

Signs of iron deficiency in copper-deficient rats are not affected by iron supplements administered by diet or by injection[☆]

Philip G. Reeves^{*}, Lana C.S. DeMars

USDA, ARS, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58203, USA

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Abstract

The goal of this study was to determine the effects of Fe supplementation on the anemia of Cu deficiency in rats. In addition, we observed changes in serum and organ Cu and Fe during the development of Cu deficiency. In Experiment 1, weanling male Sprague–Dawley rats were fed AIN-93G diets containing either <0.3 mg Cu [Cu deficient (CuD)] or 6.0 mg Cu [Cu adequate (CuA)] per kilogram diet, and 35 mg Fe/kg. Five rats from each group were killed at intervals for the analysis of hematologic parameters and mineral content of various organs. In Experiment 2, two groups of 24 rats each were fed either the CuA diet or the CuD diet for 14 days. Then, three sets of eight rats in each group received three separate Fe treatments: (1) daily intraperitoneal injections of 400 µg Fe (Cu-free ferric citrate) per rat for another 14 days, (2) fed similar diets that contained three times the normal amount of Fe (105 mg/kg) for 14 days, or (3) received no further Fe treatment. At day 21, all rats were fed a 1-g meal labeled with ⁵⁹Fe to determine Fe absorption. After 28 days, rats were killed for the analyses of Fe and Cu status. Results of Experiment 1 showed that within 14 days, CuD rats had lower blood hemoglobin (Hgb), red blood cell count, and mean corpuscular volume than CuA rats. Copper concentrations in all tissues measured were lower in the CuD rats than in controls. Serum ceruloplasmin (Cp) activity in CuD rats was only 0.8% of CuA rats at day 7. During this period, enterocyte and liver Fe concentrations were elevated and serum Fe was reduced, but there was no change in spleen Fe. Results of Experiment 2 showed that CuD rats absorbed less Fe than CuA rats. Supplemental Fe by diet or by intraperitoneal injections did not prevent anemia in the CuD rats or affect other parameters of Cu status. Serum total iron binding capacity [transferrin (Tf)] was not changed by Cu deficiency or by Fe supplementation; however, percent Tf saturation was reduced in CuD rats but was not enhanced by Fe supplementation. These data suggest that anemia of Cu deficiency occurs because of reduced Fe absorption, and it inhibits release of Fe from the liver and inefficient loading of Fe into Tf because of very low plasma Cp activity. The latter then leads to inefficient delivery of Fe to the erythroid cells for heme and Hgb synthesis.

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Abbreviations: Cp, ceruloplasmin; CpAO, Ceruloplasmin amine oxidase; Cu, Copper; CuA, Cu adequate; CuD, Cu deficient; Fe, Iron; Hgb, Hemoglobin; Hp, Hephaestin; MCV, Mean corpuscular volume; RBC, Red blood cells; Tf, Transferrin; TIBC, Total iron binding capacity; WBC, Whole-body counter.

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^{*} Corresponding author. Tel.: +1 701 795 8497; fax: +1 701 795 8395.
E-mail address: preeves@ghnrc.ars.usda.gov (P.G. Reeves).

1. Introduction

Adequate dietary Cu is required for efficient absorption of dietary iron (Fe) in rats as demonstrated by Chase et al. [1] by measuring the reduction in whole-body Fe content of Cu-deficient (CuD) rats. This was confirmed by Reeves and DeMars [2] by showing that CuD rats retained less dietary ⁵⁹Fe than Cu-adequate (CuA) rats by using whole-body counting (WBC) techniques.

Copper facilitates intestinal Fe absorption by acting through a Cu-dependent ferroxidase, hephaestin (Hp), located in the duodenal enterocyte. Bannerman and Pinkerton [3] first described a mouse model with sex-linked anemia that could be cured by injecting Fe. Vulpe et al. [4] later found that this defect was caused by a mutation in the gene of Hp,

which is a homologue of ceruloplasmin (Cp). Reeves et al. [5] then showed that the relative abundance of Hp protein was reduced in the duodenal enterocytes of CuD rats. They later demonstrated that refeeding Cu to CuD rats elevated the amount of Hp protein and returned Fe absorption to normal [6].

