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The copper-iron chronicles: The story of an intimate relationship

Paul L. Fox
Department of Cell Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA (Tel: 216-444-8053; Fax: 216-444-9404; E-mail: foxp@ccf.org)

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Abstract

During the last decade there has been a surge of interest and activity in exploring the metabolic links between copper and iron. This review describes more than a century and a half of effort that has led to our current understanding. Particular attention is given to the early events since these are less well-known and appreciated. The landmark 1928 paper of Hart, Elvehjem and coworkers is generally given credit for the start of the copper/iron field, and specifically for the discovery of the role of copper in forming hemoglobin and in overcoming anemia. However, some credit for the ideas, observations, and experiments should be shared with several investigators of the previous century. These scientists and physicians were primarily motivated to find the causes and cures of chlorosis, a common form of anemia at the time. From his chemical determination of copper in red blood cells in 1848, Millon proposed a form of chlorosis due to copper deficiency. Likewise, Pécholier and Saint-Pierre, observing the robust health of young women working in copper factories, concluded that copper was helpful in preventing and overcoming chlorosis. The first direct experimental evidence for the theory was provided by the Italian physician Mendini, who in 1862 reported that supplementation of the diet with copper salts overcame chlorosis in young women. In the 1890s Cervello and his students in Italy, using semi-quantitative hematological measurements, confirmed the beneficial effects of copper on anemia both in patients and in animal models. There was nearly a 30-year period of inactivity, but the decade of the 1930s saw renewed interest and exciting developments in the field. The Elvehjem report of 1928 was quickly verified and extended by multiple laboratories on four continents. In the 1950s and 1960s Wintrobe and Cartwright and their colleagues localized, and started to systematically evaluate, the potential sites at which copper was likely to effect iron for hemoglobin synthesis, namely, intestinal absorption, release from storage, and cellular utilization during synthesis. The copper/iron connection also has a 'flip-side', i.e., iron status can influence copper metabolism as first described by Warburg and Krebs in 1927. Thus, there are opportunities for feedback mechanisms at the cellular and physiological level that are not yet understood. The evaluation of these processes continues to this day, aided by modern molecular and genetic approaches. Studies of two copper proteins, ceruloplasmin and its recently discovered homologue hephaestin, have provided two molecular links connecting the pathways of copper and iron metabolism. The recent identification of other proteins of iron and copper metabolism, for example, copper ATPases and the membrane iron transporters DCT1/DMT1/Nramp2 and IREG1/MTP1/ferroportin1, are likely to fill crucial pathway gaps. The ongoing discovery of genes and gene mutations involved in the metabolism of copper and iron provides an important key to a deeper understanding of the connections between the pathways, and their physiological and pathological consequences. It is hoped that this historical review, by illuminating the complex paths that have led to the current state of knowledge, will contribute to our appreciation, our understanding, and perhaps our continued discovery of the connections between copper and iron.

Introduction

The farther backwards you can look, the farther forward you are likely to see

Winston Churchill

During the last decade there has been a rebirth of interest, and remarkable advances, in studies at the intersection of copper and iron metabolism. This resurgence has been accompanied by a succession of excellent reviews (Harris 1995; Kaplan 1996; Askwith & Kaplan 1998; Crichton 2001). Most reviews of the copper/iron connection begin with the well-known 1928 report by Hart, Elvehjem, and coworkers which is generally credited as the first to show that copper can facilitate hemoglobin formation and overcome anemia (Hart *et al.* 1928). Unfortunately, none of the recent reviews acknowledge the large and diverse body of relevant work beginning nearly a century before. Much of this early work focused on finding the cause and cure for chlorosis, a form of anemia common in young women. The studies of the key investigators, for example Millon, Pécholier and Saint-Pierre, Mendini, and Barabini and Cervello, have been largely forgotten. These scientists and clinicians took multiple approaches including biochemical, epidemiological, animal experimentation, and human clinical studies, but were severely hampered by the limited knowledge base and tools available at the time. It is a principal goal of this review to review these early studies. It is not our intention to critically evaluate these studies, but rather to understand the experiments in their contemporaneous context and to evaluate the impact that they had on subsequent investigators, up to the present day. The context sometimes includes the specific limitations of available knowledge and methodologies, contemporary prejudices, controversy, competition between investigators (and sometimes outright animosity), and an occasional societal influence. The original text is liberally quoted, after translation when necessary, to better transmit the flavor as well as the information. When available, relevant personal data about the investigators themselves is given, particularly their backgrounds, motivations, and other achievements. Finally, in addition to discussing the shining successes and breakthroughs, occasional dark patches and missed opportunities will be described.

Discovery of the copper/iron connection – the early years

Copper and chlorosis – rational, non-rational, and observational approaches to treatment

The role of copper in promoting overall health and its germicidal activity were recognized by early cultures including the Greek, Roman, Egyptian, Asian Indian, and possibly American Indian (Dollwet & Sorenson 1985). Investigators in the nineteenth century began to express and explore new ideas on a specific role of copper in iron metabolism and blood formation. The development of these concepts was driven primarily by studies of chlorosis. The disease, also known as the 'green sickness' or 'virgin's disease', was first described by Hippocrates (Loudon 1980). It was prominent from the mid-16th century to the beginning of the 20th century when it was commonly diagnosed in young women in Europe and America, and possibly the Middle and Far East. Chlorosis was physically characterized by a pale, greenish complexion (from which it took its name) and amenorrhea (absence of menstruation), and its victims often exhibited behavioral abnormalities including moodiness, lethargy, and sometimes pica. Studies of the disorder attracted the attention of physicians and researchers throughout Europe, but especially in France and Italy. The enthusiasm for the area can be seen in the rich publication record of the era; 324 pamphlets and books and 307 journal articles on chlorosis were published before 1879 according to the Index-Catalogue of the Library of the Surgeon-General's Office, United States Army. Nicolas Monardes from Spain first suggested iron as a treatment of chlorosis in his 1580 work on medicinal plants entitled '*Joyful news out of the new-found worlde*' (Robb-Smith 1933) Indeed, a century later, Thomas Sydenham showed that iron compounds offered a 'heroic' cure for chlorosis (Starobinski 1981). In 1745, the Italian physician Vincenzo Menghini observed that when blood was dried it could be picked up by a magnet, and thus must contain iron. He also showed that dietary iron supplements increased the amount of iron in blood. These findings led physicians to treat chlorotic women with various iron supplements, but with varying degrees of success. The Swiss pathologist Sigismond Jaccoud described a success rate of about 50% (Liégeois 1900). According to one school of thought promulgated (on surprisingly little evidence) by the influential physiological chemist Gustav von Bunge, only iron contained in food was effectively used by the body, and that chemical forms of iron were not absorbed (Josephs 1931). A second school favored the idea that other supplements were required in addition to (or in place of) iron. The latter

idea led to the consideration of multiple agents, particularly metals and metal salts, for the treatment of chlorosis.

In a rational approach to the treatment of chlorosis, and following the successful therapeutic application of the discovery of iron in blood, several investigators analyzed blood composition with the idea that supplementation with blood components could be effective. The presence of copper in blood was first reported in 1830 (Sarzeau 1830). In 1848, the eminent French metal chemist Auguste-Nicolas-Eugène Millon reported a similar finding. Using original methods he devised for the analysis, Millon showed that copper was not freely diffusible in blood but rather was 'fixed' with iron in the red blood cells (Millon 1848). Based on these observations he proposed the existence of a 'chlorose par défaut de cuivre' (chlorosis due to lack of copper). Millon is better known for the first chemical determination of protein using mercury nitrate ('Millon's reagent'), a method still in use for specific detection of tyrosine. In the same year, using essentially the same methods, Melsens in Belgium argued rather vehemently against Millon's results and conclusions. He reported that: *in 7 kilograms of blood from twenty-one different people it has been impossible to find any copper ... One must at least doubt the existence of a chlorosis due to lack of copper ... which until proven to the contrary seems to me to exist only in the imagination of the wise professor from Val-de-Grâce (Millon), while the metals themselves probably can be found in his water, reagents, glassware, filters, etc., etc.* (Melsens 1848). In a subsequent review, Béchamp analyzed the work by Millon, Melsens and others on the presence of copper in blood (Béchamp 1859). He suggested that the contradictory results were unlikely to be due to copper contamination or other methodological errors; however, he did not himself take a position and concluded that *contradictory results are in the nature of things*. His reluctance to accept the presence of copper may have been due to the contemporary philosophical view that a substance that can be toxic cannot also be a physiological component of the body. On the other hand he was clearly reluctant to challenge the view of Millon, the preeminent organic chemist of the time. A second review of copper analyses of blood and other tissues was published in a medical law journal; this issue had considerable legal significance since the presence of copper in autopsy specimens was believed to be evidence of copper poisoning (Chevallier *et al.* 1849). The existence and physiological significance of copper in blood and other tissues continued to be vigorously debated until more specific assays became available many decades later.

During the mid-19th century the use of copper as a therapeutic agent against multiple disorders became fashionable. Many of these ideas were supported at least as much by mystical belief as by scientific or clinical evidence. For example Victor Burq, a practitioner of Galvinism, i.e., the therapeutic application or detection of electricity, achieved notoriety for his studies on the external application of metals as a cure of nervous disorders (Burq 1853, 1852). He described bracelets, arm bands, corsets, brushes, lashes, and even bathtubs (Figure 1) made from alternating sections of copper and steel that he used to treat hysteria and other neuropathies. He proposed that copper and other metals exerted their curative activity through *electricity or mineral magnetism or any other cause beyond our understanding*, and were more effective when applied externally as large masses rather than internally by ingestion or injection of small amounts. He called the technique 'metallotherapy', but taking a cue from popular healing practices of the time, e.g., Mesmerism and Galvanism, also referred to it as 'Burqism'. Burq also described the successful treatment of chlorosis using a body-covering copper armature (Burq 1860). Burq, like many French physicians of that era thought that chlorosis was a nervous disorder related to hysteria, epilepsy, and paraplegia. This idea was not completely far-fetched; a recent analysis suggests that there were two distinct forms of chlorosis, one due to iron-deficiency and the other a psychogenic disorder related to modern anorexia nervosa (Loudon 1984). Although there is no supportive scientific or clinical evidence for its effectiveness, the therapeutic use of bracelets of copper and other metals remains a popular folk-medicine to this day. During this era there were also reports that bathing in, or consuming, water from certain European thermal springs that contained substantial amounts of copper improved overall health. This topic, and other contemporaneous medical uses of copper, was extensively reviewed by Giuseppe Levi and Domenico Barduzzi in the first part of their two-part manuscript (Levi *et al.* 1877a; Levi & Barduzzi 1877b). The benefits of copper in the treatment of cholera and tuberculosis were reported as well (Rademacher 1848; Burq 1853).

In the latter half of the 18th century there was much interest in reducing industrial hazards, and publication of observational studies of factory workers was commonplace. In 1848, Jean-Baptiste-Alphonse Chevallier and Jules-Louis-Charles Boys de Loury re-

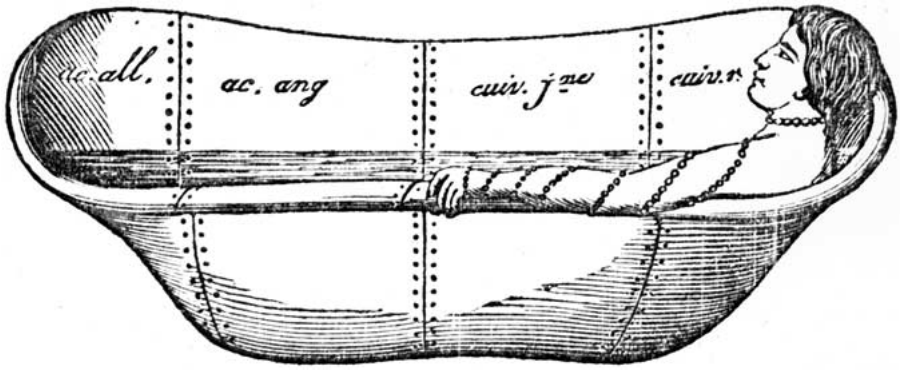

Fig. 1. Burq's metallotherapy device. The bathtub was made from four panels of different metals, from left to right: German steel (L'acier de Allemagne), English steel (l'acier de Angleterre), brass (cuivre jaune), and copper (cuivre rouge). Burq recommended that the patient cover extremities with chains of the same metals to augment surface exposure, and also that he alternate positions between ends of the tub. The bathtub was constructed by Lüer, a surgical instrument manufacturer in Paris (Burq 1853).

ported that workers in copper factories had robust health and *often lived to be octogenarians* despite the fact that they ingested so much copper that *the hair of the workers turns green and the walls on which they urinate become green* (Chevallier & Boys de Loury 1848). In contrast, Gurney Turner reported that workers subjected to copper dust in an imitation gilding process suffered muscle weakness, stomach irritation, and sometimes, behavioral problems (Turner 1839). Similarly, Perron observed that watchmakers working in an environment filled with copper dust suffered from multiple disorders, primarily affecting the lungs (Perron 1861). He attributed these health problems to an irritability property of the dust and also to a specific metabolic action of the copper. The French investigators Pécholier and Saint-Pierre undertook to explain the conflicting results on copper workers. At the outset they argued that the specific health effects of copper must depend on its dosage, method of handling, chemical form, and physical structure, and therefore each copper-containing environment must be separately investigated. They focused their attention on workers exposed to vert-de-gris, the green pigment which is a product of copper treated with vinegar. They showed that rabbits and dogs tolerated small amounts of vert-de-gris, but were poisoned by large amounts. They observed that young women working in a vert-de-gris factory in Montpellier, France exhibited a 'healthy, fresh, corpulent appearance' in contrast to the 'lean, pale faces' generally seen in female workers at other factories in the region (Pécholier & Saintpierre 1864). They further observed that menstruation of the workers was regular and that they *successfully breast-fed beautiful infants in the middle of the factory, even though we have seen their breasts sometimes covered with splashes of copper salt*. They also noted that they heard of chlorotic women whose health was restored after a few months in a vert-de-gris factory. As a control, they noted that chlorosis was very common in working women in the same city. Pécholier and Saint-Pierre indicated their wish to compare experimentally the effects of copper and iron on chlorosis, but *in the meantime, we won't hesitate to advise chlorotic young ladies to take a job as a vert-de-gris worker* (Pécholier & Saintpierre 1864). Levi and Barduzzi noted, without any detail or attribution, that similar effects of copper on chlorosis were seen in workers in an Italian mint who continuously handled copper coins (Levi & Barduzzi 1877a).

