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Mini-Review

Old Iron, Young Copper : from Mars to Venus

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Abstract

Iron and copper are metals which play an important role in the living world. From a brief consideration of their chemistry and biochemistry we conclude that the early chemistry of life used water soluble ferrous iron while copper was in the water-insoluble Cu(**I**) state as highly insoluble sulphides. The advent of oxygen was a catastrophic event for most living organisms, and can be considered to be the first general irreversible pollution of the earth. In contrast to the oxidation of iron and its loss of bioavailability as insoluble Fe(**III**), the oxidation of insoluble Cu(**I**) led to soluble Cu(**II**). A new iron biochemistry became possible after the advent of oxygen, with the development of chelators of Fe(**III**), which rendered iron once again accessible, and with the control of the potential toxicity of iron by its storage in a water soluble, non-toxic, bio-available storage protein (ferritin). Biology also discovered that whereas enzymes involved in anaerobic metabolism were designed to operate in the lower portion of the redox spectrum, the arrival of dioxygen created the need for a new redox active metal which could attain higher redox potentials. Copper, now bioavailable, was ideally suited to exploit the oxidizing power of dioxygen. The arrival of copper also coincided with the development of multicellular organisms which had extracellular cross-linked matrices capable of resisting attack by oxygen free radicals. After the initial 'iron age' subsequent evolution moved, not towards a 'copper age', but rather to an 'iron-copper' age. In the second part of the review, this symbiosis of iron and copper is examined in yeast. We then briefly consider iron and copper metabolism in mammals, before looking at iron-copper interactions in mammals, particularly man, and conclude with the reflection that, as in Greek and Roman mythology, a better understanding of the potentially positive interactions between Mars (iron) and Venus (copper) can only be to the advantage of our species.

Introduction

For a long period of time, iron and copper have exerted a fascination for human civilization. Mars, the Roman god of war, was the name given by the old chemists to iron, while Venus, the Roman goddess of love and beauty, was the alchemist's name for copper. It is well established that human societies used copper prior to iron for metallurgy. Copper and its alloy, bronze, were discovered several thousand years before iron. Fe(0) is easy to oxidize, giving rust, while Cu(0) is difficult to oxidize because of its high electrode potential, such that copper is often found in nature in the metallic state. Since the 'bronze age' has preceded the 'iron age', in this sense, it is copper that should be considered as old, and iron as young -but our subject here is not metallurgy! Iron has long been considered as a precious metal and, at the dawn of the 20th century, Rudyard Kipling wrote:

> *'Gold is for the mistress- silver for the maid-*
> *Copper for the craftsman cunning at his trade.'*
> *'Good!' said the Baron, sitting in his hall,*
> *'But Iron -Cold Iron- is master of them all.'*

Iron and copper were associated with some of the more ancient therapies used by man. 1500 years before Christ, Iphyclus, prince of Thessalia was cured of his sexual impotance by Melampus, a seer. He scraped

the rust off of an old knife into wine and administered the beverage to Iphyclus. Iron therapies were widely used by mediterranean civilizations. Hippocrate and Avicenna knew the effect of iron salts. Iron was used for the treatment of chlorosis as far back as the 17^{th} century. Bland's pills (ferrous sulfate) are prescribed for anemia since 1831. Copper therapy is also ancient: 1550 years before Christ, in the Middle Empire in Egypt, it is mentioned (Papyrus Ebers). Copper was recommended by Paracelsus (1493–1541). Today, the anti-inflammatory effects of copper salts is not questionned (while not clearly explained). Even the unusual copper bracelets worn by some people may have an efficacy, via dermal penetration of oxidized copper ions.

On the other hand, iron and copper are associated with important diseases. Iron overload, both congenital (hemochromatosis) and secondary (due to blood transfusions) occurs frequently – in W. Europe, the USA and Australia, 1 in 20 of the population may be carriers for the congenital disease – and if untreated is lethal, while iron deficiency results in anemia, cognitive impairment and immunodeficiency. The principal pathologies of copper, Menke's desease and Wilson's disease, both due to defective intracellular copper transport, are also life-threatening. The former is characterized by progressive cerebral degeneration, essentially due to insufficient copper absorption, and the latter due to excessive copper accumulation in liver, accompanied by liver disease and haemolytic crises.

The fascination exerted by iron and copper for the authors of this paper is relevant from another point of view, which is essentially biological but also has some philosophical considerations. Iron is essential for life. The only organisms which do not require iron are the well-studied genus *Lactobacillus* and some strains of *Bacillus*. Iron is used in a wide variety of essential biological processes ranging from transport, storage and activation of molecular oxygen, reduction of ribonucleotides and dinitrogen, activation and decomposition of peroxides to electron transfer via a variety of carriers. Yet iron is toxic. Trace amounts of copper are also essential for life. However, as with iron, excess of copper is also toxic. Since 'free' iron and copper are potentially dangerous, on account of their capacity to catalyse the Fenton reaction, the role of iron and copper metalloproteins, including the recently discovered metallochaperones, in achieving metal ion homeostasis must be very important.

This paper presents a brief overview of the chemistry and biochemistry of iron and copper in an evolutionary perspective. It will also try to underline the analogies and differences between these two elements successively selected by Nature as it was obliged to adapt life to the first general irreversible pollution of the earth, namely the advent of dioxygen.

"There was a primitive separation between organisms heavily dependent on iron, nickel (especially) and cobalt, the archebacteria, and other prokaryotes where the dominance of iron is more obvious. It is also clear that copper is of little significance in most of these organisms relative to its multitude of roles in multicellular eukaryotes, while in these eukaryotes the role of nickel and cobalt is further diminished. We may conjecture that biological systems did not use copper extensively before the advent of an oxidizing atmoshere based on dioxygen" (Frausto da Silva & Williams 1991).

We would therefore suggest the importance of iron from the early stages of evolution but of copper only at a later date is highly likely on the basis of their aqueous solution chemistry. The review is organised in the following sections:

- Chemistry and biochemistry of iron and copper: a brief reminder;
- Iron, copper and evolution;
- Iron and copper interactions in yeast;
- Iron and copper interactions in mammals and man;
- Conclusions.

Chemistry and biochemistry of iron and copper : a brief reminder (Frausto da Silva & Williams 1991; Huheey *et al.* 1993; Lippard & Berg 1994).

