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PYROPHOSPHATE AS A LIGAND FOR DELIVERY OF IRON TO ISOLATED RAT-LIVER MITOCHONDRIA

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Rat liver mitochondria accumulate iron mobilized from transferrin by pyrophosphate. The capacity of the mitochondria to accumulate iron is higher than the capacity of pyrophosphate to mobilize iron from transferrin: with ferric-iron-pyrophosphate as iron donor, iron uptake and heme synthesis are about 10-times that at corresponding concentrations of iron-transferrin plus pyrophosphate. Uptake of iron from ferric-iron-pyrophosphate depends on a functionary respiratory chain and involves reductive cleavage of the ferric-iron-pyrophosphate complex. Apotransferrin inhibits uptake of iron from ferric-iron-pyrophosphate by competing with the mitochondria for iron. The results focus on pyrophosphate as a possible candidate for intracellular iron transport.

Introduction

Iron can be mobilized from transferrin at neutral pH by compounds carrying a pyrophosphate group [1–4]. Pyrophosphate-containing compounds thus function as vehicles for the transfer of iron from transferrin to a number of ligands, e.g., synthetic chelators [4], apotransferrin [1], ferritin [5] and mitochondria [2,6,7]. Among the pyrophosphates tested, e.g., ATP, ADP, GTP, TTP, CTP and pyrophosphate [2,3,6], pyrophosphate was the compound most effective in mobilizing iron from transferrin.

The passage of iron from transferrin through pyrophosphate to the mitochondria is of relevance to the question of how iron is transported from receptosomes to heme [8]. Receptosomes are recently described intracellular vesicles, different from lysosomes, but with an acidic interior and the intracellular destination for compounds (e.g., transferrin) not to be digested during endocytosis [9–11]. Pyrophosphate-mediated uptake of iron from transferrin by isolated mitochondria has been studied by Konopka and Romslo [6,7]. The uptake process represents a complex series of reactions: mobilization of iron from transferrin with formation of ferric-iron-pyrophosphate, reduction and sequestering of ferric-iron-pyrophosphate and binding of ferrous iron to ligands of the mitochondria. As yet, the significance of each of these steps to the overall uptake process has not been determined. Neither is it known what is the significance of these reactions to iron metabolism in situ.

The present paper describes the relationship between mobilization of iron from transferrin to pyrophosphate and the subsequent uptake of iron by isolated mitochondria. Included are also experiments on the uptake of iron from ferric-iron-pyrophosphate without transferrin.

Abbreviation: Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Materials and Methods

Preparation of mitochondria. Rat-liver mitochondria (from male Wistar-Møll rats of weight 200–300 g) were prepared as described by Romslo and Flatmark [12]. The mitochondrial pellet was resuspended in a medium of 0.25 M sucrose and 5 mM Hepes buffer (pH 7.40), at a concentration of approx. 40 mg protein/ml.

The functional integrity of the mitochondria was determined by measuring the respiratory control ratio with ADP (RC_{ADP}), using succinate as the substrate. Only mitochondria with RC_{ADP} greater than 3 were used.

Preparation of [^{59}Fe]transferrin. [^{59}Fe]Transferrin was prepared according to Konopka and Romslo [6,7]. The specific activity was approx. 40 cpm/pmol of iron, and the saturation with iron approx. 90%.

Radioactivity was determined in an Ames Gammacord II gamma counter. All samples were counted to a precision better than ±2%.

Preparation of ^{59}Fe-pyrophosphate. To a solution of $^{59}FeCl_3$ in 0.1 M HCl, solid tetrapotassium pyrophosphate was added to a concentration of 50 mM. pH was adjusted to 7.40 and the solution was enriched with $FeCl_3$ to a final concentration of 2.5 mM Fe. The specific activity was approx. 20 cpm/pmol iron.

Accumulation of iron. Mitochondria, 2–3 mg protein/ml, were incubated at 30 °C in a medium of 225 mM sucrose/10 mM KCl/10 mM Hepes buffer (pH 7.40)/5 mM succinate/1 mM pyrophosphate.

The reaction was initiated by adding 5 μM iron as [^{59}Fe]transferrin. Further additions or ommissions were as described in the legends to the figures and Table I.

At timed intervals, aliquots of 1 ml were withdrawn and centrifuged for 2 min in an Eppendorf microcentrifuge (type 5414). The pellet was washed once with ice-cooled incubation buffer.

Radioactivity was determined as described above.