Thus investigators in the middle of the nineteenth century concluded that copper was a critical factor in chlorosis/anemia. From the chemical analyses of copper and iron in red blood cells, Millon deduced that at least one form of chlorosis could be due to copper deficiency. By observing the health of young women working in copper factories, Pécholier and Saint-Pierre arrived at a similar conclusion, that copper could help to prevent or overcome chlorosis. However, neither Millon nor Pécholier and Saint-Pierre ever tested their hypotheses by experiment. The first such experiments were reported in 1862, two years before the paper on the vert-de-gris workers, by a country doctor working in the small hillside villages near Verona in the north of Italy.

Studies of copper and chlorosis – qualitative clinical studies

L. Mendini, a physician from Trevenzuolo, Italy, was concerned about the ineffectiveness of iron in his treatmeant of some young, female chlorosis patients. In a short report entitled 'On a remedy for amenorrhea and another for hyposthenic deafness', he colorfully describes his successes with copper supplements (Mendini 1862). Mendini learned that in the village of Grezzana, near Verona, a pill was used to treat women who had chlorosis accompanied by a suspension of menstruation (described as the 'tributi mensili', i.e., monthly payment). For reasons that he doesn't disclose (he doesn't mention any of the earlier studies described above), he suspected that the pills contained copper. He sent several of the 'pills of Grezzana' for analysis to the Veronese chemist Sembenini who confirmed the presence of both iron and copper salts. Buoyed by this result, Mendini prepared the following formulation for his clinical studies:

> *Copper ammonium sulfate, 15 grains*
> *Iron sulfate, 1 drachm.*
> *Pulverize in a half drachm. of rhubarb syrup*
> *Put into 9 capsules*
> *To be taken once in the morning and once in the evening, and if possible, take a third or a fourth in the same day*

Mendini reported that using this formula he was *able to induce regular menstrual function, and cure extremely obstinate chlorotic afflications*, results previously unobtainable using iron sulfate alone. He also used a similar formulation that lacked iron, but doesn't distinguish between the results he observed. Mendini remarked that some young women could not tolerate the copper which can cause vomiting, and in these cases *it certainly helps to add a small amount of opium*. With these results, Mendini became the first investigator to report experiments showing the benefit of copper supplementation in the treatment of chlorosis/anemia. In the same 2-page paper Mendini described a rapid cure of 'hyposthenic deafness': the placement of leeches behind the ears! Nothing else is known about Mendini, his background, or his other achievements. No papers by Mendini are listed in the major medical indices; even his paper on copper and chlorosis was not listed since the journal, *Gazzetta Medica Italiana: Provincie Venete*, was not included in the indices. Unhappily, even his first name is unknown today.

Other investigators later confirmed the results of Mendini. Levi and Barduzzi in 1877 reported on the treatment of patients with a variety of symptoms with copper sulfate (Levi & Barduzzi 1877b). Several of these patients had symptoms consistent with chlorosis, and one 23-year old woman was described as having *extremely serious chloro-anemia*. All were improved or cured by the treatment as indicated by the reddish color of the skin and mucosae, return of normal menstruation, increased appetite, and even improved social behavior. Several physician-investigators in the United States also reported beneficial effects of copper both as a 'restorative' and in the treatment of chlorosis/anemia. In 1885, William Murray, a doctor in Suffolk, Virginia, colorfully described a farmer who visited him: *Skin, yellowish with a greenish tinge ... lips, tips of ears and finger nails perfectly blanched and almost as free from sanguinous hue as the paper on which I write ... he cannot walk across the street without getting almost entirely out of breath* (Murray 1885). He noted that previous treatment with tincture of iron was without benefit. Murray prescribed treatment with Rademacher's tincture (a solution of copper oxide and copper acetate, sometimes mixed with cinnamon and a *good glass of Rhine wine* (Dollwet & Sorenson 1985)), and a nutritious diet including milk, eggs, oysters, and beef. Murray described the improved condition after six weeks of treatment: *now the man can and does plow ... all the parts previously noticed as free from the tinge of blood, are now red and rosy ... The man is a living wonder to all who know him*. In a subsequent report published in 1892 in *The Therapeutic Gazette* by its senior editor H.A. Hare, he reported encouraging results on the use of arsenite of copper to treat anemia (Hare 1892). He noted that the skin *color becomes more like the normal, and ... the patients progressed rapidly towards health, provided of course, that the anaemia was functional and not organic in origin*.

The work and writings of the French physician Charles Liégeois merit some discussion. In an 1891 manuscript on the treatment of chlorosis with iron preparations (Liégeois 1891), he described a paper of his that was read before the Society of Therapeutics in November, 1890 and entitled 'Le traitement de la chlorose par le cuivre' (The treatment of chlorosis with copper). In a summary in a subsequent review article, Liégeois described his successful treatment of tuberculosis patients with copper acetate (Luton 1885). Upon obtaining a negative result for Koch's bacillus in some patients, he attributed their symptoms to chloro-

sis rather than to tuberculosis. Therefore, based on 'the old research of Pécholier and Saint-Pierre', he continued the copper treatment. Congratulating himself for his wisdom, he gushed *How many doctors would have immediately given up the copper aceto-phosphate and prescribed iron instead!* He concluded: *I have not used a red cell counter to assay the transformation of newly created hematoblasts into perfect red cells. I did not need this instrument to be sure of patient recovery: their strength returned as well as the color of their faces and mucosae ... Clinically speaking, the chlorosis was gone*. Unfortunately, it is not possible to assess the actual contribution of Liégeois to the field since the original papers are 'bibliographic ghosts', i.e., both literature citations he gave for this paper are incorrect, and a paper with the given title is not in either of the major medical indices of the time.

The mechanism by which copper helped to overcome chlorosis was not addressed in these early studies; however, several of the investigators speculated freely. Pécholier and Saint-Pierre theorized that copper, unlike iron, was not a part of the red blood cell hemoglobin 'hematosine', but rather had an unspecified positive nutritional effect on the blood. Levi and Barduzzi, suggested that copper improved the ability of red blood cells to transport oxygen and improved nutrient absorption thereby resulting in a 'hypernutritive' state (Levi & Barduzzi 1877b). They also noted that *clinicians and researchers acknowledge today that copper sulfate has the property of increasing the number of red blood cells*; unfortunately, they didn't give any attribution and it is not clear if this concept was common lore at the time or if there was an experimental basis. In his 1901 review, Liégeois reiterated a theory similar to that of Pécholier and Saint-Pierre, noting that it *is not directly, as it is for iron, that copper restores hemoglobin; it is rather indirectly: in part by stimulating hematopoiesis, and in part by helping to more perfectly assimilate the four elements that hemoglobin takes from digested matter (iron, carbon, nitrogen, and sulfur), leading to transformation of hematoblasts into red cells* (Liégeois 1901a). Liégeois added to the theory, speculating that the copper-stimulated cells are *more regular, more resistant, and less likely to die soon after their birth*. The mechanism of action of copper in erythropoiesis would remain a major area of investigation for more than a hundred years.

The clinical studies of Mendini and the others were typical of the era, lacking the rigor and standards in use today. For example, they did not quantitate specific hematological parameters, i.e., hemoglobin or red cell number or mass, they used very small treatment groups, and they did not contain a no-treatment control group. However, the description of a simple and practical method for counting red blood cells by Malassez and Potain in 1867, and the invention of clinical hemoglobinometers by Malassez and Gowers in the 1870s, soon enabled more quantitative approaches to the investigation of chlorosis and its treatment (Robb-Smith 1933).

Treatment of chlorosis/anemia with copper – quantitative studies

The first quantitative studies of the role of copper in anemia were reported in a booklet written by Vincenzo Cervello and E. Barabini at the University of Palermo in Italy (Cervello & Barabini 1894). They observed that feeding copper sulfate to dogs and roosters substantially increased blood hemoglobin as measured with a 'Fleischl hemometer'. In a series of four subsequent papers published between 1894 and 1897, Cervello and his medical students described the quantitative effects of copper salts and copper complexes on red blood cell number and hemoglobin, in both patients and animals. There are several aspects of these investigations that merit mention since they give insight into the thinking and attitudes of the investigators, and also their times. In his 1894 paper, Cervello noted that the preliminary studies he reported with Barabini in animals *seemed so promising, that before studying the detailed nature of the phenomenon, we thought it useful to do some experiments on humans* (Cervello 1894)! In addition, although he would have preferred to do experiments on patients with chlorosis/anemia, he was forced to use very sick patients with malaria since only these patients were allowed to stay in the hospital at the close of the school year. Cervello noted that *to avoid error I was careful to repeat every determination made by my assistants*. In his first clinical experiment he described results with an extremely sick patient given copper sulfate. He noted that the patient's strength appeared to improve during the first week, then the blood hemoglobin concentration decreased slightly and the patient died after 4 weeks of treatment. Cervello was not discouraged by these results, but rather he optimistically reported that *it seems that the metal successfully slowed the progression of the disease, and prevented a decrease in hemoglobin level for several days*. The experiment was followed by three others in less sick malarial pa-

tients, and in all cases the blood hemoglobin levels rose substantially. All patients survived and became healthier as determined primarily by activity and skin color. He noted in discussion that he chose to publish these studies, although incomplete, because the end of the school year prevented their completion and because he hoped that others would continue them (Cervello 1894).

Cervello's report was followed a year later by a paper by his student Giovanni Scarpinato (Scarpinato 1895). In these experiments, Scarpinato used the Merck compound 'cuproemolo' (or Kupferhämol (Kobert 1895)) instead of the copper sulfate previously used by his mentor. This compound was a mixture of copper salts with a preparation of partially-purified hemoglobin. He gave as a rationale that the combined form was less caustic than the salt since it was 'combined organically'. Scarpinato was likely swayed by the enormous influence of von Bunge and his widely-held belief in the effectiveness of food-derived matter compared to synthetic compounds. As an experimental model, Scarpinato chose roosters since their comb made the blood particularly easy to sample. He reported that cuproemolo was well-tolerated by the birds and increased hemoglobin levels after 2 days by about 25% in two birds. In another experiment he induced an 'artificial anemia' in roosters by feeding them a synthetic diet that was as free from iron as possible. The hemoglobin levels gradually decreased by about 25%, and then cuproemolo was given which restored hemoglobin to about the original level. Finally, Scarpinato gave cuproemolo to anemic patients. The first patient was described as *suffering from chronic gastritis, insufficiency of the aortic semilunar valve, and profound anemia*. The treatment essentially doubled the blood hemoglobin level and increased red blood cell counts by more than 50%. Similar success was seen in a second patient with anemia complicated by the presence of a tapeworm (which he carefully pointed out was not killed by the copper). Scarpinato also showed the presence of copper in the urine thus proving absorption of the copper supplement. He proposed an unusual mechanism of action: that copper, by saturating hydrogen sulfide in the gut, permits the free absorption of iron (Scarpinato 1895). Scarpinato apologized for the absence of some controls, and for his inability to distinguish between the activity due to the copper and to the 'emolo'. This would be addressed in the final paper in the series. The third paper in the series was by Cervello's student Simone Guagenti. This paper primarily confirms the beneficial effect of copper (and to some extent mercury) in the treatment of anemia in three additional patients (Guagenti 1896). The fourth and final paper was by still another of Cervello's students, Francesco Mercadente (Mercadante 1897). He showed that the hematopoietic activity of cuproemolo was due to the copper, because the emolo portion was ineffective by itself. Directly confronting the popular theory of von Bunge, he reported that copper salts were just as effective as the more 'food-like' cuproemolo. The experiments by Cervello and his students established many experimental 'firsts' in the history of the copper/iron connection. They were the first to show increased hemoglobin and red cell number, the first animal studies, the first dietary iron-deficiency studies, and, in fact, the first experiments with controls of any kind. A large, modern hospital at the University of Palermo, the Vincenzo Cervello Hospital, is named in honor of this early pioneer.