Iron

Iron is the second most abundant metal (after aluminium) and the fourth most abundant element of the earth's crust. Its position in the middle of the elements in the first transition series implies that iron has the possibility of existing in various oxidation states (from $-$**II** to $+$ **VI**), the principal being **II** (d^6) and **III** (d^5). Fe^{3+} is quite water insoluble ($K_{sp} = 10^{-39}$ **M** and at pH 7.0, $[Fe^{3+}] = 10^{-18}$ **M**) and significant concentrations of water-soluble Fe^{3+} species can be attained only by strong complex formation. Iron (**III**) is a hard acid which prefers hard oxygen ligands while iron (**II**) is on the borderline between hard and soft, favouring nitrogen and sulfur ligands. The coordination number of 6 is the most frequent (octahedral complexes), although four (tetrahedral) and particularly five-coordinate complexes (trigonal bipyramid or

square pyramid) are also encountered. For octahedral complexes, two different spin states (low-spin complexes with strong-field ligands and high-spin complexes with low-field ligands) can be observed. High spin complexes are kinetically labile, while low-spin complexes are exchange inert. Both oxidation states are Lewis acids, particularly the ferric state. The unique suitability of iron comes from the extreme variability of the Fe^{3+} /Fe^{2+} redox potential, which can be fine- tuned by well-chosen ligands, so that iron sites can encompass almost the entire biologically significant range of redox potentials, from about -0.5V to about 0.6V. The interaction of iron centers and oxygen is of paramount importance in biological inorganic chemistry, and we have summarized some main features in Figure 1.

Copper

Two oxidation states are usual for copper, Cu(**I**) and Cu(**II**). Although some earlier reports suggested the occurrence of Cu(**III**) in galactose oxidase, it is known that this is due to the generation of a tyrosyl radical on a tyrosine liganded to copper during the reaction cycle, and it seems unlikely that this oxidation state has any biological relevance. The Cu(**II**)/Cu(**III**) redox potential is generally high and hence, one electron oxidation of the protein ligand occurs. The $3d^9$ outer electronic configuration of Cu(**II**) lacks cubic symmetry and hence yields distorted forms of the basic stereochemistry. The coordination numbers 4 (square planar), 5 (trigonal bipyramid or square pyramid) or 6 predominate. With coordination 6, the Jahn-Teller effect excludes the regular octahedron. The stable Cu(**II**)-N bonds are often inert while the bonds with oxygen donor ligands are more labile. Cu(**I**) prefers coordination numbers 2, 3 or 4 (tetrahedral geometry) and is stabilized by soft ligands. Coordination 5 is known (square pyramid). Cu(**I**) is a closed shell d^{10} transition metal ion and thus is diamagnetic. The disproportionation of Cu(**I**) is usual:

$$2Cu^+ \rightleftharpoons Cu + Cu^{++}$$

$$E_0 = 0.182\ \text{V}$$

$$E_0(Cu^{++}/Cu^+) 0.34\ \text{V}$$

$$E_0(Cu^+/Cu) = 0.52\ \text{V}.$$

The equivalent reaction is not observed with iron. As Fe(**II**) does, Cu(**I**) catalyses the Fenton reaction with hydrogen peroxide. The Cu(**I**) state exhibits the ability to bind and activate dioxygen via $Cu_2(\mu - \eta^2 : \eta^2\text{-}O_2)$ and $Cu_2(\mu\text{-}O)_2$ species.

Iron and copper complexes of biological relevance (Holm *et al.* 1996)

Iron. (Crichton 1991; Sigel & Sigel 1998; Que & Ho 1996; Wallar & Lipscomb 1996; Sono *et al.* 1996)
Low molecular weight chelates are often encountered in the biological chemistry of iron. Bacterial siderophores (and phytosiderophores for some plants) are used for the solubilization and transport of iron. Iron proteins can be classified into haemoproteins (e.g., haemoglobin, myoglobin, cytochromes, various oxidases, peroxidases, catalases,. . .), iron-sulphur proteins containing Fe/S clusters (eg. aconitase, nitrogenase, ferredoxins, rubredoxins. . .), mononuclear non-haem proteins (lipoxygenase, various amino acid hydroxylases. . .), diiron oxo-bridged proteins (haemerythrin, methane monooxygenase, purple acid phosphatase, ribonucleotide reductase. . .). Ferritin, a fascinating protein used for storage of intracellular iron, is a member of the latter family and can contain several thousand iron atoms.

Copper (Kaim & Rall 1996; Klinman 1996; Salomon *et al.* 1996; Koch *et al.* 1997)
Copper proteins are widely distibuted in living organisms, with two main functions, electron transfer and dioxygen transport and activation. Almost all of the copper proteins are extracellular ; exceptions are cytochrome c oxidase, which is bound to the external face of the inner mitochondrial membrane and the copper-zinc superoxide dismutase found in the cytosol of eukaryotic cells. Type 1 proteins (blue copper proteins) have a highly covalent mononuclear Cu(**II**) center (even when present in oligocopper proteins) and catalyse electron transfers (plastocyanin, azurin). They are characterized by an intense $Cys^- \rightarrow$ Cu(**II**) LMCT transition. Type 2 proteins are mononuclear proteins which catalyse redox reactions (SOD, galactose oxidase, amine oxidase, Cu_B in cyt. c oxidase); no intense UV-visible absorption bands are observed for them. Type 3 proteins are binuclear, EPR silent in the oxidized Cu(**II**)-Cu(**II**) state (due to antiferromagnetic coupling between the two Cu centers). They participate in dioxygen transport (hemocyanin), dioxygen activation and oxygenation reactions (tyrosinase). The Cu(**I**)-Cu(**I**) state binds dioxygen to give $[(Cu(II))_2 O_2]$ complexes. Type 2 + 3 proteins are trinuclear proteins involved in dioxygen activation and oxidase