Synthesis of deuteroheme. Mitochondria were incubated with [^{59}Fe]transferrin (see above) and 5 μM deuteroporphyrin. At timed intervals, aliquots of 1 ml were withdrawn and transferred to 3 ml cyclohexanone containing 20 μl 8 M HCl. The mixture was stirred on a Vortex mixer for 30 s, left on ice for 30 min and the phases were separated by centrifugation at 1000 × *g* for 10 min. 1 ml of the organic phase was removed for counting of radioactivity.

Separation of transferrin and ferric-iron-pyrophosphate. Transferrin and ferric-iron-pyrophosphate were separated by chromatography on a 1.5 cm × 6 cm column of Bio-Rad AG1-X4 equilibrated with 50 mM KCl and 50 mM Hepes buffer (pH 7.5), as described by Konopka et al. [5]. The column was eluted with increasing concentrations of KCl. Transferrin was eluted with 50 mM KCl and ferric-iron-pyrophosphate with 1 M KCl. When mitochondria were present (Table I) the suspension was centrifuged at 10000 × *g* for 2 min, and the supernatant was applied to the column.

Protein determination. Protein was determined by the Bio-Rad protein assay, using bovine serum albumin as standard [13].

Chemicals. $^{59}FeCl_3$ (3–20 mCi/mg) was purchased from Amersham International (Buckinghamshire, U.K.). Human transferrin (98% pure, essentially iron-free), antimycin A (type III) and pyrophosphate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deuteroporphyrin IX was the product of Porphyrin Product (Logan, UT, U.S.A.).

Other chemicals were of highest quality commercially available.

Quartz-distilled water was used throughout.

Results

When rat liver mitochondria were incubated with transferrin plus pyrophosphate, iron was mobilized from transferrin, chelated with pyrophosphate and subsequently taken up by the mitochondria.

After an initial delay of 10–15 min uptake proceeded linearly for at least 60 min (Fig. 1).

Since mitochondria have been shown to accumulate only negligible amounts of transferrin at neutral pH [14,15], it follows that iron uptake from transferrin via pyrophosphate should depend on the concentration of ferric-iron-pyrophosphate.

As shown in Fig. 1, preincubation for 30 min of transferrin plus pyrophosphate increased the uptake of iron by the mitochondria by approx. 30%.

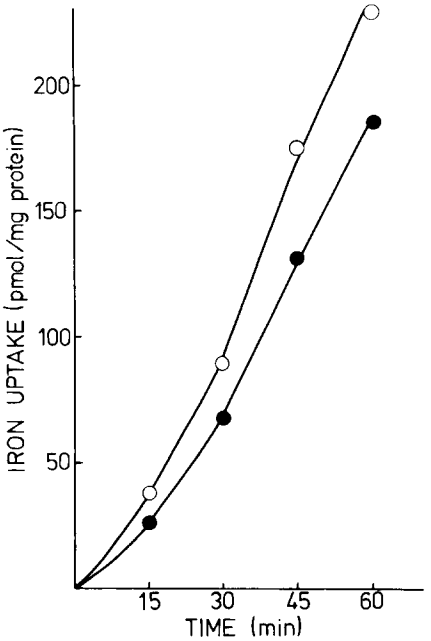

Fig. 1. Iron uptake from transferrin as mediated by pyrophosphate. Mitochondria, approx. 2.2 mg protein/ml, were incubated as described. The concentration of iron was 5 μM, and of pyrophosphate 1 mM. Temperature 30 °C. (●) Control, (○) iron uptake following preincubation of transferrin and pyrophosphate for 30 min.

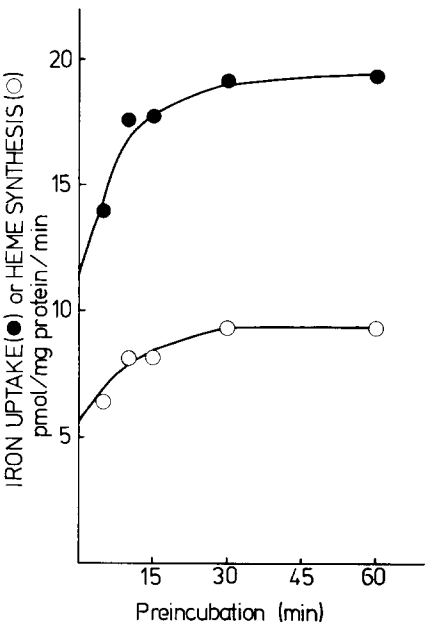

Fig. 2. Iron uptake and heme synthesis as a function of preincubation of transferrin and pyrophosphate. Experimental conditions were as described in Fig. 1 except that 5 μM deuteroporphyrin was added. Following preincubation of transferrin and pyrophosphate as indicated, mitochondria were added, and the rate of iron uptake (●) and heme synthesis (○) were determined in the interval 30–45 min.