Cervello's clinical results were supported by those of V. Giudiceandrea who described considerable patient success with copper treatment. His clinical studies in a hospital in Rome, Italy were reported in 1901 in an anonymous contribution in a medical newsletter (author anonymous, 1901). They indicated that 50 mg per day of copper acetate increased the hemoglobin and red blood cell levels in the patients, and improved their overall health. Giudiceandrea maintained that intolerance could be avoided by daily alternation of the copper and iron treatments. In contrast, two investigators of the time could not confirm the hematopoietic activity of copper. Giovanni Castronuovo, a student at the University of Naples, showed that injection of small amounts of copper salts into healthy dogs had no effect on hemoglobin level or red blood cell number, and that larger amounts were inhibitory (Castronuovo 1895). Aware of the positive studies by Cervello, he concluded that the route of administration is critical and that oral copper must increase hematopoiesis by activating 'digestion', whereas intravenous copper was ineffective. Likewise, W. Wolf (Wolf 1898) in Marburg, Germany reported that copper did not increase red blood cell number or hemoglobin level in rats. Conceding to the consensus opinion of the time, he concluded that his methods may have differed from those of the Cervello group. Neither of these investigators considered the alternative possibility, namely, that copper may only be effective in animals with below-normal hematological values, i.e., anemia.

Remarkably, three *reviews* on the treatment of chlorosis with copper were published in a single year,

1901. The competitiveness, and perhaps even animosity, between some investigators was apparent in these reviews. In that year Vincenzo Cervello was invited to read a report to a French society of physicians that was published in *Journal des Praticiens* (Cervello 1901). Cervello summarized in detail his own group's studies, but did not mention the earlier copper studies of Mendini or anyone else. In fact, none of the papers by Cervello or his students in Palermo cited any previous studies of the effects of copper on anemia. In his own comprehensive and critical 1901 review, Liégeois expressed frustration at the lack of recognition for his work. He pleads: *I firmly hope – I wrote at the end of my 1890 communication – to see the results that I have obtained with copper and chlorosis confirmed by others. These results have been claimed as their own by foreign doctors who did not know of my report any more than I knew of Mendini's. When will the doctors from our country speak up?* (Liégeois 1901a). Given their present status as 'bibliographic ghosts' one most wonder about the availability of Liégeois' papers to his contemporaries. Nevertheless, any uncertainty about the intended target of his remarks was removed by a second review written by Liégeois in the same year. This review (published in the same issue of *Journal des Praticiens* as the Cervello review) summarized the copper formulations used by investigators to treat various maladies (Liégeois 1901b). Liégeois explicitly details the chronology: *In 1890 I made known my success using copper aceto-phosphate... in chlorosis. Publication of my research on this topic (1891) is before that (1894) reported by V. Cervello*. It is abundantly clear that the needs for recognition and for establishing precedence were at least as important to some investigators a century ago as they are today.

By the close of the 19th century there was consensus among most investigators about the importance of copper as a therapy in treatment of anemia. Despite the enthusiasm of the investigators and the apparent momentum in the field, there was an unusually prolonged gap between the reviews by Liégeois and Cervello and the studies by Elvehjem and Hart – only a single paper on copper and chlorosis/anemia can be found in the quarter-century following the 1901 reviews (Chabanier *et al.* 1913). There are several explanations for this hiatus. Possibly it was due to the improved health of women in America and Europe, and the fact that chlorosis was losing credibility as a major disease at about that time. At about this time the 'hot' area in copper research seems to have switched from dietary effects on chlorosis to studies of its use as a topical bactericidal agent (known to the ancients as well). An alternative explanation is the recognition that high levels of copper can be toxic, as suggested by the appearance of many articles on the topic during this period. One unusual example is the 1908 report by Emil Felletár, Chemist of the King's Court of Hungary, on 'Cases of possible copper poisoning caused by cream puffs' (Felletár 1908). However, the recognition that a high level of copper was poisonous was not new, and in the absence of any catastrophic (but unpublished) results, it was unlikely to be responsible for the abrupt stoppage of research on chlorosis. Most likely the gap was simply a product of the normal cyclic process of scientific discovery, and that a new generation of investigators with new tools and new questions was required to bring the work to the next level. Surprisingly, when the copper/iron connection was reborn in 1927–28, it appeared as a near-total discontinuity from the previous work. The new investigators took virtually nothing from the ideas and results of the earlier investigators as evidenced by a near-total absence of citations; only the 1901 review by Liégeois survived the hiatus to be occasionally cited three decades later. The new investigators started essentially from the beginning, using new models (milk-fed rats and pigs), new biochemical and hematological methods and tools, more highly purified reagents, and even a new language (chlorosis became chloro-anemia and eventually just anemia).

Quantitation and specificity of the effects of copper on anemia – the 1930s

Studies of copper deficiency anemia

In 1928, Hart, Steenbock, Waddell, and Elvehjem ('with the cooperation of Evelyn Van Donk and Blanche M. Riising' also appeared below the title) published their seminal and now classic work entitled 'Iron in nutrition. VII. Copper as a supplement to iron for hemoglobin building in the rat' (Hart *et al.* 1928). Working at the University of Wisconsin in Madison, they took advantage of previous groundbreaking experiments by Gustav von Bunge and his student Emil Abderhalden which showed that cow's milk contained very little iron, and that rats fed an all-milk diet became severely anemic (Abderhalden 1899). (Others have taken practical advantage of this result to produce near-white, 'milk-fed veal' from calves fed only milk). They also showed that supplementation of milk

with inorganic iron salts did not restore hemoglobin to normal levels. The Elvehjem group concluded that iron was critical for hemoglobin formation, but that another component of the normal diet was also required (Hart *et al.* 1925). In the first report in their series addressing this question, they showed that the addition of an alcoholic extract of cabbage or corn meal, or chlorophyll itself, overcame anemia (Abderhalden 1899). This observation led them to conclude that a vitamin was a critical co-factor in hemoglobin synthesis. They subsequently rejected the vitamin theory after observing that supplementation of iron with the ashed residue of lettuce or beef liver also increased hemoglobin levels in milk-fed, anemic rats (Waddell *et al.* 1928). They concluded that the substance was inorganic and *active in exceedingly small amounts*. Thus the foundation was set for their classic work on copper. As a rationale for testing copper they indicated that sometimes they noticed a pale blue color in the active fractions precipitated with hydrogen sulfide from an ashed liver preparation. In their first experiment they supplemented the cow's milk diet of a single rat with copper sulfate and ferric chloride. They reported a dramatic increase in hemoglobin, from 2.7 to 13.3 g/100 ml of blood after 6 weeks of treatment, and were so pleased by its performance that a photograph of the subject, 'Rat 621', was shown below the graph. Similar results were reported for three other rats.

The paper by Hart and coworkers was received by the *Journal of Biological Chemistry* on March 23, 1928. Less than two months later, on May 19, 1928, the same journal received a paper entitled *The relation of copper to the hemoglobin content of rat blood* from McHargue and co-workers at the Kentucky Agricultural Experiment Station (McHargue *et al.* 1928). This group showed that rats fed a skim milk diet supplemented with an ash of calf liver had a higher blood hemoglobin level than rats fed the ash treated with hydrogen sulfide to remove copper. The authors infered from *the external appearance of the rats, the color of the fresh blood, together with the hemoglobin readings ... that copper has an important function in the formation of hemoglobin and in the metabolism of animals having red blood.* In an addendum they noted the publication of similar findings by Hart *et al.* after the submission of their own work. The papers immediately catalyzed a flurry of investigations and publications on the role of copper in hemoglobin formation, at a pace extraordinary even by today's standards. Three additional papers were published in 1928, six more in 1929, three in 1930, and seventeen in 1931 when interest peaked. All told, more than 60 papers were published in the field in the ten years following the initial observations in 1928. Most of these papers were confirmatory, extending the work to different species or using slightly different protocols, but one laboratory group challenged the Elvehjem group's results.

The research group led by Howard Beard and Victor Myers at Western Reserve University (now Case Western Reserve University) in Cleveland published in 1931 a series of six back-to-back papers on anemia in the same issue of the *Journal of Biological Chemistry*. In the first paper they showed that supplementation of milk-fed rats with iron by itself restored normal hemoglobin levels (Beard & Myers 1931). In the next paper they reported two areas of disagreement with the Elvehjem group: first, copper by itself (in the absence of iron) increased blood hemoglobin slightly, and second, several other metals also showed marked erythropoietic activity thus negating the specificity of the effect of copper (Myers & Beard 1931). Their concluding comment was not subtle: *The specificity of copper as a supplement to iron in the cure of the nutritional anemia of the rat receives little support from these studies*. An immediate response came from Schultze and Elvehjem in a 1933 report which showed that both copper and iron were essential for reticulocyte production in anemic rats (Schultze & Elvehjem 1933). They noted that relatively small amounts of copper are active and that contamination of reagents with copper must be carefully minimized to obtain the results they report. Without experimental evidence, and using a rather circular argument, they argued that the *fact that Beard, Baker, and Myers obtained a reticulocyte response with iron alone clearly demonstrates that their rats had some available copper with the iron.* They continued, *Whether the source of this copper was from the animal or from the milk we cannot say.* The debate did not last long, and in 1934 the Cleveland group capitulated as gracefully as they could (Bing *et al.* 1934). They reported results from the new studies *performed with milk containing as little copper as it seems possible to procure ... the product of a single prize cow in the certified milk herd of the Telling-Belle Vernon Company at Novelty, Ohio.* They concluded that *although we have not been able to obtain entirely negative results with milk and iron, the stimulation of hemoglobin production brought about by the addition of small amounts of copper has been repeatedly and uniformly verified in this laboratory.* Their use of the double-negative could not mask the fact that their earlier milk supplies had been contam-

inated with copper, and that complete restoration of hemoglobin did require both iron and copper. This paper contained groundbreaking data on the mechanism of action of copper (see below), but this aspect of the paper was over-shadowed by the controversial results. This was the group's last publication on the copper/iron connection.

Laboratories around the world soon enjoined the quest to confirm, characterize, and understand the role of copper in hemoglobin formation (Krauss 1929; Keil & Nelson 1931). Just a few highlights are described here; more complete summaries can be found in excellent reviews from that era (Elvehjem 1935; von Linden 1935; Schultze 1940). The effectiveness of copper in multiple species was shown, including chickens (Elvehjem & Hart 1929), pigs (Elvehjem & Hart 1932), and dogs (Potter *et al.* 1938), to name just a few. The specificity of copper compared to other metal salts (Waddell *et al.* 1929; Krauss 1931; Underhill *et al.* 1931) and the purity of the iron (i.e., the freedom from copper contamination) required to see a copper effect were studied intensively (Lewis *et al.* 1930). The source of copper was also addressed and protein-bound copper was found to be as effective as copper salts (Schultze *et al.* 1934). Only limited studies on the mechanism of copper action in hemoglobin formation were done during this period. Copper was shown not to be a major constituent of hemoglobin, and thus was unlikely to be a building block, but rather a catalyst required for hemoglobin synthesis (Elvehjem *et al.* 1929). Copper and iron were shown to be required for reticulocyte production suggesting a tight link between this process and hemoglobin formation (Schultze & Elvehjem 1933). In addition to the animal experiments, some human studies were done. Copper supplementation of iron was reported to be beneficial to anemic infants and to adults (see section below on clinical studies). Even royalty contributed to the field as the Countess Maria von Linden, in her erudite 1935 review, noted that copper salts cured anemia in game animals (von Linden 1935). Sadly, the specific species treated, and the undoubtedly unique experimental protocols, were not described. Much more sadly, these studies were reported from her exile in Schaan, Liechtenstein after she was permanently removed from her tenured position at the University of Bonn by the Nazis, as a 'politically unreliable or non-aryan enemy of Germany' (von Linden 1933). She possibly was punished for her association with the family of Heinrich Hertz (she lived in their home), the Jewish-German physicist whose name became the unit of electromagnetic frequency.

Impact of the Hart and Elvehjem studies

The seminal paper published by Hart and co-workers in 1928 had, and continues to have to this day, an enormous impact on research in the copper/iron field. Its publication was followed by a frenzy of investigative activity in the area. Whereas there were at most a handful of reports on the connections between copper and iron metabolism in the preceding quarter century, there were at least 65 papers published in the decade following the report, nearly all on the effect of dietary copper on hemoglobin formation. Significant contributions were made by investigators from many laboratories in the United States and from countries on four continents including Canada (Mills 1930), the Netherlands (Gorter *et al.* 1931), England (Parsons 1933), Scotland (Hutchison 1938), Liechtenstein (von Linden 1935), New Zealand (Cunningham 1931), and Japan (Sarata 1934). The paper was cited extensively during the succeeding decade, and it continues to be cited at an extraordinary rate to this day; between 1974 (the start of record-keeping) and late 2001 the Hart paper has been cited about 140 times, more than 5 times per year. In addition, the original article was reprinted in its entirety in two different journals (no author 1987; Hart *et al.* 2001), and summarized and analyzed in detail in an article by Leslie Klevay as part of a minisymposium on 'Experiments That Changed Nutritional Thinking' (Klevay 1997).

A whimsical portrait of Conrad Elvehjem done by Aaron Bohrod shows the Lasker award he received in 1952 for his many seminal contributions in nutrition and biochemistry (Figure 2); a more austere portrait currently hangs in the Elvehjem Museum, named in his honor, in Madison, Wisconsin (not shown). The McHargue paper which came to a similar conclusion, but was submitted 2 months later, has been cited only once since 1974 (in a paper from this author's laboratory). It should not be too surprising that the McHargue group is little remembered for their work on copper – the Elvehjem group was first (if only by 2 months) and by far the more well-known of the two research groups. Perhaps more importantly, the Elvehjem group tackled the problem tenaciously and published about twenty papers in the next eight years, while the McHargue group did not pursue this avenue and published no additional papers. The early papers describing the role of copper in chlorosis/anemia by

Fig. 2. 'Portrait of Conrad Elvehjem', Aaron Bohrod, 1963, oil, commissioned by the Wisconsin Union Directorate. Items associated with Elvehjem's personal life and scientific career are shown, including the Albert Lasker Medical Research Award (for Clinical Research, 1952), some favorite experimental animals, and a tip he used to teach the correct pronunciation of his name, i.e., "LVM". Courtesy, Wisconsin Union Collection, University of Wisconsin, Madison.