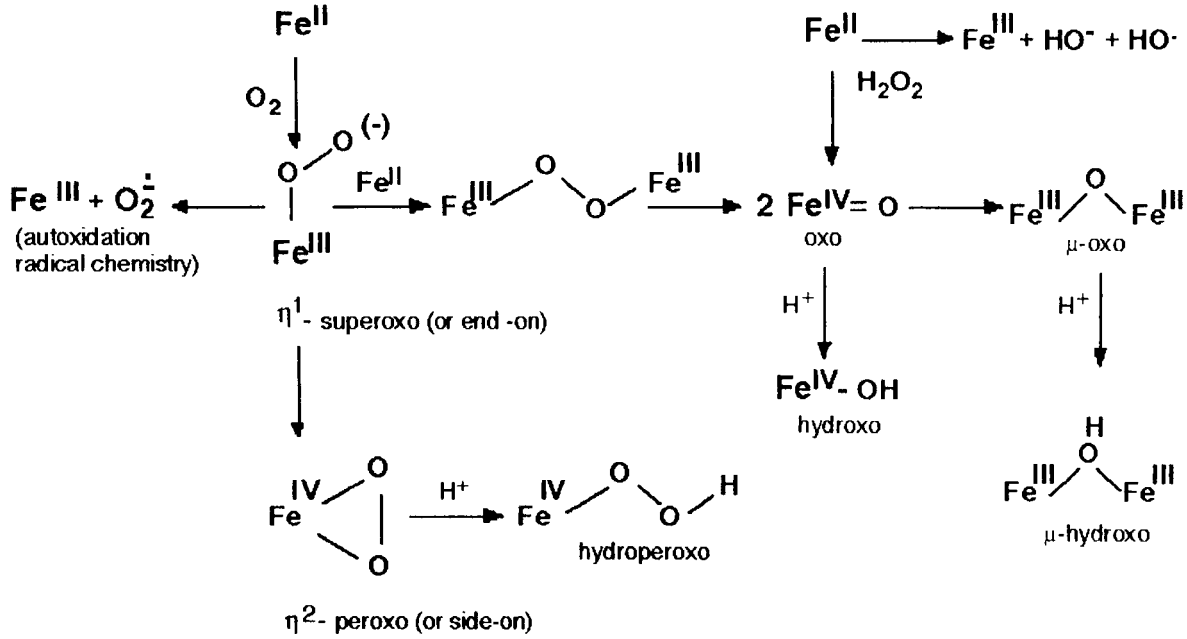

Fig. 1. Iron-oxygen chemistry (multi-bridged species have been omitted).

functions (ascorbate oxidase, laccase). Ceruloplasmin contains Type 1, 2 and 3 centers.

Iron and copper proteins participate in many of the same biological reactions (electron transfers, monooxygenase, dioxygenase and oxidase activities). C-H bond activation is catalyzed by iron or by copper methane monooxygenases.

Iron and copper : some differences

Copper and iron display distinct features in terms of their coordination chemistry and their redox properties. One of the major differences resides in their electrochemical properties (Figure 2). The redox potential Cu(**II**)/Cu(**I**) of copper enzymes are usually higher than the Fe(**III**)/Fe(**II**) potential of iron enzymes, most copper enzymes working between + 0.25 and + 0.75V. This high potential can be utilized for a direct oxidation of certain substrates easy to oxidize, such as superoxide (superoxide dismutase), ascorbate (ascorbate oxidase) or catechol (tyrosinase). As copper is difficult to oxidize, the Cu(**III**) state is probably not biologically relevant. In contrast, a number or iron-dependent monooxygenases generate high-valent Fe(**IV**) or Fe(**V**) reactive intermediates during their catalytic cycle. Iron seems to be much more suited to the oxidation of resistant substrates, such as hydrocarbons (althout an ill-defined copper methane monooxygenase is known). In the case of copper centers, the difficulty to reach the highly oxidizing Cu(**III**) state has up till now limited the range of substrates that can be oxidised. In the few examples of **C-H** bond oxidation by copper enzymes (dopamine β-hydroxylase, tyrosinase), an intermediate copper peroxo Cu-OOH or $Cu(O_2)Cu$ complex has been suggested as the oxidizing agent. Some Cu enzymes have exploited yet another molecular strategy for the design of efficient catalysts of oxidative reactions, namely the association of a Cu(**II**) center with a non-metallic redox center (organic radical) (Klinman 1996). Galactose oxidase is a typical example of this strategy.

Another aspect which differentiates iron and copper biology may be related to *biomineralization* (Mann 1997; Konhauser 1997). Many structures formed by plants and animals are minerals (calcium-phosphate in bones and teeth, calcium in the shells of marine organisms, silicon in grasses and the shells of invertebrates). Biomineralization involves the formation of these inorganic materials under the influence of proteins, carbohydrates and lipids. This fascinating problem includes initiation of nucleation, growth of the inorganic crystalline phases, definition of the volume and shape of the inorganic material. The formation of the ferritin core and its transformation in haemosiderin is one example of a biomineralization process. The formation of magnetite (Fe_3O_4) particles by magne-

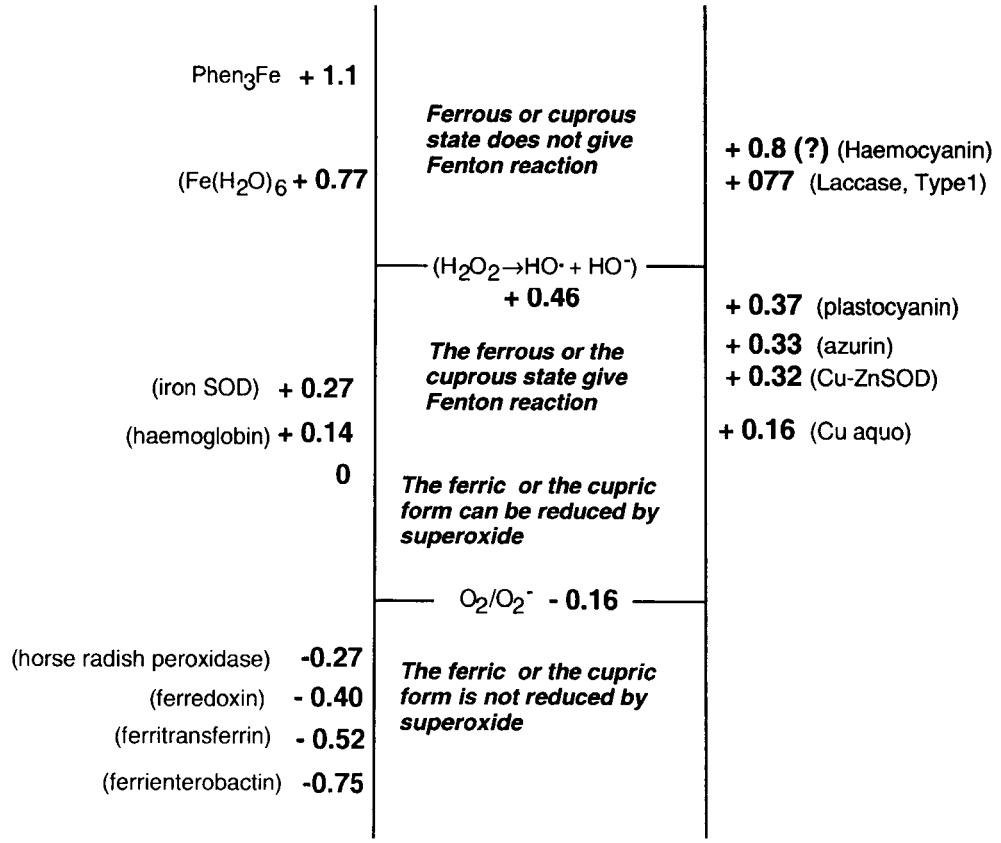

Fig. 2. Some reduction potentials at pH 7 (V relative to the standard hydrogen electrode).

totactic bacteria is another. To our knowledge, copper is not implicated in biomineralization processes.