The shape of the uptake curve, however, remained unchanged with an initial delay followed by a linear uptake.

The relationship between iron uptake by the mitochondria and preincubation of transferrin and pyrophosphate is shown in Fig. 2. At concentrations of iron and pyrophosphate as shown in Fig. 2, iron uptake increased with preincubation to reach a level of approx. 20 pmol/mg protein per min at 30–45 min preincubation.

Since ferric-iron-pyrophosphate is very little soluble at neutral pH [16], it could be speculated if the enhanced uptake with preincubation (Fig. 2) simply reflects coprecipitation of ferric-iron-pyrophosphate. This, however, seems unlikely because synthesis of heme strictly parallelled uptake (Fig. 2) and the shape of the time progress curve with ferric-iron pyrophosphate was identical to that from transferrin (Fig. 1 compared to Fig. 5).

Direct proofs for the transfer of iron from transferrin to pyrophosphate during preincubation are the results presented in Figs. 3 and 4. Incubation of transferrin plus pyrophosphate for 60 min followed by chromatography resulted in the transfer of radioactive iron moving from peak I typical of transferrin [5] to peak II typical of ferric-iron-pyrophosphate (Fig. 3). Also, the time progress for spectrophotometric disappearance of transferrin at 466 nm is compatible with generation of ferric-iron-pyrophosphate (Fig. 4).

Mobilization of iron from transferrin and generation of ferric-iron-pyrophosphate increased by approx. 100% by lowering pH from 8 to 7. By comparison, iron uptake and heme synthesis were maximal at pH 7.4–7.6, with optimum for heme synthesis slightly above that of iron uptake (data not shown).

Our results thus far are strong evidence that uptake of iron from transferrin as mediated by pyrophosphate is limited by the concentration of ferric-iron-pyrophosphate, i.e., the capacity of the mitochondria to accumulate iron surpassed the ability of pyrophosphate to mobilize iron from transferrin. Hence, it should be of importance to

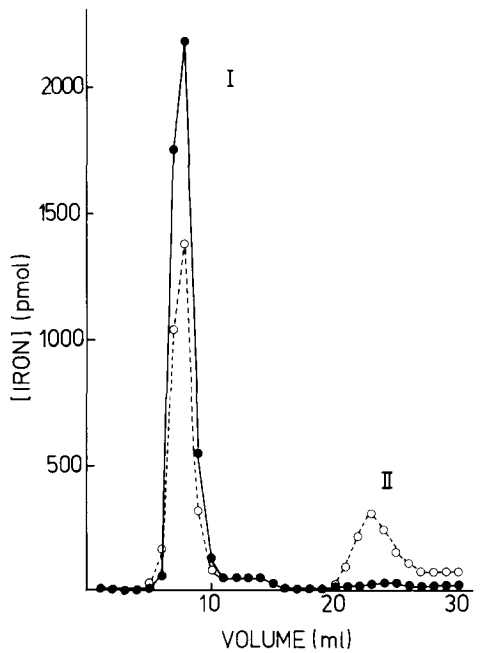

Fig. 3. Elution profiles on an AG1-X4 column of a mixture of 5 μM [^{59}Fe]transferrin and 1 mM pyrophosphate at zero time (●) and after 60 min incubation at 30 °C (○).

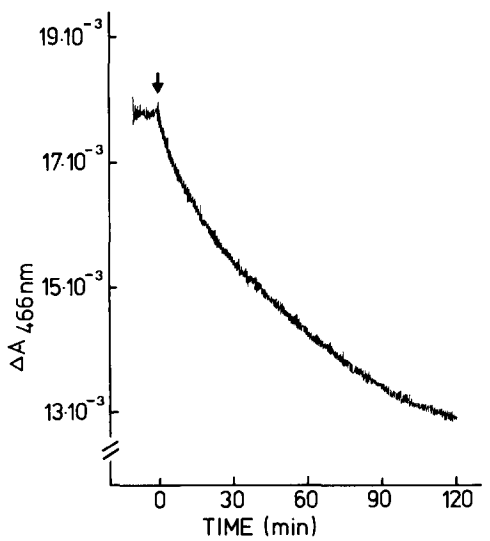

Fig. 4. Disappearance of transferrin as determined from the decline in absorbance at 466 nm. The incubation mixture was 5 μM diferric transferrin in 0.25 M sucrose and 5 mM Hepes buffer (pH 7.40). At the arrow, 1 mM pyrophosphate was added. Temperature 30 °C.