Mendini and by Cervello and his students have not been cited since 1974, and are little known even to workers in the field.

The studies by Hart, Elvehjem and coworkers were the first rigorous, controlled, quasi-modern experiments on the role of copper in overcoming anemia. However, given the earlier groundbreaking studies by investigators in Europe, it is a little surprising, and perhaps inequitable, that the entire credit for the discovery of the copper/iron connection is commonly given to the 1928 paper. It is interesting to speculate on the possible reasons that the earlier studies have been essentially forgotten. Certainly the discontinuity caused by the long lapse in research between 1901 and 1928 didn't help the earlier investigators. By the time that interest in the area was renewed, the methods and concepts of hematological research had been much advanced, but even more importantly, the approaches to research (especially attention to repetition and controls) were far more sophisticated. Thus there may have been a common tendency to ignore earlier work. The prodigious output of the Elvehjem laboratory, as evidenced by continuous publications in excellent journals for more than a decade, certainly helped to establish them as the premier laboratory in the copper/iron field. Elvehjem's well-deserved reputation as a world-class biochemist also contributed. Finally, Hart and Elvehjem aggressively asserted their

precedence in their published papers while at the same time virtually ignoring the previous work. For example, they stated in their 1928 paper that *We think this is the first experiment in the literature giving to copper in association with iron the specific function of hemoglobin building in a mammal on an otherwise satisfactory diet* (Hart *et al.* 1928). There are so many qualifications here that one might hazard that they were tiptoeing around the previous studies. None of the work from the previous century was mentioned in the 1928 paper or in any of the dozen or so papers by Elvehjem and coworkers in the next few years. Finally, in his 1935 review Elvehjem briefly noted that the *early medical literature contains several references to the use of copper in animals (reviewed by Liégeois 1901) but no attempt was made to use a combination of available iron and copper until (our) experimental work* (Elvehjem 1935). Elvehjem here appears to discount the earlier studies because of the lack of purified materials or the absence of the appropriate control experimental group, which is largely correct; systematic studies comparing copper and iron to iron alone were not done by Mendini or Cervello. Nonetheless, at least partial credit for the discovery of the copper/iron connection should go to these investigators: to Mendini for showing that supplementation of iron with copper salts could help to overcome chlorosis/anemia and to Cervello and his students for semi-quantitative analysis of the effects of copper on hemoglobin and red blood cell formation. And what credit should go to Liégeois? Without examining the original reports, it is not possible to determine whether his experiments advanced the field. However, we have Liégeois to thank for acknowledging the study by Mendini whose work was otherwise never cited and would certainly be lost today.

Physiological mechanisms by which copper overcomes anemia – identification of control points

The presence of copper in red blood cells was known as early as 1848 (Millon 1848). The possibility that copper was an essential part of the hemoglobin molecule was considered but discarded by Pécholier and Saint-Pierre and then by Liégeois who favored a nutritional or indirect effect of copper (Liégeois 1901a; Pécholier & Saintpierre 1864). This conclusion was supported by experiments by Elvehjem and co-workers who showed that copper was not a stoichiometric constituent of hemoglobin (Elvehjem *et al.* 1929), and thus began the quest for an enzymatic role of copper in erythropoiesis.

Most attention was given to the role of copper as a catalyst in iron metabolism. This focus was likely the result of observations that copper deficiency anemia and iron deficiency anemia exhibited similar hematological features, namely, bone marrow morphology and microcytic, hypochromic circulating red blood cells (Smith & Medlicott 1944; Cartwright *et al.* 1956). Hugh Josephs at Johns Hopkins University identified six control points of iron metabolism that were potentially regulated by copper, including gut absorption ('retention'), formation of 'function iron of tissues', deposition into storage pools, release from storage, and formation (or degradation) of hemoglobin (Josephs 1932). In the first of their remarkable series of about 30 papers on the topic, Maxwell Wintrobe and his collaborators George Cartwright, Eugene Lahey, and Clark Gubler at the University of Utah subdivided the likely functions of copper in hemoglobin formation into three major mechanisms (Figure 3A). They remarked that *one could wonder whether copper acts in some way by ... facilitating absorption of iron from the intestinal tract, by releasing iron from stores in the liver and elsewhere, or that copper is necessary for the incorporation of iron into hemoglobin* (Wintrobe *et al.* 1951). Wintrobe and his students and colleagues spent the better part of three decades investigating the importance of each of these three mechanisms, primarily in the copper-deficient swine model (Figure 4). Their work and experiments by others will be described in subsequent sections, subdivided into the three mechanisms. However, a detour into the discovery of ceruloplasmin is appropriate here since it occurred at about the time that these mechanistic studies were getting underway, and it had a marked impact on all three proposed mechanisms of copper action.

The discovery of ceruloplasmin and its ferroxidase activity

Despite extensive studies of copper-containing polyphenol oxidases in plants, the presence of a comparable oxidase in plasma was hotly debated during nearly the entire first half of the nineteenth century (Holmberg & Laurell 1951). Seeking such a copper oxidase, Mann and Keilin at the University of Cambridge were the first to attempt to identify a copper protein(s) in the blood (Mann and Keilin, 1938). They purified

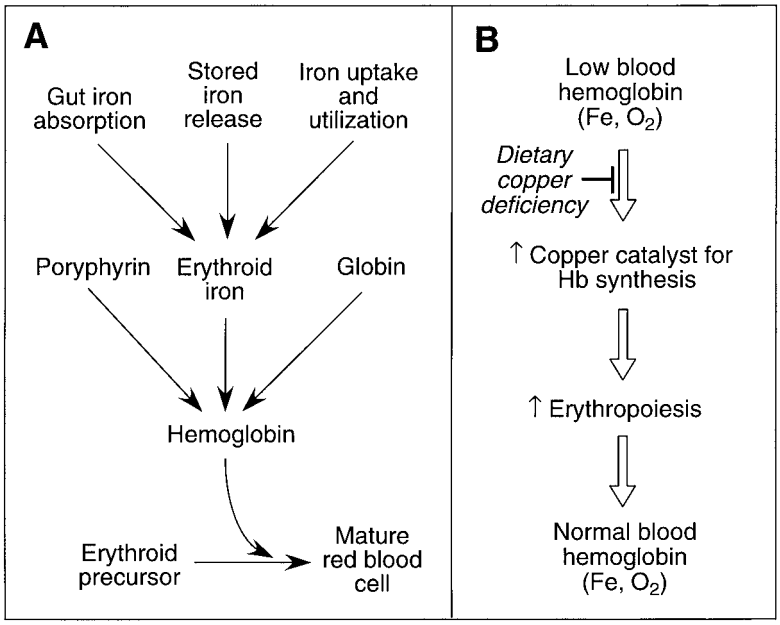

Fig. 3. Erythropoietic pathways involving copper. (A) Iron-dependent and iron-independent pathways of hemoglobin formation and erythropoiesis. (B) Interdependence of iron and copper in erythropoiesis.

Fig. 4. Photograph of M.M. Wintrobe, J.G. Palmer, W.N. Jensen, and G.E. Cartwright with experimental animal, c. 1956. Courtesy, J. Willard Marriott Library, University of Utah.

and crystallized a blue protein of molecular weight of about 35,000 from serum and blood and named it haemocuprein. The fact that the copper-to-weight ratio (0.34%) was identical to that of ceruloplasmin (which has a molecular weight of 132,000 Da) suggests that they may have purified a degraded form of ceruloplasmin. Unfortunately, the purified protein lacked all enzymatic activities tested, including the known polyphenol oxidase activity of plant copper oxidases. In 1944, Carl Holmberg and C.-B. Laurell at the University of Lund in Sweden found a serum *p*-phenylenediamine oxidase activity that co-fractionated with copper (Holmberg 1944). In 1948, they purified the protein on the basis of its blue color and found a large, 151,000 Da protein containing about 8 copper atoms per molecule, and which retained the a *p*-phenylenediamine oxidase activity of the crude fraction (Holmberg & Laurell 1948). Holmberg and Laurell proposed that the new protein be named 'coeruloplasmin' (i.e., sky-blue plasma protein), and they are generally credited with the discovery of the protein.

There has been some inconsistency in the spelling (not to mention the pronunciation) of ceruloplasmin. Holmberg and Laurell spelled it as 'coeruloplasmin' in all of their papers, using the 'o-e' ligature (œ) in their original report. Unfortunately, due to poor typography, the vertical line connecting the two letters had a small pip at the top, making it essentially indistinguishable from the 'a-e' (æ) ligature. Wintrobe and coworkers, possibly misreading the ligature, denoted it as 'caeruloplasmin', a form still in common use in England (Wintrobe *et al.* 1951). The spelling most often used today, 'ceruloplasmin', first appeared in the paper by Herb Scheinberg and David Gitlin in which they made the seminal observation that ceruloplasmin levels were low in plasma from Wilson's disease patients (Scheinberg & Gitlin 1952).

An important clue to the function of ceruloplasmin was related to the discovery of transferrin. Arthur Schade and Leona Caroline reported the discovery of this iron-binding protein in 1944 in egg whites (Schade & Caroline 1944), and later in plasma (Schade & Caroline 1946). Soon thereafter, Bernard Koechlin reported that binding of ferrous iron to the 'β_1-metal-combining protein' of plasma, i.e., transferrin, required oxygen, and was *enhanced by certain catalytic factors present in plasma* (Koechlin 1952). These results provided additional motivation for the search for a serum oxidase, and in particular, a ferroxidase. The presence of a catalyst in blood able to oxidize ferrous salts was shown independently by two groups working at the German University in Prague, Czechoslovakia (Hendrych *et al.* 1933; Starkenstein & Harvalik 1933). Curzon and O'Reilly at the National Hospital in London, England were the first to recognize that ceruloplasmin had a ferrous ion oxidase activity (Curzon & O'Reilly 1960). They described a coupled iron-ceruloplasmin oxidation system in which ferrous ions increased ceruloplasmin-mediated oxidation of a phenylenediamine substrate. They proposed a mechanism in which Fe^{2+} was oxidized by ceruloplasmin to form Fe^{3+}, which in turn oxidized the organic amine. In a second paper characterizing ceruloplasmin oxidation of substrates, Curzon mentioned the earlier work on plasma iron oxidases and the requirement for iron oxidation for loading of transferrin (Curzon 1961). Although he clearly recognized the catalytic nature of the reaction with respect to the Fe^{2+} substrate, he did not suggest that ceruloplasmin had an important role in iron metabolism but instead focused on the ability of ferric ions to enhance phenylenediamine oxidation. In fact, Curzon specifically cautioned that ceruloplasmin was unlikely to be the plasma ferroxidase described previously since ceruloplasmin had a lower K_m for Fe^{2+} and was insensitive to an inhibitor which blocked the activity in plasma (Curzon 1961).

Osaki, Johnson, and Frieden were the first to recognize the physiological significance of iron oxidation by ceruloplasmin in their classic 1966 publication (Osaki *et al.* 1966). This collaboration began in 1960 when Earl Frieden visited the Tokyo Institute of Technology in Japan and recruited Shigemasa 'Gem' Osaki to his laboratory at Florida State University as a postdoctoral fellow (Figure 5). Frieden had a long-standing interest in trace elements, specifically, copper protein biochemistry; Osaki had just developed a new procedure for ceruloplasmin purification in the laboratory of Michiyuki Shimizu at Showa University in Tokyo (Osaki *et al.* 1961). Together they embarked on a collaboration spanning a 15-year period that would unveil major insights into ceruloplasmin function, and which provided a foundation that persists in current models of iron metabolism. They showed that purified ceruloplasmin markedly increased the rate of iron loading into transferrin by a mechanism that consumed oxygen stoichiometrically (Osaki *et al.* 1966). They also provided evidence, both correlative and by co-fractionation from serum, that ceruloplasmin was the plasma factor responsible for oxidation of both iron and *p*-phenylenediamine. They noted that of all

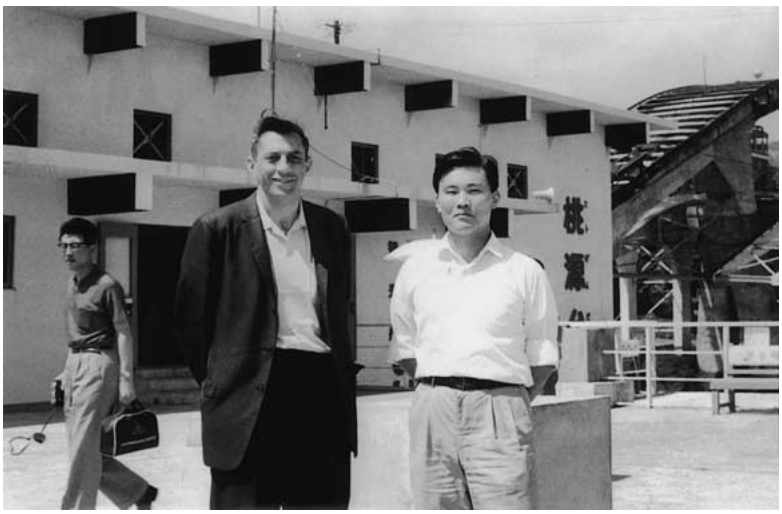

Fig. 5. Earl Frieden (left) and Shigemasa 'Gem' Osaki (right). Photograph taken at Togenkyo aerial rope-way station in Fuji-Hakone-Izu National Park, Japan, 1961. Courtesy, S. Osaki.

the substrates oxidized by ceruloplasmin, Fe^{2+} had the highest activity, and they proposed that ceruloplasmin should be re-classified as a ferro-O_2 oxidoreductase and be newly designated as 'serum ferroxidase'.