Signs of Fe deficiency such as low serum Fe and anemia appear in weanling rats within days after feeding them a CuD diet [7–9]. This suggests a very important role for Cu in iron absorption. However, Cu also might act through other systemic mechanisms that affect the metabolic fate of iron such as in the formation of heme and/or hemoglobin (Hgb) or trafficking of Fe inside cells. Recent work by Morrison et al. [10] suggests that the homeostatic regulation of the different forms of heme in yeast and bacteria is not controlled by Cu. Whether this is true for the regulation of heme and Hgb in erythroid cells is not known for certain [6,11–13]. However, it is widely accepted that Fe is delivered to the erythroid cells by Fe³⁺-loaded transferrin (Tf) by endocytosis of the Tf receptor 1 complex on the cell surface [14]. It is also believed that the Cu-dependent ferroxidase activity of Cp in plasma maintains Fe in the oxidized state. In CuD rats, Cp activity is reduced to near zero and the rats are anemic. However, curious observations in some studies [15,16] show that Cp^{-/-} mice have low serum Fe but little or no incidence of anemia, but in others [17], there is about a 15% to 20% lower Hgb and a 10% lower hematocrit than Cp^{+/+} mice. Yet, if the mice are stressed by bleeding, it takes them longer to recover to near normal blood Hgb concentrations than Cp^{+/+} mice [16].

If the anemia of Cu deficiency were caused primarily by reduced Fe absorption, then injecting Fe would cure the anemia. On the other hand, if the anemia were caused by reduced Fe³⁺ loading into Tf because of low Cp activity, then feeding high amounts of Fe in the diet or administering high doses of injected Fe should have no effect on the signs of anemia in these animals. In some studies, the latter was indeed the case, but in others, it was not. Gubler et al. [18] administered high amounts of dietary Fe to CuD pigs and found that anemia was not cured. In addition, these investigators as well as Cartwright et al. [19] administered Fe intraperitoneally or intravenously to CuD pigs and showed no effect on anemia. These data suggested that reduced absorption and low serum Fe were not the only factors in causing anemia in CuD animals. Conversely, Weisenberg et al. [20] fed 80 to 100 mg Fe/kg diet to anemic CuD rats and reversed the condition. Cohen et al. [21] fed 120 vs. 25 mg Fe/kg diet, and low or adequate Cu, to female rats during gestation and lactation and found no signs of anemia in the CuD dams or the offspring consuming the high Fe diet, but there was anemia in CuD rats fed 25 mg Fe/kg diet. In addition, Prohaska [22] injected CuD mice with Cu-free Fe and found a complete reversal of anemia, lending more uncertainty to the Cu–Fe connection related to anemia.

One of the following experiments was designed to revisit this area of investigation in an attempt to resolve the question of whether high Fe supplements to CuD rats improve the signs of Cu deficiency anemia. Preliminary to this, however, we describe the results of an experiment that assessed changes in organ concentrations of Cu and Fe during the development of Cu deficiency.

2. Materials and methods

This study was approved by the Animal Use Committee of the USDA-ARS, Grand Forks Human Nutrition Research Center. The procedures followed the guidelines of the National Institutes of Health for the experimental use of laboratory animals [23].

2.1. Experiment 1

Sixty-five weanling male Sprague–Dawley rats [strain: SAS:VAF (SD), Charles River/Sasco, Wilmington, MA] at 3 weeks of age were used in this study. Five of the rats were killed initially to obtain baseline values before the experiment began. The remaining 60 rats were randomly divided into two groups of 30 rats each and fed either of two diets. The diets used were based on the AIN-93G formulation [24,25] and contained 6.0 mg Cu/kg in the CuA group and <0.3 mg Cu/kg in the CuD group. The diet contained ferric citrate as the source of Fe (35 mg Fe/kg). Rats were housed individually in stainless steel wire-bottom cages in a temperature/humidity (72°C/50%) controlled room with a 12-h light/dark cycle. Food was offered ad libitum in glass containers with stainless steel screw caps that had holes through which the rat could obtain food and, at the same time, prevent food wastage. Deionized water was offered ad libitum in glass bottles with silicon stoppers and stainless steel sipper tubes. Food and water were monitored daily for freshness, and body weights were recorded weekly.