Iron, copper and evolution

The basic principles involved in the bioselection of elements are governed by four fundamental rules (Frausto da Silva & Williams 1991; Orchiai 1986) related to: (i) the abundance of the element, (ii) its efficiency, (iii) its basic fitness for a given task and (iv) the evolutionary pressure. Water soluble ferrous iron was present during prebiotic times and was the form used in the first stage of life. The natural abundance and the redox properties of iron allowed the chemistry that was suited for life. At the same time, copper was in the water-insoluble Cu(**I**) state, in the form of highly insoluble sulphides, and was not available for life. *The early chemistry of life used iron (II).*

The chemoautrophic theory (Haber & Wächtershauser 1997; Maurel & Décout 1999) represents a fascinating hypothesis of the origin of life in an iron-sulfur world. CO and CO_2 were produced at volcanic hydrothermal sites. The reductive formation of CH_3SH and its carbonylation into activated thioester CH_3COSCH_3 can play the role of a primitive acetyl-coenzyme A. The reducing power necessary for this metabolism arises from the oxidative formation of iron pyrite from iron sulphide and hydrogen sulphide. The surface of iron-sulfur mineral serves as a matrix for all subsequent chemical conversions.

It has often been suggested that ferrous iron could be stabilized inside ferritin, long enough for it to be used in some types of cells. Ferritin is certainly an ancient protein, which could be thought to represent the precursor of several forms of iron in living organisms.

About 10^9 years ago, an evolution of dioxygen into the earth's athmosphere began to develop, due to the metabolism of a prokaryote (Cyanobacteria). A lag of 200–300 millions years is estimated to have been required between the first production of O_2 and the appearance of a significant O_2 concentration in the athmosphere, because the O_2 produced was initially consumed by the oxidation of ferrous ions in the

oceans. *The advent of O_2 was a catastrophic event for most living organisms and can be considered as the first general irreversible pollution of the earth.* Iron was oxidized and transformed into the insoluble iron(**III**) state. Iron hydroxides precipitate and the bioavailability of iron was lost. On the contrary, the oxidation of insoluble copper (I) led to soluble copper (II). While the proteins and enzymes involved in anaerobic metabolism were designed to act in the lower portion of the redox potential spectrum, the presence of dioxygen created the need of a new redox active metal with E_0 M^{n+1}/M^n from 0 to 0.8V. Copper, now biovailable, is quite ideally-suited! Copper began to be used in energy-capture systems like cytochrome oxidase. Increasing radical attacks by oxygen radicals were observed which destroyed the extracellular matrix of unicellular organisms. Evolutionary development of cross-linked connective tissue (collagen, chitine) in free radical polymerization, led to multicellular organisms which use copper. Concurrently, the use of iron now required bioenergetically costly strategies: complexation and/or reduction.

In fact, two solutions were possible for Nature: (i) either to use iron with adaptation to dioxygen or (ii) to use copper. Both evolutionary responses arose (as did mixed responses):

- *Iron* being in an insoluble and not available form, microorganisms 'invented' strong iron(III) chelators (siderophores) and secreted them into the surrounding. These chelators bind Fe^{3+} and the soluble chelates are ingested by the organisms via a sophisticated mechanism. Many organisms are able to maintain an intracellular concentration of iron(III) several orders of magnitude higher than simple aqueous solutions permit. Plant roots secrete organic acids solubilizing and chelating Fe^{3+}, or specific phytosiderophores The toxicity of iron (free radical chemistry) requires that the ability to store and release iron is controlled, and this requirement was fulfilled by the ubiquitous iron storage protein, ferritin and its degradation product haemosiderin.. *A new iron biochemistry became possible after the advent of dioxygen*.
- Copper became used by enzymes, firstly in the extracellular space (lysine oxidase, laccase, phenol oxidase, amine oxidase...). While the new role of copper evolved, the role of iron changed. Inside cells, iron could still be used for catalytic functions. For aerobic metabolism, proteins and enzymes with higher redox potentials came to be utilized, taking advantage of the oxidizing power of dioxygen.

Biological systems did not used copper before the advent of O_2. The event coincides with the development towards multicellular organisms. So, the 'catastrophe' turned out to be a piece of good luck for the progress of life!

It is clear that the first age of life is the 'iron age'. The subsequent age is not a 'copper age' but rather an 'iron-copper age'. Iron and copper metabolism are often involved together. As an example, the mammalian plasma glycoprotein ceruloplasmin, which is the principal copper binding protein in serum, acts as a multicopper oxidase which catalyzes the oxidation of iron(II) into iron(III), coupled to the reduction of O_2 to water. This oxidation is necessary for high affinity iron transport can take place. Another example is given by heteropolynuclear copper-iron enzymes : Cytochrome c oxidase involves two heme iron centers and two copper centers (Cu_A and Cu_B).

Iron and copper interactions in yeast

The common yeast, *Saccharomyces cerivisia* is an extremely attractive eukaryotic model system on account of its short generation time, the ease with which it can be grown, the facility with which it can be submitted to the powerful techniques of moleular biology and the fact that its complete genome has now been completely sequenced. We showed in early studies that iron uptake in yeast requires reduction of Fe(III) by an NADPH-dependent enzyme, transport of Fe(II) into the cell, and storage of iron, not in ferritin, but in vacuoles, from which the iron can be mobilised for the synthesis of mitochondrial haem (Lesuisse *et al.* 1987; Raguzzi *et al.* 1988). More recently, using the powerful modern techniques of molecular genetics, several American groups have confirmed some of these proposals and considerably extended them to finally provide a mechanistic link between the uptake of iron and copper in yeast (Askwith & Kaplan 1998). In many ways, this breakthrough is epitomized by two back-to back publications which appeared in Cell in January, 1994 (Dancis *et al.* 1994; Askwith *et al.* 1994), in which the groups of David Eide, Jerry Kaplan and Rick Klausner established unequivocally that iron uptake by yeast requires two proteins, one, Ctr1p required for high affinity copper transport across the plasma membrane and the second, Fet3p, a member of the family of blue multicopper oxidoreductases, involved in cellular iron uptake through its ferroxidase activity. Both extracellular ferric iron and cupric copper are reduced by plasma-membrane fer-