The results of Osaki, Johnson, and Frieden were controversial, and considerable evidence was raised against ceruloplasmin as a physiological ferroxidase (Rydén 1984). The arguments include the rapid auto-oxidation of Fe^{2+} (thereby eliminating the need for catalysis), the inability of Fe^{3+} to bind apo-transferrin (Bates *et al.* 1973), the normal iron metabolism in Wilson's disease patients with low ceruloplasmin levels (Roeser *et al.* 1970), and the inverse relationship between serum ceruloplasmin and transferrin saturation, for example in newborns (Shokeir 1972). Recently, the important role of ceruloplasmin in iron homeostasis *in vivo* has been clearly demonstrated in patients and mice with genetic defects in ceruloplasmin (see next section); however, the specific role of ceruloplasmin ferroxidase activity and its role in iron loading into transferrin have not yet been rigorously shown *in vivo*. Additional landmark events have greatly enriched our understanding of ceruloplasmin structure and function. For example, the complete sequencing of the human ceruloplasmin protein (Takahashi *et al.* 1984), cloning of the full-length cDNA (Koschinsky *et al.* 1986), determination of the X-ray crystallographic structure (Lindley *et al.* 1997), and the discovery of glycosylphosphatidylinositol-linked ceruloplasmin in the brain (Patel & David 1997).

Role of copper in iron release from storage tissues

In 1931, Cook and Spilles in Berkeley provided the first experimental evidence, albeit indirect, that copper stimulated release of iron from storage tissues during erythropoiesis (Cook & Spilles 1931). They showed that addition of copper to an iron-poor diet fed to rats not only increased hemoglobin formation, but also depleted the iron stores in the spleen. Similarly, Josephs reported that the 'function iron' of the tissues increased during copper deficiency, and that replenishment of copper caused the iron to shift from the tissues to plasma hemoglobin (Josephs 1932). Iron transfer from the liver to red blood cells in anemic rats given copper was reported by others (Elvehjem & Sherman 1932; Muntwyler & Hanzal, 1933; Marston & Allen 1967). Clark Gubler and members of the Wintrobe group published a landmark paper (Gubler *et al.* 1952) which provided experimental evidence for (and in some cases, against) all three of the proposed mechanisms of copper-stimulated iron transfer. In one experiment they showed a time course in which oral copper induced maximal iron mobilization in copper-deficient pigs after about 8 hours. Interestingly, this is the only report from the Wintrobe group providing evidence for an iron-mobilizing activity of copper. Subsequent studies by the Wintrobe/Cartwright group did not support this conclusion, for example, the temporary nature of the plasma iron deficit even in the presence of continued anemia (Lee *et al.* 1968b) and the inability of injected iron to overcome anemia

(Gubler *et al.* 1952). A ferrokinetic analysis by the group led them to conclude that *our previous postulate that the mobilization of iron is impaired in copper deficiency must be incorrect* (Bush *et al.* 1956).

For almost four decades following the initial 1931 report there was little experimental or conceptual progress towards understanding the role of copper in tissue iron mobilization. A critical breakthrough occurred in 1966 when Osaki and co-workers (Osaki *et al.* 1966) proposed a model in which ceruloplasmin played a central role in driving iron from the gut, through the plasma, and to the erythroid marrow for hemoglobin synthesis and red blood cell formation (Figure 6). According to their model, the ferroxidase activity of ceruloplasmin, by facilitating the binding of iron to apo-transferrin, created a negative iron gradient with respect to the cell surface that increased cellular iron efflux. This model, linking the copper protein ceruloplasmin to iron mobilization, provided the first molecular connection between copper and iron metabolism. Experimental support for their hypothesis followed soon afterward from work in two laboratories using markedly different approaches. The Cartwright laboratory in 1969 showed that injection of ceruloplasmin into copper-deficient pigs increased the rate of iron movement into the plasma (Ragan *et al.* 1969). They suggested that the ferroxidase activity was critical since rat ceruloplasmin, which had low ferroxidase activity, was much less effective than human or pig ceruloplasmin in inducing iron mobilization. They also showed that substantial iron flux occurred in the presence of as little as 10% of the normal plasma level of ceruloplasmin. This finding was crucial since it helped to overcome the objection raised by several workers that ceruloplasmin could not be important in iron metabolism since iron status was normal in Wilson's disease patients with low levels of ceruloplasmin. The fact that most Wilson's patients have more than 10% of the normal level of ceruloplasmin suggests that gross changes in iron metabolism should not be expected. This finding was confirmed in a subsequent paper by the same group in which defective iron metabolism was observed only in those Wilson's disease patients who had ceruloplasmin plasma levels less than 5% of normal (Roeser *et al.* 1970). Osaki and Johnson, working with Earl Frieden, provided *ex vivo* evidence for ceruloplasmin-mediated iron efflux using perfused liver preparations. They showed that addition of ceruloplasmin and apo-transferrin to a perfusate of a normal dog liver increased the rate of iron release by up to 10-fold (Osaki & Johnson 1969). They subsequently reported similar results in perfusates of livers from copper-deficient pigs, and also confirmed iron mobilization at ceruloplasmin levels about 10% of the normal human plasma concentration (Osaki *et al.* 1971).

Perhaps the most convincing evidence for a role of ceruloplasmin in iron transport from storage tissues comes from recent studies in humans and mice with defects in the ceruloplasmin gene. Hereditary hypoceruloplasminemia has been long-recognized as a rare syndrome distinct from Wilson's disease (Cox 1966; Edwards *et al.* 1979). However, credit for connecting familial ceruloplasmin deficiency with defects in iron metabolism must go to Hiroaki Miyajima and his co-workers at Hamamatsu University in Japan (Miyajima *et al.* 1987). They described in 1987 a patient with bilateral blepharospasm and retinal degeneration (and also mild anemia). Plasma ceruloplasmin in the patient, and in two relatives, was very low or undetectable. Tissue iron overload was suggested by the extremely high serum ferritin level which was confirmed by a computed tomographic scan of the brain and by chemical analysis of a liver biopsy specimen. Likewise, a ferrokinetic analysis after injection of ^{59}Fe showed prolonged radioiron accumulation in the liver and spleen. Similar findings of tissue iron overload accompanied by mild microcytic, hypochromic anemia, and also dementia, cerebellar ataxia, and diabetes mellitus, were subsequently reported by several laboratories (Logan *et al.* 1994; Morita *et al.* 1995). The complete absence of plasma ceruloplasmin was due to frame-shift or truncation mutations in the ceruloplasmin gene (for review, see (Harris *et al.* 1998)). Together, these clinical data suggested an important role of ceruloplasmin in iron release from storage tissues, and consequent iron accumulation in its absence. This function of ceruloplasmin was confirmed by studies by Leah Harris and co-workers in mice with targeted ceruloplasmin gene disruption (Harris *et al.* 1999). The mouse model recapitulated several characteristics of the human disease, and also permitted the direct demonstration of ceruloplasmin-stimulated iron release from iron-loaded tissues.

After the landmark work by Osaki and Cartwright and their coworkers, there has been slow progress in understanding the cellular mechanism of iron efflux from stores, and a substantial amount of controversy and contradictory results. For example, the studies by Erica Baker and co-workers showed that perfusion of isolated rat liver preparations with apo-transferrin by itself was sufficient to release iron at the same rate

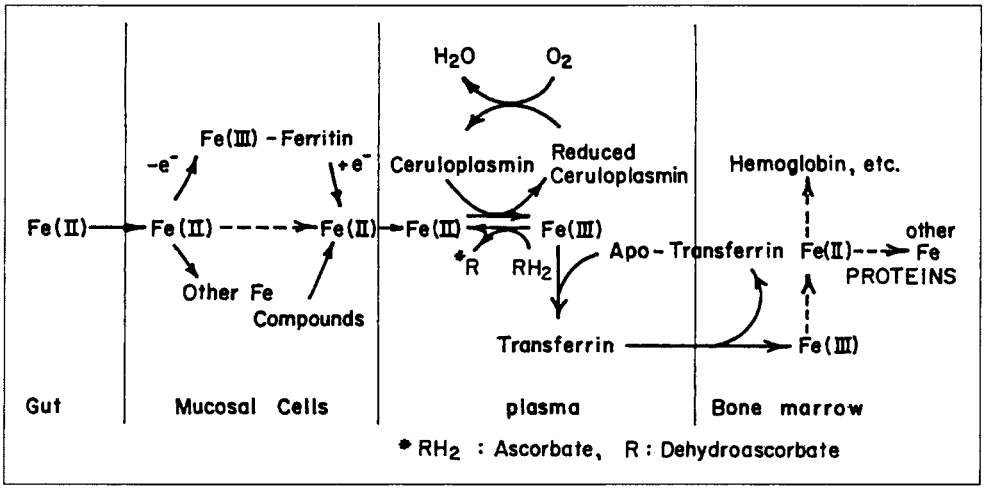

Fig. 6. 'Prevailing Theory of Iron Metabolism showing the Possible Role of Ceruloplasmin in Promoting Iron Utilization' proposed by Osaki and coworkers (Osaki *et al.* 1966). Courtesy, American Society for Biochemistry and Molecular Biology.

Über locker gebundenes Kupfer und Eisen im Blutserum.

Von

Otto Warburg und H. A. Krebs.

(Aus dem Kaiser Wilhelm-Institut für Biologie, Berlin-Dahlem.)

(Eingegangen am 14. September 1927.)

Tabelle III.

Wirkung der Blutentziehung.

Nr.	Art des Serums	Pro ccm Serum		Pro g Serumeiweiß	
		mg Cu .10⁻³	mg Fe .10⁻³	mg Cu .10⁻³	mg Fe .10⁻³
1	Taube, normal	0,08	0,66	0,22	1,80
	Das gleiche Tier, 3 Tage vorher und 1 Tag vorher je 8 ccm Blut entnommen . .	0,25	0,32	0,79	1,02
2	Gans, normal	0,41	0,71	0,98	1,70
	Das gleiche Tier, 4 Tage vorher 90 ccm Blut entnommen	1,87	0,98	4,52	2,38
3	Gans, normal	0,13	0,79	0,38	2,30
	Das gleiche Tier, 3 Tage vorher 90 ccm Blut entnommen	0,75	0,28	2,10	0,75

Fig. 7. Details from paper by Warburg and Krebs showing influence of bleeding on blood copper. Top, the translated title is 'On the loosely bound copper and iron in serum'. Bottom, Table III. Effect of Bleeding. Taube, pigeon; Gans, goose; Serumeiweiβ, serum protein. Text in row 1 is Pigeon, normal; The same animal, 8 ml of blood removed 3 days and 1 day before measurement.

as whole plasma, thus excluding a need for ceruloplasmin (Baker *et al.* 1980). Recent in vitro studies have been inconclusive as well. Steve Young and co-workers showed that ceruloplasmin stimulated iron release from HepG2 cells, but inconsistent with the Osaki model, also showed that iron release was the same in the presence of either apo- or holo-transferrin (Young *et al.* 1997). Des Richardson showed that ceruloplasmin stimulated iron release from HepG2 cells but that apo-transferrin was not required (Richardson 1999). Chinmay Mukhopadhyay and Zouhair Attieh and their co-workers in Cleveland found that iron release from HepG2 or K562 cells was not enhanced by ceruloplasmin in the absence or presence of apo-transferrin (Mukhopadhyay *et al.* 1998; Attieh *et al.* 1999). These contradictory results may be condition- or reagent-dependent and will require persistent effort to unravel.

Quantum advances in our identification of iron transporters and other iron regulatory proteins during the last few years should be enormously helpful in elucidating the molecular mechanism of iron export. Particularly exciting was the near-simultaneous identification by three laboratories of a putative iron export protein variously called ferroportin1, IREG1, and MTP1. The possibility that ferroportin1 is the transporter that works in concert with ceruloplasmin and apo-transferrin was investigated in reconstitution studies by two of these laboratories. Unfortunately, the results were not consistent despite the similar approaches using transfected, iron-loaded *Xenopus* oocytes. In one laboratory, ceruloplasmin, but not apo-transferrin, was required for iron export (McKie *et al.* 2000), whereas in the other apo-transferrin was sufficient for iron export (Donovan *et al.* 2000). Although most attention has focused on the role of ferroportin1 in gut iron transport, its abundance in reticuloendothelial cells and in hepatocytes also suggests a possible role in iron export from storage tissues (Abboud & Haile 2000). The physiological importance of this gene in iron transport has been confirmed by the finding of autosomal dominant hemochromatosis in patients with defects in the ferroportin1 gene (Montosi *et al.* 2001; Njajou *et al.* 2001).