follow iron uptake from ferric-iron-pyrophosphate. A typical experiment is shown in Fig. 5. At 20 μM iron and 1 mM pyrophosphate, uptake of iron followed the same curvilinear pattern, but the amount of iron taken up was 5–10-times that observed with transferrin plus pyrophosphate. Note that not only had the mitochondria a considerable capacity to accumulate iron, but also a significant reserve for heme synthesis. As shown in Fig. 5, the rate of heme synthesis in the time interval 60–90 min amounted to 54 pmol/mg protein per min.

The concentration-progress curve for the rate of iron uptake from ferric-iron-pyrophosphate is shown in Fig. 6. The uptake increased curvilinearly with the concentration of ferric-iron-pyrophosphate. Vmax ≈ 200 pmol/mg protein per min and $K'_m \approx 20$ μM (with respect to iron). Synthesis of heme apparently parallelled iron uptake. Vmax ≈ 150 pmol/mg protein per min, and $K'_m \approx 20$ μM (with respect to iron).

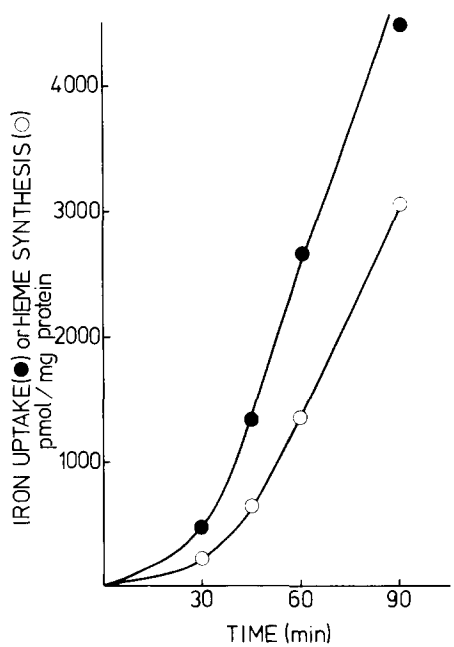

Fig. 5. Iron accumulation and heme synthesis with ferric-iron-pyrophosphate as substrate. 1.7 mg/ml mitochondrial protein was incubated with ferric-iron-pyrophosphate (20 μM with respect to iron, and 1 mM with respect to pyrophosphate) and 7.5 μM deuteroporphyrin. Temperature 30 °C. Iron accumulation (●) and heme synthesis (○) were determined at the time intervals indicated.

Since iron taken up from ferric-iron-pyrophosphate can be passed into heme, iron must be reduced. Reduction of iron, however, is obligatory not only for the synthesis of heme, but also for the uptake of iron [6,7]. Thus, inhibition of mitochondrial respiration by antimycin A reduced the uptake of iron from ferric-iron-pyrophosphate, by approx. 90% (Fig. 6).

Mobilization of iron from transferrin as mediated by pyrophosphate represents a complex series of equilibria, being dependent on the amount of transferrin and pyrophosphate, pH, competing ligands and reductants [1–7]. It has been shown that iron may pass from transferrin via pyrophosphate to a number of ligands: ferritin [5], chelators [4] and mitochondria [2,6,7]. It would also be expected that iron may pass from pyrophosphate back to apotransferrin. This is shown in the experiments reported in Figs. 7 and 8. Apotransferrin competitively inhibited the uptake of iron from ferric-iron-pyrophosphate. In the experiments reported in Fig. 7, K_i was found to be approx. 8 μM. Direct proof that apotransferrin actually trapped iron from pyrophosphate is shown in Fig. 8. During 60 min incubation, peak II (pyrophosphate) markedly declined and at the same time [^{59}Fe]transferrin in peak I increased. Apotransferrin similarly depressed mitochondrial uptake of iron from ferric-iron-pyrophosphate (Table I).

