient centrifugation, the iron-loaded mitochondria were separated from the residual Fe(III)–sucrose complex as well as from the small amount of iron hydroxide formed during the incubation period (Fig. 1b). The recovery of protein in the mitochondrial interphase band (Fraction III) was influenced by the composition of Solution B.

TABLE II

DISTRIBUTION OF PROTEIN AND ENZYMIC ACTIVITIES FOLLOWING DENSITY GRADIENT CENTRIFUGATION OF THE MITOCHONDRIAL INCUBATION MIXTURE

For experimental details and specification of fraction numbers, see Methods and Fig. 1b. All enzymic activities were expressed as relative specific activities; the specific activity of the initial incubation mixture was set equal to 1.0. Only the particle bound (latent) activities of acid phosphatase and urate oxidase were calculated. The average recoveries ($n=7$) were 92.2% for acid phosphatase, 110.1% for urate oxidase, 105.3% for glucose-6-phosphatase, 103.2% for monoamine oxidase, 95.7% for glutamate dehydrogenase and 89.1% for cytochrome oxidase.

| *Fraction No.* | | *Protein recovery* (%) | *Acid phosphatase* | *Urate oxidase* | *Glucose-6-phosphatase* | *Monoamine oxidase* | *Glutamate dehydrogenase* | *Cytochrome oxidase* |
|---|---|---|---|---|---|---|---|---|
| I | Mean | 9.0* | 3.2 | 3.8 | 2.8 | 0.4 | 0 | 0 |
| | Range | | 1.9– 4.3 | 2.4– 5.2 | 1.9– 5.0 | 0 –0.7 | | |
| II | Mean | 1.5 | 12.6 | 13.9 | 24.0 | 2.1 | 1.6 | 1.1 |
| | Range | 0.9– 2.4 | 5.9–19.5 | 6.1–18.9 | 10.6–44.7 | 1.6–4.1 | 0.9–2.3 | 0 –2.1 |
| III | Mean | 89.0 | 0.54 | 0.63 | 0.51 | 1.08 | 1.05 | 0.97 |
| | Range | 86.0–94.8 | 0.39– 0.70 | 0.48– 0.79 | 0.41– 0.67 | 1.04–1.11 | 0.98–1.09 | 0.95–1.04 |
| IV | Mean | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Range | — | | | | | | |

* The protein concentration could not be determined accurately due to a high concentration of EDTA in fraction I and is based on a yield of 100%.

Thus, in the absence of $Mg^{2+}$, Fraction II was slightly opaque, whereas in the presence of 5 mM $MgCl_2$ no turbidity was observed. In this case, the recovery of protein in the mitochondrial interphase band was 86–95% if the total amount of protein applied did not exceed 8–12 mg (Table II). Besides being a convenient way of separating iron-loaded mitochondria from the residual iron–sucrose complex, the gradient provided other properties of great importance in the present study. First, the total iron accumulation was inhibited more than 99% by 20 mM EDTA (*i.e.* the concentration in Solution C). Secondly, during prolonged storage (20 min) only approx. 2% of the iron accumulated leaked out from the mitochondria. Thirdly, from Table II it is seen that the density gradient centrifugation resulted in a considerable purification of the mitochondria, especially prominent from the measurements of the particle-bound (latent) peroxisomal and lysosomal enzymic activities.

*Energy-dependent and energy-independent accumulation of iron*

When freshly prepared liver mitochondria, respiring on endogenous substrates, were incubated with $^{59}Fe$(III)–sucrose, iron was rapidly accumulated. During a 1-min incubation period, a 4-fold increase in the mitochondrial iron content was observed (Table III), and within an incubation period of 10 min 62–70% of the iron present was accumulated (Fig. 2), *i.e.* 12–117 nmoles iron per mg of protein. A saturation level (180–240 nmoles iron per mg of protein; $n=10$) was reached at 200–300 $\mu$M

TABLE III

IRON CONTENT OF NATIVE AND IRON-LOADED RAT LIVER MITOCHONDRIA

Total mitochondrial iron was determined before and after incubation with iron(III)–sucrose at a final iron concentration of 0.3 mM for 1 min. For experimental details, see Methods.

| | *Iron content (nmoles iron per mg of protein)* | |
|---|---|---|
| | *Mean** | *Range* |
| Native mitochondria | 7.2 ($n=6$) | 4.3– 9.1 |
| Iron-loaded mitochondria | 29.1 ($n=6$) | 21.3–36.6 |

* The $n$ values represent the number of experiments on different mitochondrial preparations.

of iron (Fig. 3), and half-maximal accumulation was obtained at an iron concentration of 90 $\mu$M.