Role of copper in cellular iron utilization, hemoglobin formation, and red blood cell development

Role of copper in iron utilization and hemoglobin formation

Although the catalytic (indirect) role for copper in iron incorporation into hemoglobin was proposed in the nineteenth century, the first experimental evidence was provided much later by Clark Gubler and co-workers in the Wintrobe laboratory (Gubler *et al.* 1952). They showed that intraperitoneal or intravenous injection of iron failed to prevent anemia in copper-deficient pigs. Since this mode of iron delivery bypassed gut iron absorption and iron release from stores, the results suggested that copper was critical in a distal pathway step, e.g., iron utilization. In a second experiment that directly measured iron utilization, they showed that conversion of intravenously delivered radioiron into circulating hemoglobin was about 75% lower in copper-deficient pigs compared to controls. The radioiron was prebound to the 'free iron-binding protein of the plasma' thereby eliminating the possibility that the decreased iron utilization was due to slow binding to apo-transferrin. Additional evidence for defective iron utilization during copper deficiency was provided by Richard Lee and co-workers in 1968 (Lee *et al.* 1968b). They showed that an unusually large proportion of red cell precursors (normoblasts) in the bone marrow of copper-deficient pigs contained large amounts of granular, stainable iron (sideroblasts). They speculated that the increase in normoblast iron indicated a defect in a step in intracellular iron metabolism. This role of ceruloplasmin itself in hemoglobin formation and erythropoiesis was examined by Mitiyuki Shimuzu and his students at Showa Medical School in Tokyo, Japan. Repeated injection of purified human ceruloplasmin into non-anemic rats or rabbits increased incorporation of ^{59}Fe into circulating erythrocytes (Yamaguchi 1965; Yamamoto 1969).

Searching for a possible intracellular defect in iron metabolism caused by copper deficiency, several laboratories focused on the mitochondria and its enzymes. Mitochondria are the major site of heme formation in erythroid precursor cells, and thus likely targets for regulation by copper deficiency. An early study from Elvehjem's laboratory showed that copper deficiency decreased the content of the cytochromes in liver and other tissues, but no link was made to erythrocytes (Cohen & Elvehjem 1934). This connection

was first shown by M.O. Schultze, a previous trainee of Hart and Elvehjem, who reported decreased activity of cytochrome oxidase (which contains 2 copper atoms per molecule) in the bone marrow of copper-deficient rats (Schultze 1941). He also showed a correlation between marrow cytochrome oxidase activity and red cell formation under various conditions. Schultze proposed that cytochrome oxidase, by an unknown mechanism, was essential for erythropoiesis thereby explaining the copper requirement. Later Joseph Goodman and Peter Dallman, working in San Francisco and primarily using morphological approaches, demonstrated the presence of enlarged and possibly iron-deficient mitochondria in copper-deficient rats (Goodman & Dallman 1969; Dallman & Goodman 1970). By a process analogous to that proposed for cells and tissues, they postulated that copper deficiency impaired iron release from intracellular storage vesicles. Noting that these data also were consistent with impaired iron uptake by mitochondria, Darryl Williams and his co-workers used a biochemical approach to study the effects of copper on mitochondrial iron metabolism in reticulocytes, and like previous investigators found reduced cytochrome oxidase activity (Williams *et al.* 1976). They also showed that mitochondria from copper-deficient animals synthesized heme from Fe^{3+} and protoporphyrin at a reduced rate. They proposed that cytochrome oxidase and an intact electron transport system were required for Fe^{3+} reduction to provide the Fe^{2+} required as substrate (with protoporphyrin) for ferrochelatase, the terminal enzyme in heme synthesis. In other studies, Williams and his colleagues showed that copper-deficiency resulted in cellular accumulation of an unidentified form of iron that can be used for heme synthesis by isolated mitochondria, thus providing additional evidence for a role of copper in iron utilization by mitochondria (Williams *et al.* 1978).

Recent evidence for a role for ceruloplasmin-like proteins in iron flux came from an unexpected source, namely, genetic studies of iron metabolism in yeast. In back-to-back papers published in 1993, Jerry Kaplan's and Rick Klausner's laboratories described a novel protein, Fet3, required for high-affinity iron transport in *S. cerevisiae* (Askwith *et al.* 1994; Dancis *et al.* 1994). Fet3 was shown to be a membrane-spanning, multicopper protein interacting with an iron transport protein, Ftr1 (Stearman *et al.* 1996). Both Fet3 and Fio1, its homologue in *S. pombe* (Askwith & Kaplan 1997), have limited sequence homology to human ceruloplasmin, primarily in the copper-binding motifs. Also like ceruloplasmin, fet3 has ferroxidase activity, which was shown to be required for high-affinity iron uptake. These studies provided evidence for a multi-step pathway in which Fe^{3+} was first reduced by a membrane-bound reductase, Fre1/Fre2, then reoxidized to Fe^{3+} by ferroxidase activity of Fet3, followed by transport into the yeast by the ferric ion transporter, Ftr1. The convergence of the yeast and mammalian pathways of iron transport on a copper-containing ferroxidase suggested a universality of the copper/iron connection (see Kaplan & O'Halloran 1996 for review). However, the proposed direction of ferroxidase-mediated iron transport was opposite in the two systems – inward in yeast and outward in vertebrates.

The possibility that ceruloplasmin, like Fet3 in yeast, also facilitated inward iron transport in mammalian cells was investigated (Mukhopadhyay *et al.* 1998; Attieh *et al.* 1999). Ceruloplasmin was shown to stimulate uptake of low molecular weight iron complexes, in a ferroxidase-dependent manner, in iron-deficient erythroleukemic K562 (and HepG2) cells. The apparent paradox with respect to the directionality of ceruloplasmin-mediated iron transport may be in part resolved by the recognition that ferroxidases themselves are non-directional with respect to iron transport, and that the net flux is determined by available partners that recognize the ferric ion product. The requirement for a ferrireductase in this process was not examined (ascorbate was present to maintain the ferrous state), but the requirement for ferrireductase activity for iron uptake in K562 cells was reported previously (Inman *et al.* 1994). The identity of the putative erythroid ferrireductase is not known, but a mammalian intestinal ferric reductase, Dcytb, has been recently described (McKie *et al.* 2001). The possible physiological role of transferrin-independent iron uptake by erythroid cells is not known. The severe anemia in hypotransferrinemic mice clearly shows a critical role for transferrin for iron transport in erythropoiesis (Craven *et al.* 1987), but it does not exclude an auxiliary role by low molecular weight iron forms. The finding that mice defective in the transferrin receptor have intravascular erythrocytes is consistent with transferrin-independent uptake mechanisms, at least during embryogenesis (Levy *et al.* 1999). Ceruloplasmin may also be involved in iron transport by non-erythroid cells. In intriguing results indicating an important role of ceruloplasmin in brain iron metabolism, Richard Qian and co-workers have shown that ceruloplasmin promotes iron uptake rather than release in

cultured glioblastoma cells (Qian *et al.* 2001; Qian & Ke 2001). While the focus of this section has been on the role of ceruloplasmin and copper in transport of low-molecular weight iron forms, it should be mentioned that there is some evidence that copper may be involved in cellular uptake of transferrin-bound iron (Williams *et al.* 1976).

Role of copper in iron-independent erythropoietic mechanisms

In his 1901 review Liégeois noted that copper could influence red blood cells by at least three distinct mechanisms, by *helping to more perfectly assimilate* iron into hemoglobin, by *stimulating hematopoiesis* and by formation of red blood cells *less likely to die soon after their birth*. Most attention has focused on the first of these – the iron-dependent pathway. However, the possible role of copper in the latter iron-independent increases in hemoglobin and red blood cells has received some attention as well (Stein & Lewis 1933; Van Wyk *et al.* 1953). In 1952 the Cartwright/Wintrobe group (Lahey *et al.* 1952) started a systematic dissection of the hemoglobin synthesis pathway in copper-deficient animals, expecting to find a defect in the synthesis or metabolism of one of the three major components of hemoglobin: protoporphyrin, iron, or globin (Figure 3A). They reported that anemia in copper-deficient animals was not due to defective synthesis of the porphyrin precursor of hemoglobin. This observation was confirmed by Richard Lee in the same laboratory, who showed that copper deficiency did not reduce the in vitro activity of the heme biosynthetic enzymes, and concluded that copper must have a role in either globin synthesis or in iron metabolism (Lee *et al.* 1968a). To this day, there does not appear to be any reports of experiments on the role of copper in globin synthesis.

The third mechanism proposed by Liégeois, namely, that copper helps in the formation of *red cells which are more regular, more resistant, and less likely to die soon after their birth*, has not received much attention. The possibility that copper influenced erythrocyte life-span was first investigated by James Bush in the Cartwright/Wintrobe laboratory. Bush showed that red blood cell half-life was reduced from 17 days in control swine to 9 days in copper-deficient swine (Bush *et al.* 1956). Cross-over experiments suggested that the defect was in the copper-deficient red cells themselves rather than a plasma or systemic defect of the pig. Bush's work with the Utah group appeared in 5 outstanding papers, all published in 1956. Sadly, all of the papers were published posthumously as the career of this extraordinarily promising post-doctoral fellow was cut short by his death in October, 1955. The protective effect of ceruloplasmin against transition metal ion-induced lysis of erythrocytes *in vitro* was reported by Rolf Løvstad in Norway (Lovstad 1981), and then investigated in some detail by Saenko and Yaropolov and their colleagues in Moscow (Saenko & Yaropolov 1990) and by James Caffrey in Earl Frieden's laboratory (Caffrey *et al.* 1990). The specific role of ceruloplasmin and other copper proteins in extending red cell life *in vivo* has not been investigated.

Role of copper in intestinal iron absorption

Copper-deficiency models and the role of ceruloplasmin in iron absorption

The role of copper in iron absorption by the gut has had a checkered history characterized by contradictory findings. Cunningham in 1931 and then Josephs in 1932 were the first to appreciate and experimentally test the possibility that copper increased hemoglobin formation by enhancing gut iron absorption or 'retention' (Cunningham 1931; Josephs 1932). Measuring total carcass iron, they both observed that addition of dietary copper did not increase iron absorption in rats fed a cow's milk diet supplemented with iron. In 1934, Bing and co-workers in Cleveland were the first to report increased iron absorption by copper (Bing *et al.* 1934). They showed that dietary copper increased the body iron content of anemic rats by about 50%. By subtraction of the initial iron mass they calculated that copper essentially doubled the rate of gut iron absorption. In the same set of experiments, they showed that oral iron supplementation did not increase hemoglobin levels, whereas intraperitoneal injection of iron, which bypassed the gut absorption process, completely restored hemoglobin. They also showed that oral iron plus copper restored hemoglobin levels, confirming the original work by the Elvehjem group. An appropriate conclusion from these results is that the increase in iron absorption by copper is a major mechanism explaining increased hemoglobin formation. Surprisingly, they did not offer this as a major conclusion of the paper, but instead focused their discussion on less crucial areas, for example, the variable copper content of milk and copper utilization. This strategy was possibly a defense for their

shift in position on the copper contamination issue since this is the same paper in which they conceded to the Elvehjem group the important role of copper in overcoming nutritional anemia. This paper should be credited as the first to show a positive role of copper in iron absorption. However, their unfortunate focus on explaining away their previous position masked the new and important discovery, and may have lessened the impact of their work. The results of Bing and coworkers were later confirmed by others (Houk *et al.* 1946), but were contradicted in iron balance studies which showed that copper substantially decreased iron absorption in anemic patients (Barer *et al.* 1937). A difference between the studies (besides the obvious species difference) which may account for the inconsistent results, is that the rats were copper- and iron-deficient while the patients were most likely iron-deficient only. The Elvehjem group, which seemed to be at or near the leading edge of most experimental avenues, did not participate in this particular arena, and the 1930s ended without a clear consensus on the role of copper in iron absorption.

Taking advantage of the higher sensitivity and accuracy of isotopic studies, the Wintrobe group in 1952 showed that copper supplementation increased radio-iron absorption by the gut by about 25% in copper-deficient rats (Chase *et al.* 1952), and also in pigs (Gubler *et al.* 1952). In their 1966 paper, Osaki and Frieden proposed that ceruloplasmin-mediated iron loading of transferrin could generate an iron gradient sufficient to drive iron out of tissues, including gut mucosal cells. This idea was first tested by Richard Lee in the Cartwright group, who described a breakthrough result that foreshadowed our current understanding. They reported massive Prussian-blue detectable iron accumulation in the gut of copper-deficient pigs, both in epithelial cells and in macrophages of the lamina propria. They found that when ^{59}Fe was fed to the pigs, the duodenal mucosa absorbed iron at the normal rate but subsequent transfer to the blood was impaired (Lee *et al.* 1968b). They concluded that *the barrier to absorption of iron in the copper-deficient animals did not lie between the intestinal lumen and the duodenal mucosa, but at a subsequent site in the absorption pathway*. Based on the Osaki model, they speculated that the defect may be due to the absence of ceruloplasmin.

The role of ceruloplasmin in iron absorption has not been resolved conclusively. Brittin and Chee did not find a correlation between plasma ceruloplasmin levels and gut iron absorption under various conditions, and concluded that ceruloplasmin was not involved in a rate-limiting event in iron absorption (Brittin & Chee 1969). Other negative results were reported by Coppen and Davies in experiments measuring iron transport in a perfused *ex vivo* intestinal preparation (Coppen & Davies 1988). They found that ceruloplasmin, added together with apo-transferrin, did not increase iron uptake, retention, or transfer in tissue from copper-deficient or control rats, and concluded that ceruloplasmin was not involved in gut iron absorption. These results were disputed by Wollenberg and co-workers who, using a perfused gut loop in live rats, showed a marked increase in iron absorption and transfer to the portal blood by ceruloplasmin (Wollenberg *et al.* 1990). They suggested that the discrepancy between their results and those of Coppen and Davies was due to the fact that the effect of ceruloplasmin was only seen in animals with severe copper deficiency. Experiments using mice with targeted ceruloplasmin gene defects are likely to resolve this important question. Leah Harris and coworkers, when comparing ceruloplasmin "knock-out" mice to controls, did not observe a significant difference in ^{59}Fe absorption, thus ruling out a major role of ceruloplasmin in absorption (Harris *et al.* 1999). However, the low number of mice used and their mixed background leaves open the possibility that a small difference would have gone undetected, and that ceruloplasmin may have a minor role of in iron absorption.