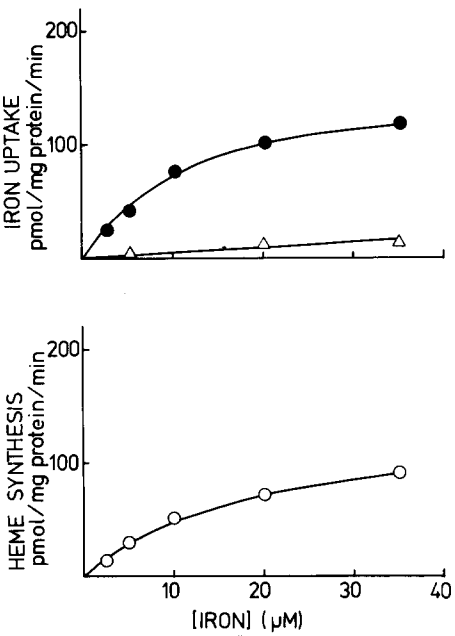

Fig. 6. Iron accumulation and heme synthesis at increasing concentrations of ferric-iron-pyrophosphate. Experimental conditions: 2.4 mg/ml mitochondrial protein was incubated with ferric-iron-pyrophosphate as indicated. The rate of iron accumulated between 45–60 min of incubation was determined with (△) and without (●) 2.5 μg/ml antimycin A. The rate of heme synthesis (○) was determined (see Materials and Methods) in the presence of 5 μM deuteroporphyrin.

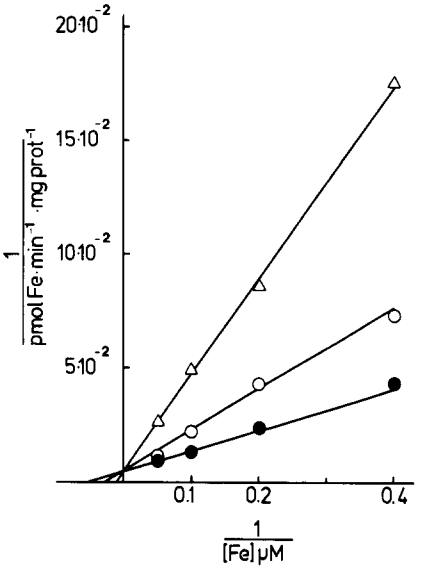

Fig. 7. Effect of apotransferrin on the uptake of iron. Mitochondria, 2.4 mg protein/ml were incubated as described with increasing concentrations of ferric-iron-pyrophosphate. (●) control; (○) with 10 μM apotransferrin; (△) with 30 μM apotransferrin. Incubation was at 30 °C and the rate of iron uptake was determined in the interval 45–60 min.

Discussion

According to Konopka and Romslo [6,7], rat-liver mitochondria accumulate iron from transferrin at a rate of 10–30 pmol/mg protein per min, but only if a pyrophosphate-containing compound is present. Our results (Fig. 1, and Refs. 14 and 15) are in agreement with these observations.

The rate constants as given by Konopka and Romslo [6,7] do not reflect the true ability of the mitochondria to accumulate iron, but rather express the overall rate of the mobilization of iron from transferrin, formation of ferric-iron-pyrophosphate, sequestering of ferric-iron-pyrophosphate and uptake of iron by the mitochondria. As

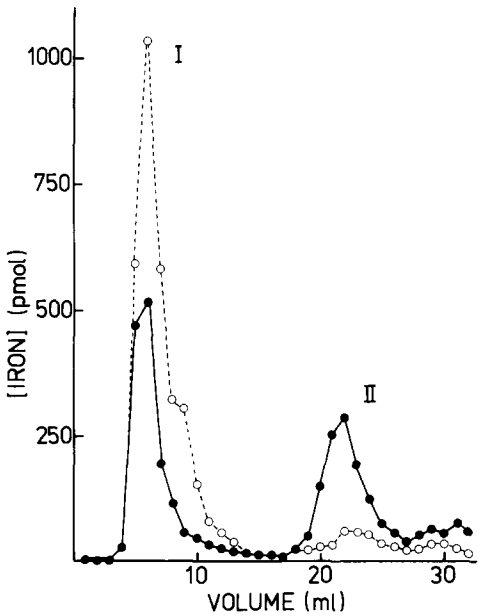

Fig. 8. Mobilization of iron from pyrophosphate to apotransferrin. The incubation medium contained 50 μM apotransferrin, 5 μM iron (as ferric-iron-pyrophosphate) and 1 mM pyrophosphate. The elution was on an AG1-X4 resin column at zero time (●) and after 60 min incubation at 30 °C (○).

shown here (Figs. 3 and 5), the capacity of the mitochondria to accumulate iron largely surpasses the rate of mobilization of iron from transferrin, the rate-limiting reaction being the generation of ferric-iron-pyrophosphate.