ric reductases, encoded by the gene products Fre1p and Fre2p (Figure 3). There is a low affinity Fe(II)-transporter, Fet4p, which probably corresponds to the transport system that we observed earlier (Lesuisse *et al.* 1987). Iron can also enter the cell via a bipartite high affinity iron transport system consisting of Ftr1p, a transmembrane iron permease, and Fet3p, an integral membrane protein with an extracellular multicopper oxidase domain (Stearman *et al.* 1996). Fet3p has sequence homologies with the family of multicopper oxidases, a family of enzymes that oxidise substrates with concomitant reduction of molecular oxygen to water, and which includes ceruloplasmin, which seems to play an important role in human iron homeostasis (see below). From its amino acid sequence Fet3p is predicted to have three distinct copper binding sites : one type 1, or blue, Cu(**I**) site, one type 2, non-blue, Cu(**II**) site and one dinuclear type 3 site, the latter undetectable by EPR spectroscopy. Consistent with this, recombinant Fet3p co-purifies with 4 copper ions, two of which are EPR silent (Kossman *et al.* 1998). It is postulated that Fet3p mediates iron transport by acting as a ferroxidase, converting ferrous iron to ferric iron, which is then transported by Ftr1p. Fet3p expression is required for the Ftr1p protein to be transported to the plasma mermbrane, and Ftr1p expression is required for apo Fet3p protein to be loaded with copper and aquire its oxidase activity. Muations in Ftr1p altering either of the glutamic acid residues of the conserved sequence motif REGLE interferes only with iron transport, specifically implicating the Ftr1p protein in the transport process.

Reduction of environmental Cu(**II**) to Cu(**I**) by Fre1p (Figure 3) is followed by high affinity copper uptake across the yeast plasma membrane mediated by Ctr1p and Ctr3p (Dancis *et al.* 1994; Knight *et al.* 1996). A low affinity system also exists involving Ctr2p (Kampfenkel *et al.* 1995). Once copper has entered the cell it is transported to specific destinations by cytosolic copper chaperones (Harrison *et al.* 1999, 2000). Whether they interact directly with the CTR copper import pumps directly at their cytosolic face, or whether an as yet unidentified protein facilitates copper transport between them is not known. The 74 residue cytoplasmic protein Atx1p (Lin *et al.* 1997) delivers copper to Ccc2p (Figure 4), the post Golgi vesicular copper transporter which is responsible for copper loading and hence activation of Fet3p (Askwith & Kaplan 1998; Harrison *et al.* 1999). Ccc2p has all the characteristics of a Cpx-type ATPase, a sub-group of a family of ATP-driven ion pumps, which transport transition metal ions, and has copper chaperone-like sub-domains in its N-terminus (Harrison *et al.* 1999). The proposed path for intracellular transfer of copper by Atx1p is presented in (Figure 4), which also includes a proposed mechanism for the exchange of Cu(I between Atx1p and Ccc2p involving two and three coordinate Cu-bridged intermediates (Pufahl *et al.* 1997).

Metallothioneins are ubiquitous, low molecular weight, thiol-rich proteins which have a selective capacity to bind metal ions, such as zinc, cadmium, mercury and copper. In the yeast *Saccharomyces cerevisiae* a family of metallothioneins, encoded by the amplified CUP1 gene locus and the single copy CRS5 gene, both under the regulation of their copper-dependent transcriptional activator ACE1, play a key role in mediating copper resistance (Strain & Culotta 1996; Jensen *et al.* 1996). Activation of metallothioneins gene expression is not only involved in protective responses to the toxicity of copper (and other metals) but also of oxygen-derived radicals (Liu & Thiele 1997).

In response to copper, the expression of the high affinity Cu(I) uptake genes CTR1, CTR3 and FRE1 are all regulated by the nutritional copper sensor Mac1p, while the transcriptional activation of the detoxification genes CUP1 and CRS5 (the metallothioneins) and SOD1 (the cytosolic Cu/Zn superoxide dismutase) are regulated by the toxic copper sensor Ace1p (Pena *et al.* 1998).

Lys7p, a 249 residue cytosolic copper chaperone, appears to deliver copper *in vivo* to the cytoplasmic copper/zinc superoxide dismutase (Horecka *et al.* 1995; Gamonet & Loaquin 1998), whereas Cox 17p, another cytoplasmic protein of 69 residues, appears to supply copper to the Cu_A site of mitochondrial cytochrome c oxidase (Glerum *et al.* 1996a). Cox 17p, 60% of which is found in the intermembrane space in mitochondria, does not have a classical mitochondrial import sequence, and probably transfers its copper directly to the Cu_A binuclear site, which protrudes into the mitochondrial intermembrane space (Harrison *et al.* 1999). The active site of cytochrome c oxidase (Cu_B) is buried in the mitochondrial inner membrane and it appears that the proteins Sco1p and Sco2p are required for copper transport into the mitochondrial lumen for insertion into the Cu_B site (Glerum *et al.* 1996b). To date however, the prediction that other redox-active metal ions such as iron and cobalt will also be transported by corresponding chaperone pro-

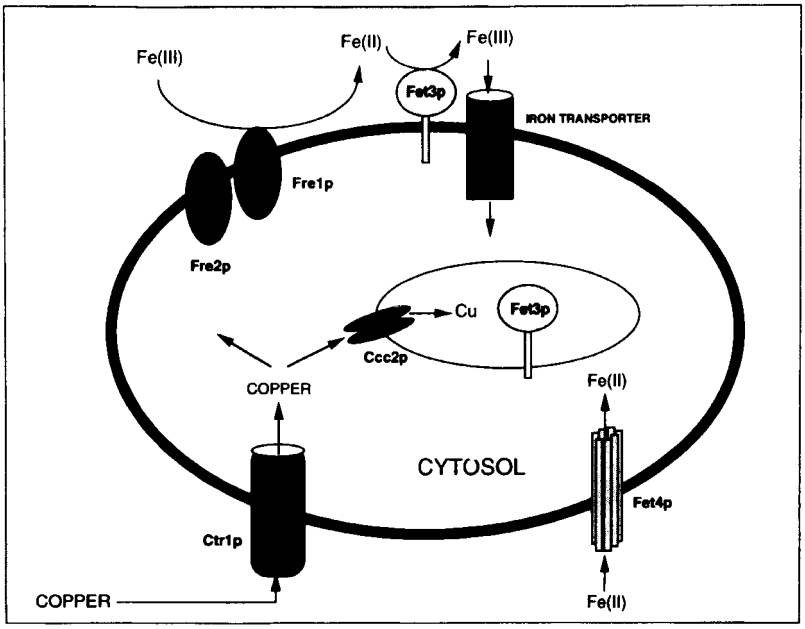

Fig. 3. A model of iron accumulation in Saccharomyces cerevisiae. Fre1p and Fre 2p are membrane-associated ferrireductases, Ctr1p is a copper transporter, Fet4p is a low affinity Fe^{2+} transporter, Ccc2p is a P-type ATPase with homology to the Wilson's disease protein in humans, Fet3p is an extracellular multicopper oxidase and the high affinity iron uptake system is completed by the Fe^{3+} transporter Ftr1p (adapted from De Silva *et al.* 1996).

teins to their sites of utilisation (Harrison *et al.* 1999) remains to be established.