The role of hephaestin in iron absorption

An important clue to understanding copper's role in iron absorption came from studies of mice subjected to mutagenesis by X-irradiation. In 1958 D.S. Falconer from the Institute of Animal Genetics in Edinburgh observed that descendants of an irradiated male mouse were born anemic, distinguishable by their pale color (Falconer & Isaacson 1962; Grewal 1963). The gene was shown to be sex-linked and recessive, and the genotype described as 'sex-linked anemia' (*sla*). The anemia was temporary and gradually disappeared as the mice aged. Experiments by Peter Pinkerton, Robin Bannerman and their co-workers in Buffalo showed that the red blood cells in the *sla* mouse were microcytic but that erythropoiesis was normal (Bannerman & Cooper 1966; Pinkerton & Bannerman 1967). Tracer studies using oral administration of ^{59}Fe showed that the primary defect was in iron absorption (Pinkerton & Bannerman 1967). Defective absorption was confirmed by other studies which showed that

iron injection led to rapid (but incomplete) regression of the anemia (Bannerman & Pinkerton 1967), and also by histological evidence which showed striking iron deposits in epithelial cells of the small intestine (Pinkerton 1968). From these studies, Pinkerton concluded that the defect in the *sla* mouse was in the transport of iron from the intestinal mucosa to the tissues, a conclusion verified by autoradiographic studies (Bédard *et al.* 1976). Experiments using isolated intestinal epithelial cells indicated that the defect was not in the mucosal cell itself, but rather between that cell and the vascular compartment (Peppriell *et al.* 1982). James Manis showed in 1970 that low serosal transfer was due to a defect in the active transport mechanism for iron in the gut of the *sla* mouse (Manis 1970). Three years later he reported that intestinal mucosal cells contained a ferroxidase activity that was likely due to a novel enzyme since even partially purified gut extracts had higher specific activity than ceruloplasmin (Manis & Schwartz 1973). Unfortunately, neither Manis nor other investigators compared the gut ferroxidase activity in normal and *sla* mice, and a potentially revealing clue to the defect in the *sla* mouse was not unveiled. The specific molecular defect remained a mystery for more than 25 years.

An important step towards understanding the defect in the *sla* mouse came in 1998 when Greg Anderson and coworkers mapped the *sla* locus between two microsatellite markers on the X-chromosome (Anderson *et al.* 1998). The breakthrough event occurred the following year when Chris Vulpe, Anderson and their collaborators identified the specific gene mutated in the *sla* mouse (Vulpe *et al.* 1999). The *hephaestin* gene (named for the god of metal-working) was highly expressed in the small intestine and colon of the wild-type mouse (Vulpe *et al.* 1999) and rat (Frazer *et al.* 2001). The encoded protein had high sequence homology with ceruloplasmin, and in particular exhibited conservation of amino acid residues essential for copper binding, and thus for ferroxidase activity. A unique structural feature of hephaestin is the C-terminal, hydrophobic tail that anchors it to membranes, whereas ceruloplasmin is a secreted protein found primarily in plasma. Compound mouse knockouts of *hephaestin* and the hereditary hemochromatosis gene, *Hfe*, showed less iron loading than mice lacking *Hfe* alone, suggesting that *hephaestin* may be a modifier gene for the hemochromatosis phenotype (Levy *et al.* 2000). The mechanism of action of hephaestin is not yet understood, and the specific cellular location and iron transport function have not yet been determined. As described above, the ferrous iron transporter ferroportin1 may couple with hephaestin to drive cellular iron efflux from intestinal epithelial cells.

Clinical studies of copper-deficiency anemia and its treatment

The 1928 report by Hart and Elvehjem awakened clinical interest in copper as a therapeutic agent for the treatment of anemia. Multiple investigators repeated and extended the early trials by Mendini and Cervello, but were armed with better reagents and far more knowledge on the etiology of multiple forms of anemia. Successful treatment of hypochromic, microcytic ('idiopathic') anemia with copper and iron was reported by Edward Mills in Montreal (Mills 1930). In contrast, Barer and Fowler reported that copper did not increase hemoglobin levels when patients were given moderate amounts of iron (Barer & Fowler 1937). They suggested that sufficient copper may be present in foods to mask the contribution by additional copper. Similarly, Bethell and co-workers found that purification of the dietary iron to remove all traces of copper, did not reduce its efficacy, and concluded that there was no advantage to adding copper in treatment of anemia (Bethell *et al.* 1934). These early investigators concluded that anemia is a collection of disorders, that copper stores in adults may mask any requirement for dietary copper, that effective treatment of anemia with copper may be limited to cases of copper deficiency, and that copper deficiency in adults is likely to be rare since copper is abundant in many foods.

Several studies in the 1930s on infants and children, who are likely to have lower copper stores than adults, were promising. A rigorous and extended series of studies by Hugh Josephs in 1931 showed that treatment of anemic infants with copper and iron was significantly more effective than iron alone, particularly in cases of very low hemoglobin (Josephs 1931). He noted that copper seemed to accelerate hemoglobin formation, but not reticulocyte production. Perhaps intentionally thumbing his nose at the out-dated ideas of von Bunge, he noted that *medicinal iron was found to be far superior to food iron*. The effectiveness of copper in children was verified by other investigators. Milton Lewis reported that copper was particularly effective in children with chronic nutritional anemia (Lewis 1931). Elvehjem tried his hand in the clinical arena and in 1935 reported that iron plus copper in-

creased the hemoglobin level in infants suffering from severe nutritional anemia, but unfortunately did not show data for iron alone (Elvehjem *et al.* 1935). In 1938 James Hutchinson also showed the effectiveness of copper in treatment of infants suffering from nutritional anemia. Using a strategy in which iron was given first, followed by a non-treatment period, and then copper, he concluded that copper facilitates the utilization of iron, possibly by release from storage sites (Hutchison 1938).

The evidence for successful treatment of anemia by copper, particularly in children, was persuasive. However, the mechanism of copper action was not at all clear since the occurrence of copper deficiency in humans was very controversial. In fact, nutrition and hematology textbooks as recently as the 1960s expounded on the complete absence of evidence for copper deficiency in humans. This controversy was partly resolved by observations by Angel Cordano and George Graham at the British-American Hospital in Lima, Peru who reported the first evidence for copper deficiency in humans. They studied four infants with several conditions in common: short breast-feeding period, followed by a near-starvation diet, which was complicated by repeated episodes of diarrhea or vomiting (Cordano *et al.* 1964). They reported that the anemia was accompanied by low plasma copper levels that were raised to near-normal by copper supplementation. In a larger series they reported that 8% of the malnourished children admitted to the hospital were copper-deficient at the time of admission, but even worse, an additional 63% became copper-deficient during the course of hospital rehabilitation on a milk diet (Graham, *et al.* 1969). Most had nearly absent circulating ceruloplasmin. Today, copper deficiency anemia in children is rare, but is occasionally observed in infants nourished solely on a cow's milk diet (Levy *et al.* 1985).

Two other causes of copper-deficiency in humans have been reported: patients receiving long-term parenteral nutrition and cases of zinc poisoning. The initial observation was made by John Karpel and Virginia Peden on a baby receiving total parenteral nutrition after surgical repair of an ileal defect (Karpel & Peden 1972). A severe hypochromic, microcytic anemia (and neutropenia) ensued which was unresponsive to intramuscular iron. Following the observation that serum copper and ceruloplasmin were about 10% and 1% of the normal level, respectively, oral copper therapy was administered, and all hematological parameters returned to near-normal level within weeks. Essentially identical observations have been made in adults on long-term parenteral nutrition, primarily after bowel surgery (Dunlap *et al.* 1974; Spiegel & Willenbucher 1999). The first report of anemia following zinc-induced copper deficiency was by William Patterson and co-workers (Patterson *et al.* 1985). The patient had profound sideroblastic anemia with low plasma levels of copper and ceruloplasmin. He had taken megadoses of zinc supplements for 2 years as a self-prescribed treatment for 'prostate trouble'. Since zinc was known to inhibit copper absorption and increase copper secretion (Prasad *et al.* 1978), the authors hypothesized that the anemia was caused by the excess zinc. Discontinuation of the zinc supplements led to rapid recovery of the copper and ceruloplasmin levels as well as all hematological parameters. These findings have been verified by other investigators on a patient population that has been expanded by the increasingly popular use of zinc supplements among certain food faddists, and also by recent reports of the effectiveness of zinc in treatment of the common cold (Broun *et al.* 1990). A highly unusual mode of excess zinc accumulation was described by Randolph Broun and co-workers in a case report on a patient with severe sideroblastic anemia and pica (Broun *et al.* 1990). The patient had almost undetectable plasma copper and ceruloplasmin, and an abdominal x-ray revealed a large radiopaque mass. The patient's history was notable for chronic paranoid schizophrenia, and he reported that he had ingested coins for the past 12 years. Surgery was done to remove $22.50 of coins, many of which were pennies that since 1982 contained 97.6% zinc. Normal hematological values were observed four months after surgery.

Animal and clinical studies on the role of ceruloplasmin in anemia were a primary focus of Mitiyuki Shimuzu's laboratory at Showa Medical School in Tokyo, Japan. His group published several dozen interesting articles between about 1960 to 1980 on the erythropoietic activity of ceruloplasmin. However, this work has received little attention in the west, undoubtedly because most of the articles are in Japanese. The group showed that injection of purified ceruloplasmin into animals caused a rapid increase in ^{59}Fe incorporation into circulating erythrocytes, followed by an increase in reticulocytes, and finally increased hemoglobin and erythrocytes (Yamaguchi 1965; Yamamoto 1969). In 1979 Shimizu reported extraordinary results from a clinical study by the Ceruloplasmin Study Group consisting of 24 institutions in Japan (Shimizu 1979). Purified human ceruloplasmin was

injected intravenously (15 mg day) into 73 patients with aplastic anemia. They reported a marked improvement in hematological parameters in 20% of the patients, and some benefit in the majority. Unfortunately, the ceruloplasmin levels in the patients were not measured and similar experiments have not been pursued in Japan or elsewhere.

The 'flip' side of the copper/iron connection: regulation of cuproprotein metabolism by iron

Effect of anemia on plasma copper and ceruloplasmin

There are two sides to the copper/iron connection. The above sections describe the aspect most often considered – the effect of copper on iron metabolism. The obverse relationship has been shown, but is perhaps less well-recognized, i.e., the influence of iron on copper metabolism. This relationship was first observed by one future Nobel laureate working in the laboratory of a second future laureate. After receiving his M.D. degree in 1925, Hans Krebs took a position as research assistant in the laboratory of Otto Warburg at the Kaiser Wilhelm Institute for Biology in Berlin, where he remained until 1930. In 1927, Warburg reported a new method for measurement of copper and iron in blood using a Warburg manometer (which he called a 'Wassermanometern', or water manometer) to measure cysteine oxidation (Warburg 1927). That same year, he and Krebs used the method to measure the amount of 'loosely bound' iron and copper in the blood from several species and in patients with various diseases (Warburg *et al.* 1927). More importantly, they reported that acute bleeding of birds caused a 3- to 5-fold increase in the amount of loosely bound copper in the blood (Figure 7).

Since loosely bound material was defined as oxidizable by cysteine in the presence of oxygen, and since ceruloplasmin is the primary cysteine oxidant in plasma (Albergoni & Cassini 1975; SenGupta *et al.* 2001), it is probable that the copper increase they measured was due to increased ceruloplasmin. This observation was largely ignored by Elvehjem and most other contemporaneous investigators, since it appeared to present a paradox antithetical to the findings on copper deficiency. To wit, the absence of copper caused anemia, but according to Warburg and Krebs, copper increased during anemia. In their classic 1928 paper, Hart and Elvehjem cited the 1927 Warburg and Krebs paper, but only in the context of copper being a normal constituent of the blood; the reciprocal relation between copper and iron was not mentioned.