When the mitochondria were preincubated either with the uncoupler FCCP (12.5 $\mu$M) or with cyanide (4 mM), the accumulation was largely reduced (Fig. 3) indicating that part of the iron accumulation is dependent on the mitochondrial "energy potential". Similar results were obtained with CCCP (0.2–20 $\mu$M) and 2,4-dinitrophenol (125 $\mu$M). Contrary to the energy-independent accumulation, the energy-dependent accumulation was a rapid process, *i.e.* completed within 45 s (Fig. 4), and contributed with a 2-fold increase in the total iron content of the mitochondria. It should be stressed, however, that the tightly coupled liver mitochondria had to be preincubated with the uncoupler for 10 min to obtain complete deenergiza-

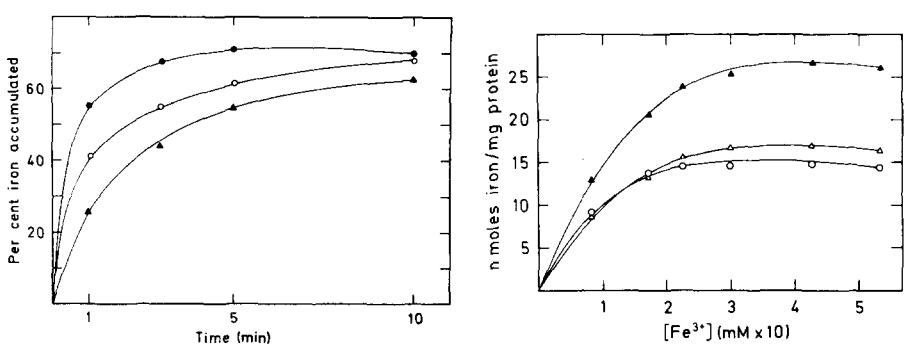
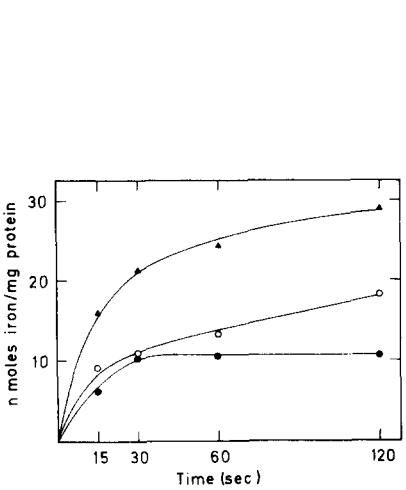



Fig. 2. Time-course of the total iron accumulation in isolated rat liver mitochondria at various concentrations of iron(III)–sucrose. The mitochondria were suspended in the standard incubation medium (see Methods) at a protein concentration of 1.36 mg/ml; 20 °C. The iron concentrations were 16 $\mu$M (●), 80 $\mu$M (○) and 160 $\mu$M (▲), respectively.

Fig. 3. Effect of iron(III)–sucrose concentration on the iron accumulation in rat liver mitochondria. The mitochondria were suspended in the standard incubation medium (see Methods) at a protein concentration of 1.36 mg/ml. The incubation period was 1 min; 20 °C. (▲), total iron accumulation; △, iron accumulation in the presence of 4 mM KCN; ○, iron accumulation in the presence of 12.5 $\mu$M FCCP; the mitochondria were preincubated for 10 min with the uncoupler before the iron(III)–sucrose was added.

tion using the energy-dependent redox changes of the cytochrome *b* complex as an internal probe of the "energy potential". This result is in good agreement with previous observations in liver mitochondria of guinea pigs (Flatmark, T. and Pedersen, J. I., unpublished).