Disruption of any one of the genes involved in the cellular copper transport system composed of Ctr1p, Atx1p, Ccc2p results in a deficiency of both Fet3p activity and high-affinity iron transport (Askwith & Kaplan 1998).

Of the copper chaperones described above for yeast, functional homologues have been found for Atx1p, Lys7p and Cox17p in human cells which complement the function of the yeast chaperones, while Ctr1p also has a functional human homologue (reviewed in Harrison *et al.* 1999), underlining the likely similarity of at least some aspects of both copper and iron homeostasis in yeast and human cells.

Iron and copper interactions in mammals and man

A historical overview

Copper was found to be necessary for haemoglobin formation in rats (Hart *et al.* 1928) fed on a milk-based diet. In rapidly growing piglets, copper deficiency lowered body iron content and interfered with iron distribution to tissues (Gubler *et al.* 1956), and it was subsequently shown that reticulocytes from copper deficient pigs took up iron poorly and were deficient in haem synthesis (Williams *et al.* 1976). While it is now clearly established that copper deficiency is associated with anemia in animals, acquired copper deficiency in human is mainly a pathology of infants: most cases of clinical manifestations have been described in malnourished children (Olivares & Uauy 1996). The most constant clinical manifestations are anemia, neutropenia and bone abnormalities. Haemolysis is associated with copper overload in domestic animals (dogs, sheep, cattle), in acute copper sulphate poisoning in humans, and it appears that the haemolytic episodes in Wilson's disease are similar to those in animals with heavy copper overload, and that the periodic release of copper from the liver is responsible for these acute episodes (Johnson 1995). There seems to be no documented evidence of interference with copper metabolism in conditions of either iron deficiency or overload.

A brief overview of iron metabolism in mammals

Transferrins are an important class of iron-binding proteins found in the physiological fluids of many vertebrates which ensure iron transport to tissues. The

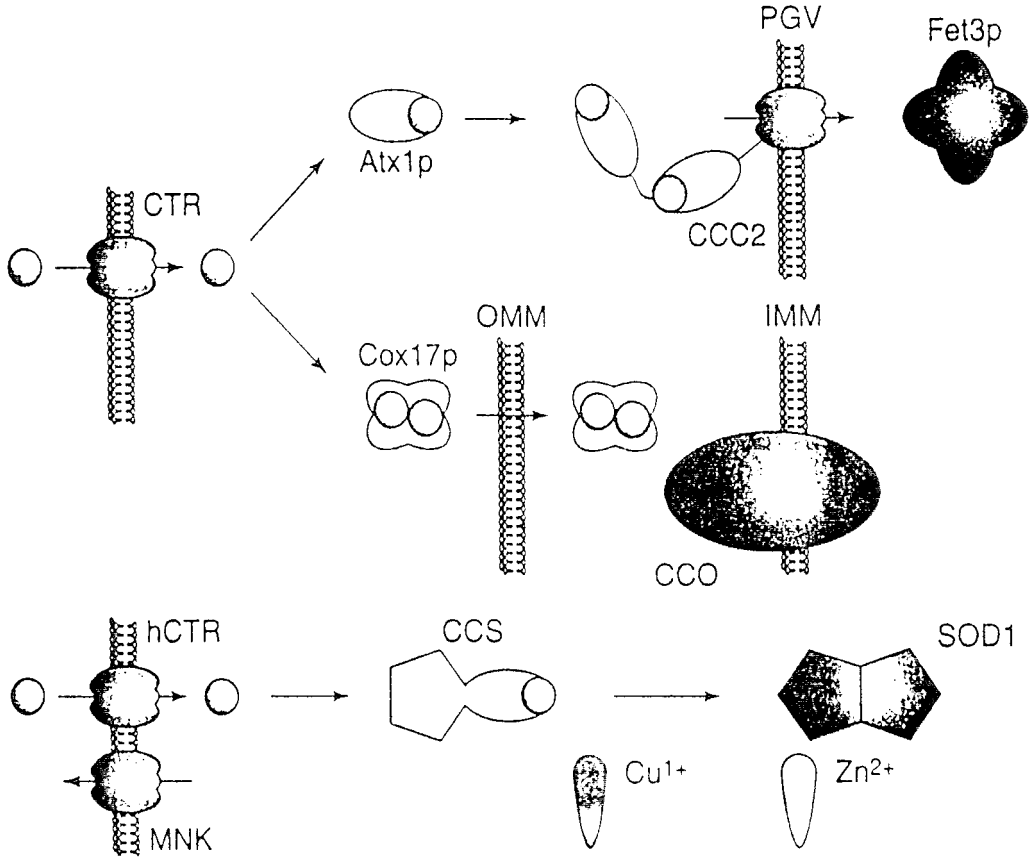

Fig. 4. Copper chaperone function. The CTR protein family functions to transport copper ions into yeast cells. Atx1p delivers copper to the CPx-type ATPases resident in the post-Golgi apparatus for the maturation of Fet3p and other copper-dependent proteins. Cox17p delivers copper to the mitochondrial intermembrane space for incorporation into cytochrome c oxidase (CCO). hCTR, a human homologue of CTR, mediates copper-iron uptake in human cells. CCS delivers copper to cytoplasmic copper/zinc superoxide dismutase (SOD1). The function of CCS has been inferred from its sequence similarity to yeast proteins with known function and ability to complement $Lys7^{-}$ yeast cells. Abbreviations: IMM – inner mitochondrial membrane; OMM – outer mitochondrial membrane; PGV – post Golgi vesicle.

transferrin molecule consists of two identical lobes each of which tightly binds one atom of ferric iron at the inner end of deep interdomain clefts within the molecule. Iron coordination involves four ligands from the protein and two bidentate oxygen ligands from an associated carbonate anion (Crichton 1991). The *H. influenzae* periplasmic ferric binding protein together with many other bacterial periplasmic binding proteins are all members of the transferrin superfamily (Bruns *et al.* 1997).