The observations by Warburg and Krebs were soon buttressed by two types of studies: by similar bleeding experiments in animals and by reports that copper levels increased in many human anemias. The first experimental confirmation was by Uichiro Sarata and Akio Suzuki working at Tohoku Imperial University in Japan. They showed that acute bleeding of rabbits led to a rapid increase in blood copper, reaching a peak after about 2 days and then gradually declining to the normal level (Sarata & Suzuki 1934). They noted that the kinetics were essentially identical to reticulocyte formation. They essentially resolved the paradox raised by the Warburg and Krebs studies, proposing that *the possibility remains that the increase in plasma copper is not a mere by-product of the rapid reformation of plasma, but is an important factor in recovering from anaemia, as a stimulant for haematopoietic organs for instance, and that accordingly after bleeding the metal is mobilized for this purpose from its depots* (see Figure 3B for a current interpretation of this idea). Likewise, Adolph Sachs in 1938 found that acute bleeding of dogs caused a rapid increase in blood copper (Sachs 1938). He indicated that the *most likely conclusion to be drawn from the finding of a high blood content in almost all cases of anemia, is that the copper is brought into use from the body reservoirs to stimulate hematopoiesis.* He memorably expounded that *hypercupremia is the usual response to hypoferronemia*. Although arriving a little late to this game, Elvehjem and Hart (with Potter) also made an important contribution. In 1938 they reported that bleeding of dogs caused a mobilization of copper into the blood, but only when there was sufficient iron available for erythropoiesis (Potter *et al.* 1938). After the discovery in 1948 that ceruloplasmin was the primary copper protein in blood, several investigators measured its response during anemic conditions. In 1961, Yoshihide Kuwatsuru at Showa Medical School showed that induction of anemia in rabbits, whether by acute bleeding or by phenylhydrazine hemolysis, caused dramatic increases in plasma copper and ceruloplasmin (Kuwatsuru 1961). Increased plasma ceruloplasmin in response to anemia was confirmed by others in iron-deficient rats (Iwanska & Strusinska 1978) and in bled rabbits (Mainero *et al.* 1996).

The reciprocal relation between iron and copper was confirmed in multiple observational studies of patients, first in studies measuring serum copper, then in studies of ceruloplasmin. Recognizing the potential

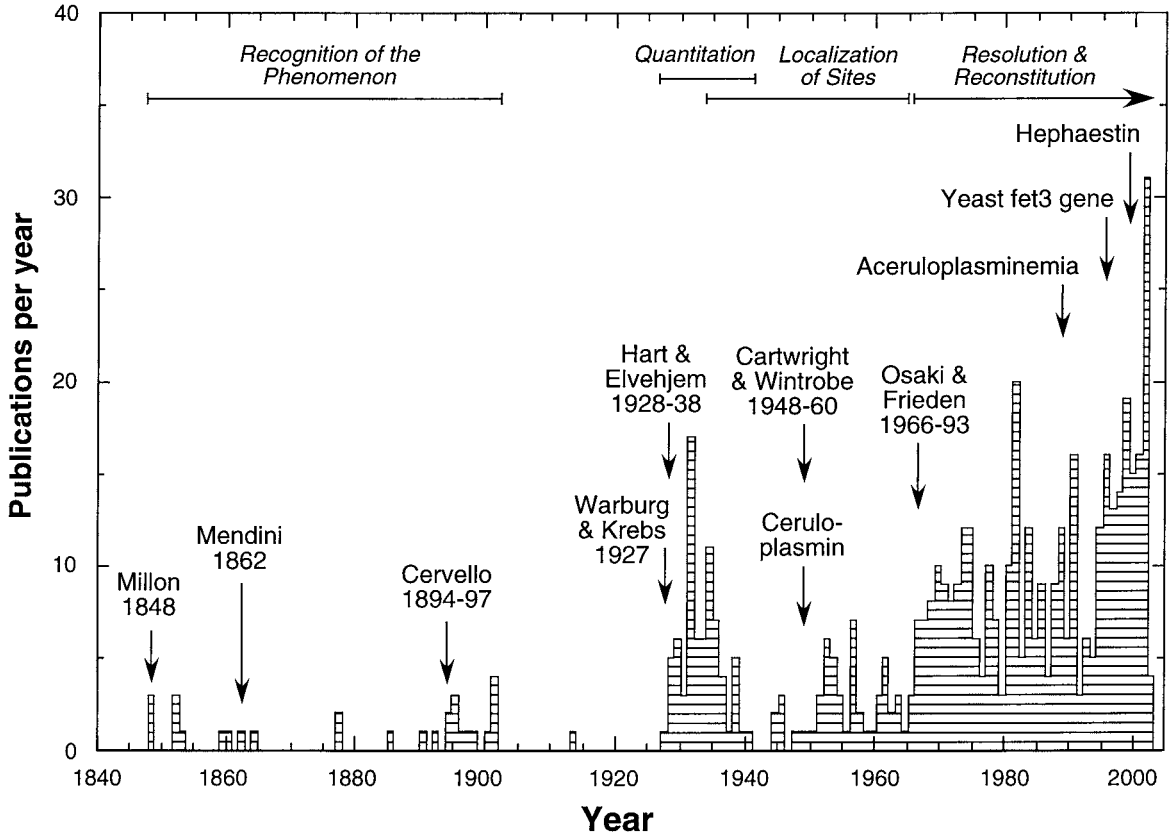

Fig. 8. Time course of papers published on the copper/iron connection. The papers represent the author's non-systematic, and certainly incomplete, collection of references that primarily concern both iron and copper metabolism (534 articles through February, 2002).

clinical importance of the work of Warburg and Krebs, Evert Görter with Grendel and Weyers at the University of Leiden in Holland were first to investigate this relationship in humans. They reported in 1931 that anemic children had significantly higher blood copper levels than new-borne or 'recovering children' (Gorter *et al.* 1931). Görter, himself a pediatrician, appears to have been somewhat reluctant to present his work to an audience of American 'experts' at the Chicago Pediatric Society and starts by saying that he will *begin with the transport of owls to Athena*, a charming, earlier version of the modern *...coals to Newcastle* (Gorter 1933). In addition to Görter's career as a pediatrician, he was also an accomplished basic scientist, and Görter and Grendel are much better known for their classic 1925 experiment from which they deduced that red cell membranes were composed of lipid bilayers (Gorter & Grendel 1925). This concept remained controversial for at least 40 years when it was finally verified by electron microscopy.

Görter's work on childhood anemia was somewhat less controversial than his membrane studies, and elevated serum copper was later confirmed in iron-deficient children (and to a lesser extent in iron-deficient adults) (Cartwright *et al.* 1948; Lahey *et al.* 1953). Elevated copper was also shown after massive hemorrhage in adults (Pagliardi *et al.* 1957; Sachs 1938). Wintrobe's laboratory reported that blood copper levels were highly elevated during the anemia of inflammation (and in various malignancies) (Fay *et al.* 1949; Cartwright *et al.* 1946), but given current knowledge about ceruloplasmin induction by cytokine agonists of the acute phase reaction (Baumann & Gauldie 1994), these results may reflect the state of inflammation rather than the iron status of the blood. Likewise, the increase in copper observed during the anemia of pregnancy is likely to be a response to steroid hormones rather than to changes in iron status (Lahey *et al.* 1953; Fay *et al.* 1949). The increase in serum copper was shown to reflect elevated serum ceruloplasmin in several conditions of anemia

including dietary iron deficiency anemia (Venakteshwara Rao *et al.* 1975; Iwanska & Strusinska 1978), hemorrhagic anemia (Johnson *et al.* 1967), renal failure (Taylor *et al.* 1994), anemia of inflammation (Markowitz *et al.* 1955; Beaumier *et al.* 1984; Morimoto *et al.* 1987), and pregnancy (Kalra *et al.* 1989). Again, the increased ceruloplasmin in anemia due to pregnancy and inflammation is most likely due primarily to hormone and cytokine levels; however, the findings in dietary anemia and hemorrhage provide evidence for a direct relationship between iron deficiency and plasma ceruloplasmin concentration.

Mechanism of ceruloplasmin induction

An early explanation for the reciprocal relation between plasma iron and copper was suggested by Holmberg and Laurell. Based on Edwin Cohn's observation that the metal-binding protein in plasma bound both copper and iron, they proposed that competition for the ions may be responsible for their reciprocal relationship in plasma (Holmberg & Laurell 1947; Cohn 1947). However, their experiments showed that iron was the preferred binding partner, and Holmberg and Laurell concluded that *the hypothesis about iron and copper competing for the same protein in serum cannot be correct.* In the same report they proposed that the iron-binding component of serum be named 'transferrin'. Thus in a one-year period Holmberg and Laurell coined the lasting names of two principal serum proteins involved in the copper/iron connection: transferrin and ceruloplasmin.

An alternate mechanism for the inverse relation between plasma iron and copper involves regulatory mechanisms of ceruloplasmin synthesis by iron status. The liver is the principal site of plasma ceruloplasmin synthesis in adults. The molecular mechanisms underlying the regulation of ceruloplasmin production were investigated in human hepatocellular carcinoma HepG2 cells, which constitutively secrete ceruloplasmin. Mukhopadhyay and co-workers showed that treatment of HepG2 cells with iron chelators increased the steady-state level of ceruloplasmin mRNA and the rate of ceruloplasmin protein production by about 4-fold (Mukhopadhyay *et al.* 1998). They also showed that the regulation was due to increased transcription of the ceruloplasmin transcript. Subsequent studies from the same laboratory showed that transcriptional activation of ceruloplasmin required a single hypoxia-inducible factor-1 (HIF-1) binding site in the distal 5′-flanking region of the ceruloplasmin gene (Mukhopadhyay *et al.* 2000). HIF-1 is the transcription factor primarily responsible for erythropoietin gene activation, and thus for increased erythropoiesis, during conditions of iron deficiency and hypoxia. Thus it is likely that anemia increases plasma ceruloplasmin level by hepatic activation of HIF-1. The HIF-1 element in ceruloplasmin was found to be similar to that in erythropoietin, and likewise was activated by hypoxia. Interestingly, ceruloplasmin levels were shown to be mildly elevated during hypoxic conditions, for example in soldiers transported to the summit of Pikes Peak, Colorado (Surks 1966) and in patients with chronic obstructive pulmonary disease (Pedersen *et al.* 1987; Erel 1998). The discovery of transcriptional activation of ceruloplasmin by HIF-1 provides molecular, albeit indirect, evidence for an important role of ceruloplasmin in erythropoiesis. Given the similarity in sequence and function between ceruloplasmin and hephaestin, it is possible that they may undergo similar transcriptional regulation. However, in the limited studies to date it appears that hephaestin expression in the gut is not significantly influenced by iron status (Frazer *et al.* 2001).

Effect of iron overload on plasma ceruloplasmin

There is evidence for the converse regulation of copper by iron, i.e., decreased plasma ceruloplasmin in the presence of excess iron. A 1978 report showed that rats made polycythemic by red blood cell injection had decreased plasma ceruloplasmin (Iwanska & Strusinska 1978). Likewise, plasma ceruloplasmin was substantially decreased during diet-induced iron overload in rats (Johnson & Murphy 1988; Johnson & Hove 1986; Soyars & Fischer 1998; Klevay 2001). In human studies, oral iron therapy was shown to decrease plasma ceruloplasmin in children (Morais *et al.* 1994). Finally, a recent report by Gaetano Cairo and co-workers showed that ceruloplasmin levels were low in hereditary hemochromatosis patients (Cairo *et al.* 2001). This result was confirmed by Pierre Brissot and his colleagues who showed further that Cp levels were normalized in hemochromatosis patients subjected to regular phlebotomy to reduce iron, thus indicating a direct role for serum iron in regulating Cp in these patients (Lainé *et al.* 2002). Very little is known about mechanisms underlying the decrease in ceruloplasmin level during iron overload, but one *in vitro* study showed that iron-loading of HepG2 cells decreased the rate of ceruloplasmin synthesis by half (Mukhopadhyay *et al.* 1998).

End-note

The discovery of the role of copper in overcoming anemia has captured the imagination and effort of hundreds of investigators, and resulted in a dogged pursuit of the mechanism that has continued unabated for more than a century and a half. In his marvelous book of lectures on bioenergetics, Efraim Racker from Cornell University defined four stages in the process of discovery of oxidative phosphorylation (Racker 1965). The discovery of the copper/iron connection fit well into these stages (Figure 8). The initial discovery stage or 'recognition of the phenomenon' was made by physician-scientists in the second half of the nineteenth century, primarily by Millon, Pécholier and Saint-Pierre, Mendini, and Barabini and Cervello. The second stage of 'quantitative evaluations' was started by Barabini and Cervello (and his students), and then rigorously addressed by Hart and Elvehjem and coworkers and by others of the period. The specificity of the effect of copper was rigorously addressed at the same time. The third stage, 'localization of sites', was defined and investigated by Cartwright, Wintrobe and their colleagues. The fourth and final stage, 'resolution and reconstitution' was begun by Osaki and Frieden with their discovery of ceruloplasmin as a molecular link between copper and continues today at an ever-increasing rate in many laboratories. The recent discovery of novel genes involved in iron and copper metabolism, and the likely continued identification of other genes via the human genome project is likely to contribute to the 'resolution' step. The 'reconstitution' step is only just beginning; it is highly likely that mice with targeted gene disruption(s), combined with new imaging techniques, and perhaps improved biochemical methods for measuring iron in specific metabolic compartments (non-transferrin bound iron comes to mind) will finally allow us a simultaneous molecular and global view of the copper/iron connection.

Where does the field stand after 150 years of effort? Gubler and his coworkers perhaps best summarized the iron/copper interaction as follows: *copper may, in some basic manner, be concerned wherever and whenever iron moves* (Gubler *et al.* 1952). The pursuit of the meaning of 'in some manner' has led to the identification of many of the molecular components and at least a partial understanding, at the cellular and biochemical level of the pathways at the intersection of copper and iron metabolism. Many gaps still remain. For example: what is the molecular mechanism of copper-stimulated iron release from tissues? Is iron release facilitated or gradient-driven? What are the specific roles of ceruloplasmin, hephaestinand ferroportin1 in iron release? Is there a role for copper in iron utilization by red cell precursors? Does copper facilitate metabolism of low molecular weight iron complexes? Do copper enzymes play a regulatory role in the anemia of inflammation? These and other questions will continue to drive research in the copper/iron area for decades to come.

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