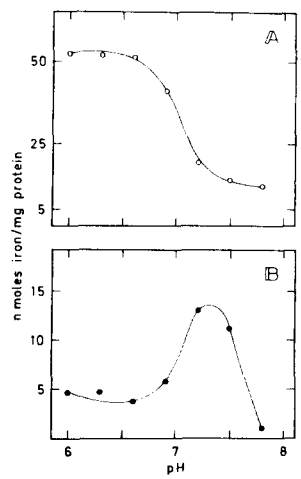




Fig. 4. Time-course of the uncoupler-insensitive and the uncoupler-sensitive iron accumulation. For experimental details, see Fig. 3. The concentration of iron was 0.3 mM. ▲, total iron accumulation; ○, iron accumulation in the presence of 12.5 $\mu$M FCCP; ●, the difference between the iron accumulation in the absence and presence of 12.5 $\mu$M FCCP.

Fig. 5. Effect of pH on the energy-independent (A) and the energy-dependent (B) accumulation of iron. For experimental details see Fig. 3, except that a fixed concentration of iron (0.3 mM) was used.

*Effect of pH*

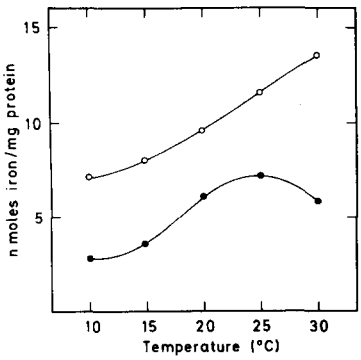
The accumulation of iron was strongly dependent on pH (Fig. 5). Whereas the energy-independent accumulation increased with increasing proton concentration, reaching a level at pH 6–6.5, the energy-dependent accumulation revealed a pH optimum at 7.2–7.4.

*Effect of temperature*

The initial rate (as well as the maximal amount) of iron accumulation was strongly influenced by changes in the temperature (Fig. 6). Thus, the energy-independent accumulation increased almost linearly from 10 to 30 °C, whereas the energy-dependent accumulation revealed a maximum at around 25 °C. The apparent energy of activation was calculated to be 4.6 kcal and 12.6 kcal for the energy-independent and the energy-dependent accumulation, respectively, using the temperature range 10–20 °C.

*Effect of ions*

As seen from Fig. 7A the energy-independent as well as the energy-dependent iron accumulation was stimulated by $Mg^{2+}$, being optimal at around 5 mM. Furthermore, it is also seen that $P_i$ inhibited both type of reactions at all concentrations



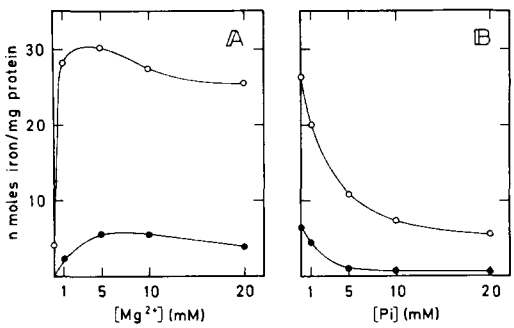




Fig. 6. Effect of temperature on the energy-independent (○) and the energy-dependent (●) accumulation of iron. For experimental details, see Fig. 3. The incubation period was 30 s and the concentration of iron was 0.3 mM.

Fig. 7. Effect of $Mg^{2+}$ (A) and $P_i$ (B) on the total (○) and energy-dependent (●) accumulation of iron. (A) The mitochondria were incubated in a medium containing 225 mM sucrose and 5 mM HEPES buffer, pH 7.2, at a protein concentration of 1.24 mg per ml; 20 °C. (B) The mitochondria were incubated in the standard incubation medium (see Methods) at a protein concentration of 1.24 mg/ml, 20 °C. For experimental details, see Fig. 3.

tested (Fig. 7B). Arsenate, acetate, nitrate, succinate and sulphate had all properties similar to that of $P_i$. Citrate and tartrate were even stronger inhibitors, and EDTA almost completely abolished the mitochondrial iron accumulation.

## DISCUSSION

The selection of $^{59}$Fe(III)–sucrose as a model complex for the present purpose appears to have several advantages as compared to the previously used iron chelate complexes of 2,3-dihydroxybenzoylserine[34], citrate[34], D-fructose[34], ferrioxamine G[35], 8-hydroxyquinoline[11] and adenine nucleotides[10]. Thus, the sucrose complexes (see Methods) are stable and highly soluble in water at neutral pH and have favorable dissociation constants. Furthermore, they are only slightly negatively charged at pH 7 (Romslo, I., unpublished results). Finally, the mitochondrial inner membrane is impermeable to the complexes as well as to sucrose[36], which is used in the isolation and incubation medium.