Early studies with doubly-labelled transferrin, showed that whereas the iron was rapidly cleared from the circulation, the protein recycles many times. Most if not all, mammalian cells have transferrin receptors located on their plasma membranes. The transferrin to cell cycle has been well established (Crichton 1991), and is illustrated in Figure 5. After binding to the receptor the diferric transferrin-receptor complex is internalised within an endosomal vesicle in which an ATP-dependent proton pump establishes a pH of 5.0–5.5. Iron release from transferrin at mildly acidic pH values is substantially increased when it is bound to its receptor compared to release from free diferric-transferrin: thus within the endosome the transferrin receptor facilitates iron release from transferrin. At acidic pH values, the apotransferrin has a high affinity for its receptor, and is then recycled back to the plasma membrane: at the slightly alkaline extracellular pH it dissociates from the receptor and is released into the circulation in search of iron. Other systems of iron uptake such as phagocytosis from ferritin or non-transferrin iron are also known to operate in certain mammalian cells.

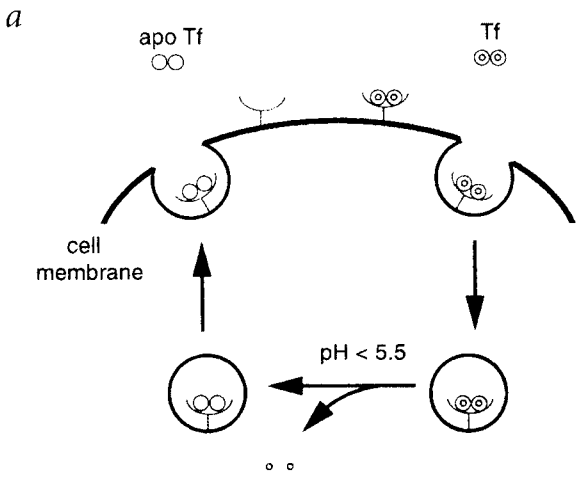

Fig. 5. Schematic representation of the transferrin-mediated pathway of iron uptake in animal cells which involves receptor-mediated endocytosis of diferric transferrin (Tf), release of iron at the lower intraendosomal pH and recycling of apo-transferrin (apo Tf) (from Baker 1997).

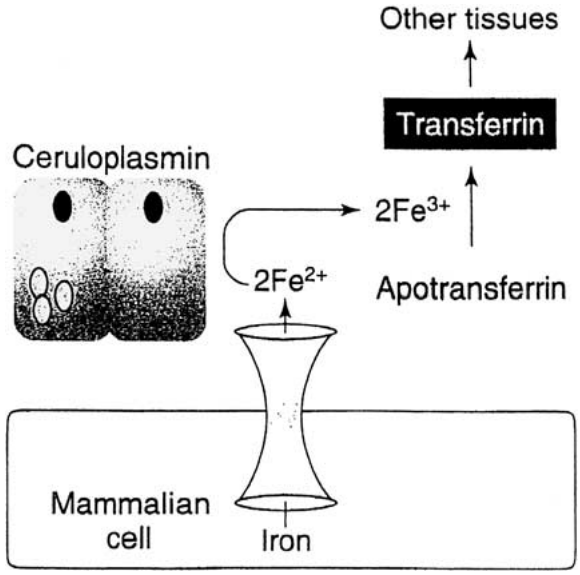

Fig. 6. Proposal for oxidase-dependent iron transport in mammals. It is proposed that ceruloplasmin mediates iron oxidation, facilitating iron export from cells. Small circles represent copper atoms within the multicopper oxidase (from Askwith & Kaplan 1998).

Intracellular iron storage involves two proteins, ferritin, and haemosiderin (Crichton 1991). Haemosiderin is a water insoluble protein complex, characterised by a higher iron to protein ratio than ferritin, and typically found in lysosomes, where it is almost certainly formed from ferritin by aggregation and proteolytic degradation. Ferritin is a cytosolic protein consisting of a soluble, hollow protein shell within which an iron-rich core consisting of a ferrihydrite biomineral phase, containing up to 4,500 atoms of ferric oxyhydroxide together with additional variable amounts of phosphate is deposited (Crichton 1991). Apoferritin, the iron-free protein, is made up of twenty four subunits of two types, H-chain and L-chain. Access to the interior of the molecule for iron is probably via the eight 3-fold channels which are extremely polar. Iron deposition in ferritin involves the protein itself, at least in the early stages. Initially Fe^{2+} is oxidised by the protein to Fe^{3+}, which then migrates to nucleation sites where biomineralisation to ferrihydrite proceeds. The H-subunits have a much greater ferroxidase activity than the L subunits, although the latter predominate in most tissues where large amounts of iron are stored (Crichton 1991). The putative ferroxidase sites of two *E. coli* ferritins have been identified directly by X-ray crystallography (Frolow *et al.* 1994; Hempstead *et al.* 1994). They are dinuclear iron sites in which the two iron atoms are linked by one or two bridging carboxyl groups, in a geometry reminiscent of a number of other proteins which have dinuclear μ-oxo-bridged diiron centers, incuding haemerythrin, methane monooxygenanse and ribonucleotide reductase.

A brief overview of copper metabolism in mammals

All of the genes required for copper loading of Fet3p in yeast have homologues in man with considerable sequence conservation (Askwith & Kaplan 1998). Although the transport of copper into mammalian cells is not well established, a functional human homologue, *HCTR1* of the yeast copper transporter gene, *CTR1*, has been identified by complementation (Zhou & Gitschier 1997). The human homologue to Atx1p, the chaperone which delivers copper to the CPx-type ATPases in the post-Golgi apparatus, HAH1, a 68 residue protein, can restore Fet3p function in yeast mutants lacking functional Atx1p (Klomp *et al.* 1997), and it is inferred that in man HAH1 delivers copper to a trans-Golgi compartment where it is required for copper loading of ceruloplasmin (Sato & Gitlin 1991). Despite strikingly different clinical phenotypes, both Wilson's and Menkes disease result from the absence or dysfunction of copper-transporting ATPases located in the trans-Golgi network of cells. Both proteins involved in Menkes and Wilson's disease, respectively ATP7Ap and ATP7Bp, are CPx-type ATPases, homologous to yeast Ccc2p (Yuan *et al.* 1995), which is responsible for Fet3p copper loading. The Menkes