Initially it was thought that the delay to reach steady-state rate was due to the building up of ferric-iron-pyrophosphate. However, the delay bears no relationship to the concentration of ferric-iron-pyrophosphate (Fig. 1 compared to Fig. 5), and a similar delay has also been reported in experiments with iron uptake from ferritin [17]. The delay could neither be explained by a limited capacity for ferric-iron reduction, because a similar delay is observed when antimycin A is present [18].

Uptake of iron from transferrin as mediated by pyrophosphate involves reduction and dissociation of ferrous iron from the ferric-iron-pyrophosphate complex to suitable ligands of the mitochondria. In agreement to this is the finding that the rate of heme synthesis increases in parallel with the rate of iron uptake (Fig. 2). The rate of heme synthesis shown in Fig. 2 is less than that reported by Koller and Romslo [19]. However, our results are from experiments with low concentrations of iron. At higher concentrations of iron, heme synthesis increases to values (Fig. 6) close to those reported by Koller and Romslo [19].

Ferric-iron-pyrophosphate is very stable and highly insoluble at neutral pH [16]. With excess pyrophosphate, a stable, but yet soluble complex is formed. As shown in Fig. 5, uptake of iron from ferric-iron-pyrophosphate amounts to approx. 4 nmol/mg protein at 90 min. This figure should be compared to the iron content of normal mitochondria, approx. 5–10 nmol/mg protein [20] and to the energy-dependent uptake of 6–10 nmol/mg protein as reported by Romslo and

TABLE I

EFFECT OF APOTRANSFERRIN ON THE PARTITION OF IRON BETWEEN TRANSFERRIN, PYROPHOSPHATE AND MITOCHONDRIA

Mitochondria, 2.0 mg/ml were incubated with 5 μM iron (as [^{59}Fe]transferrin and 1 mM pyrophosphate, 60 min at 30 °C. In one series of experiments, 50 μM apotransferrin was added. Following incubation, the mitochondria were spun down, and the uptake of iron was determined. The partition of iron between pyrophosphate and transferrin in the supernatant was determined by column chromatography. The results are given as percent (mean and ranges given in parentheses) relative to the total amount of iron added.

	Percent iron in			Recovery (%)
	Transferrin	Pyrophosphate	Mitochondria	
Control ($n = 3$)	48 (55–39)	18 (14–25)	16 (13–21)	82 (85–77)
With 50 μM apotransferrin ($n = 3$)	70 (73–66)	5 (10–2)	9 (9–8)	84 (83–85)

Flatmark [12]. It could be argued that the uptake as shown in Fig. 5 does not reveal saturation kinetics. However, incubation of mitochondria beyond 90 min hardly reflects physiology because oxidative phosphorylation and mitochondrial integrity are seldom preserved for more than 45–60 min incubation. What then is the physiological significance of the present results? Could pyrophosphate function as iron donor to mitochondria in situ?

According to Howard et al. [21], pyrophosphate is not found in detectable concentrations in eucaryotic cells. On the other hand, Flodgaard [22] found that rat liver contained 10–15 nmol/g liver wt., and Veech [23] reported that the cellular pyrophosphate concentration was approx. 12 μM, i.e., the cellular concentration of pyrophosphate is below that found effective in the present study. However, local high concentration of pyrophosphate may exist, but as far as we are aware, data on compartmentalization of pyrophosphate within the cell have not been reported. Mitochondria are known to contain a membrane-bound pyrophosphatase which through coupling to the respiratory chain may generate approx. 8 nmol pyrophosphate/mg protein per 10 min [24]. Moreover, it should be remembered that the amount of iron taken up by the hepatocyte averages $9 \cdot 10^6$ molecules/hepatocyte per h [25] and with 70% paved into ferritin and 20–30% into heme, the mitochondria have to take up approx. 0.4 pmol iron/mg protein per min which could perhaps be obtained with pyrophosphate concentrations as low as 5–10 μM.

It remains, however, to be shown that pyrophosphate penetrates the receptosomal membrane and after having picked up iron re-enters cytosol where the major part passes into ferritin and some 20% to the mitochondria.

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