The commonly used techniques for separate measurements for intra- and extra-mitochondrial metabolic states could not be used in the present study. Thus, attempts to separate the $^{59}$Fe(III)–sucrose complexes from mitochondria by a sieve filtration technique (Millipore filter[37,38]) was unsuccessful since the high molecular weight iron(III)–sucrose complexes were largely trapped on the filter as previously reported for ferric hydroxide polymers[34]. Furthermore, the technique of quick chilling of the incubated mitochondria at 0 °C followed by centrifugation, washing and analysis of the pellet[10,11,39–41] was unsuccessful; coprecipitation and aggregation of the iron(III)–sucrose complexes to mitochondria could not be ruled out. The density gradient centrifugation technique selected in this study (Fig. 1) was, however, found to be well suited for several reasons. First, by rapid mixing with Solution C containing excess EDTA, the iron accumulation was instantaneously terminated. Secondly, in the absence of added mitochondria, all radioactivity was retained in

the top layer (Fraction 1) which could be easily removed without contamination of the interphase A/B (where the mitochondria form a band). Thirdly, the iron accumulated did not leak out after the termination of the reaction or during the centrifugation process.

As discussed in the introduction section, several lines of evidence point to the presence of a mitochondrial transport system of iron. From the results presented in this paper it is evident that the accumulation of iron in rat liver mitochondria proceeds by two different mechanisms, *i.e.* by an energy-independent and an energy-dependent mechanism. First let us consider the energy-independent iron accumulation.

That mammalian mitochondria accumulate iron in some energy-independent process, has been demonstrated using either ferric adenine nucleotide complexes[10] or a ferric/ferrous 8-hydroxyquinoline complex[11]. Considering the normal amount of mitochondrial iron (Table III and ref. 42), it is hard to conceive that the results thus obtained[10,11] represent physiological iron accumulations. In the present study only small variations in the total iron accumulation with external iron concentrations above 0.3–0.4 mM were observed and the maximal amount accumulated was less than 1/25 of that reported by Cederbaum and Wainio[11] for iron(II)–8-hydroxyquinoline as the iron source. Still, however, the iron accumulation is approx. 30-fold higher than the total iron content of native mitochondria. When the external iron concentration was increased beyond 0.6–0.8 mM, a transient increase in the energy-independent and a decrease in the energy-dependent iron accumulation was observed, and simultaneously the mitochondria tended to aggregate. These effects are probably caused by a deleterious influence by the high concentrations of iron on the integrity of the mitochondria, similar to that seen at high concentrations of calcium[43]. Thus, to avoid damaging of the mitochondria and thereby introducing artifacts, studies on mitochondrial iron metabolism should be limited to external iron concentrations of less than 0.3 mM. This value is approx. 10 times lower than those reported to reach saturation levels of some other cations as $Ca^{2+}$ (1.5–2.0 mM)[40,44], $Sr^{2+}$ (2–3 mM)[41] and $Cu^{2+}$ (4 mM)[11]. However, except for the $Cu^{2+}$ accumulation[11], these studies[40,41,44] were all carried out in the presence of $P_i$, whereby the cations accumulated in the matrix were removed as insoluble salts.

In contrast to the uptake of other cations[45–47] is the observation (Fig. 5) that a low pH favours the energy-independent accumulation of iron. Thus, based on the determination of the isoelectric point of the iron(III)–sucrose complex (see Materials and Methods) it may be concluded that the energy-independent accumulation can not be caused by simple electrostatic interactions.

The addition of the uncoupler FCCP resulted in a marked decrease of the iron accumulation (Fig. 3) showing that at least a certain percentage of the iron uptake is dependent on the "energy potential" of the mitochondrial inner membrane. That a part of the iron accumulation is energy-dependent is also shown by the fact that the respiratory inhibitor $CN^-$ (Fig. 3) revealed the same degree of inhibition as did the uncoupler FCCP. Furthermore, the progress curve as well as the pH and temperature dependence of this energy-linked accumulation was very different from the energy-independent uptake. An unanswered question is how the iron ions enter the energized mitochondria. The presence of a carrier system, is however, suggested since there is an approach to saturation kinetics with a half-saturation $K_m$ value of about 140 $\mu$M.

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