protein, ATPAp, which is expressed in most tissues except the liver, causes defective intestinal copper absorption and decreased tissue copper mobilization leading to copper deficiency. In Wilson's disease, the tissues that normally express ATPBp are unable to export copper, and accumulate it, causing tissue damage, particularly in liver and brain. The loss of ATPBp leads to defective copper transport into the intracellular vesicles in which ceruloplasmin is normally loaded with copper. Since the liver is the principal site of ceruloplasmin synthesis, plasma ceruloplasmin levels are low (Scheinberg & Gitlin 1952), in some cases resulting in anaemia similar to that observed in copper-deficient pigs. Since the Menkes and Wilson's proteins can complement yeast CCC2 knockout mutants, it is likely that these ATPases play a similar role to Ccc2p, a view which is supported by their localisation in the trans-Golgi network (Petris *et al.* 1996; Nagano *et al.* 1998). It has been shown recently (Hamza *et al.* 1999) that HAH1 can be detected in lysates of human cell lines and tissues and that HAH1 interacts with both the Wilson and Menkes proteins *in vivo*. When similar studies were repeated using three disease-associated mutations in the amino terminus of the Wilson protein, a marked diminution in HAH1 interactions was observed, suggesting that impaired copper delivery by HAH1 constitutes the molecular basis of Wilson's disease in patients harbouring these mutations. This (Hamza *et al.* 1999) provides a mechanism for HAH1's function as a copper chaperone with an essential role in copper homeostasis, and suggests further that the defect, at least in Wilson's disease, probably involves transfer of copper to the CPx-type ATPase, rather than from the ATPase to ceruloplasmin..

In mammals, as in yeast, several different metallothionien isoforms are known, each with a particular tissue distribution (Vasak & Hasler 2000): their synthesis is regulated at the level of transcription not only by copper (as well as the other divalent metal ions cadmium, mercury and zinc) but also by hormones, notably steroid hormones which affect cellular differentiation. Intracellular copper accumulates in metallothionein in copper overload diseases, such as Wilson's disease, forming two distinct molecular forms; one with 12 Cu(I) equivalents bound, in which all 20 thiolate ligands of the protein participate in metal binding; the other with 8 Cu(I)/metallothionein molecule, with between 12–14 cysteines involved in Cu(I) coordination (Pountney *et al.* 1994). Although the role of specific metallothionein isoforms in zinc homeostasis and apoptosis is established, its primary function in copper metabolism remains enigmatic (Vasak & Hasler 2000).

Iron and copper interactions in mammals and man

The biochemical links between dietary copper and iron metabolism in animals which were outlined in physiological terms earlier have tended to focalise on the principal copper-containing protein of plasma, ceruloplasmin. Ceruloplasmin is a blue copper protein sythesized mainly in the liver. In the pioneering studies of the Utah group dietary copper restriction was reflected in decreased plasma levels of ceruloplasmin, defective mobilization of iron into the plasma with hypoferraemia and accumulation of iron in parenchymal tissues (Lee *et al.* 1968). Injection of ceruloplasmin repaired the hypoferraemia (Ragan *et al.* 1969). It was proposed that it was the ferroxidase activity of ceruloplasmin (Osaki *et al.* 1966) which was necessary for the oxidation of Fe^{2+} to Fe^{3+} to enable its uptake into apotransferrin (Williams *et al.* 1974). The importance of ceruloplasmin in normal human iron homeostasis has been confirmed by the recent description of the human congenital disease, aceruloplasminaemia, in which mutations in the ceruloplasmin gene leads to its absence from plasma (Yoshida *et al.* 1995; Harris *et al.* 1995). No detectable changes in copper transport are observed, but iron loading is found in parenchymal tissues, principally liver and pancreas, similar to that in copper-deficient pigs. While the anaemia observed in aceruloplasmic humans is not as severe as in the copper-deficient pigs, severe iron loading is also found in brain (accompanied by neurological symptoms such as extrapyrimidal disorders and cerebral ataxia), indicative of a role for ceruloplasmin in iron export from human brain (Harris *et al.* 1995; Klomp & Gitlin 1997). A murine model of aceruloplasminaemia showed no abnormalities in cellular iron uptake but a striking impairment in the movement of iron out of reticuloendothelial cells and hepatocytes (Harris *et al.* 1999). In order to explain both the findings in copper deficient animals and in man, it is proposed that plasma ceruloplasmin mediates iron mobilization from cells (after reduction and transport of Fe^{2+} out of the cell), facilitating iron binding to apotransferrin and hence its delivery to other iron-requiring cells (Askwith & Kaplan 1998).

It has been reported that ceruloplasmin increases iron uptake into cultured human cells (Mukhopadhay *et al.* 1998), although this apparently contradictory *in vitro* observation must be set against the *in vivo*

evidence presented above from both animal and human studies. Plasma ceruloplasmin levels increase markedly in anaemia, consistent with a physiological role in tissue iron mobilisation, and this effect is due to transcriptional activation of ceruloplasmin mRNA synthesis (Mukhopadhay *et al.* 2000).

Recently a genetic approach has been used to identify the gene mutant in *sla* (sex-linked anaemia) mice, which have a block in intestinal iron transport. Mice carrying the sla mutation develop moderate to severe anaemia, and although they take up iron from the intestinal lumen into mature epithelial cells normally, subsequent exit of iron into the circulation is diminished, and iron accumulates in the enterocytes and is lost during turnover of the intestinal epithelium. The mutant gene in sla mice, Heph (hephaestin) has been identified as a transmembrane-bound ceruloplasmin homologue, which is highly expressed in the intestine (Vulpe *et al.* 1999). It is proposed that the hephaestin protein is a multicopper ferroxidase necessary for the egress of iron from intestinal enterocytes into the circulation, establishing yet another important link between copper and iron metabolism in mammals. In recent studies a reciprocal relationship between liver copper and iron status has been observed in iron-loaded rats (Ward *et al.* 1998). Whether this is related to a possible role of the proton-coupled divalent cation transporter (DCT1) (Gunshin *et al.* 1997), in intestinal copper uptake, and its regulation by the IRP system remains to be established.

Conclusions

It is interesting to observe that in Roman mythology allusions not only to interactions, but even to connivances, between Mars and Venus, going further even to seduction, abound. There are, despite our growing awareness of the important interdependence between the two metals across biological evolution even now unanswered questions. When did copper really start to be used extensively, why was it necessary to have superoxide dismutases in strict anaerobes (although one explanation may lie in the definition of 'strict'), why does copper appear necessary for iron uptake and metabolism even in primitive eukaryotes like yeast, but not in bacteria? We can only hope that the newly developing impulses to apply the cutting edge of molecular biology to the cellular biology and physiology of iron and copper metabolism will provide at least some of the explanations.

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