On days 4, 7, 14, 21, 28, and 35, blood and organs were collected for the determination of various parameters of Cu and Fe status. Five rats from each treatment group were anesthetized without fasting with a 1.37:1 mixture of ketamine/xylazine (1.0 ml/kg BW, ip), and blood was withdrawn from the abdominal aorta. EDTA-treated (50 µl of a 30% solution of NaEDTA, pH 7.4, per milliliter) whole blood was analyzed with a Cell-Dyn 3500 automated hematology cell counter (Abbott Lab., Abbott Park, IL) to assess the effects of Cu deficiency on Hgb, red blood cell (RBC) number, and mean corpuscular volume (MCV) (data reported in Ref. [2]). To evaluate Cu and Fe status, we collected a second sample of blood from each rat, and the serum was separated for the analysis of Cp amine oxidase (CpAO) activity [26,27] and Cu and Fe concentration (data reported in Ref. [2]). A 10-cm section of the duodenum, beginning at the pylorus, was removed and cut open, and the mucosal layer was scraped off with the edge of a glass slide. Whole liver was collected and a 2-g piece at the end

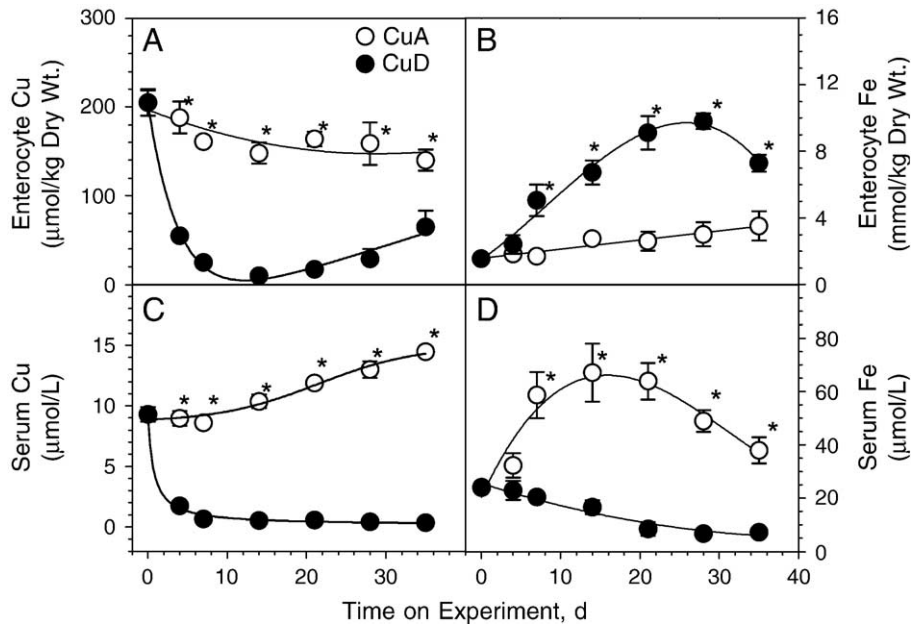


Fig. 1. Over time, Cu deficiency (CuD) reduced Cu concentrations in rat intestinal mucosa (A) and serum (C), and reduced Fe in serum (D), but elevated Fe in enterocytes (B) compared with Cu adequacy (CuA). Values are means±S.E.M., *n*=5. An asterisks (*) indicates a significant (*P*<.05) difference between CuA and CuD groups on a particular day.

of the right lateral lobe was removed and perfused with ice-cold normal saline (0.85% NaCl in deionized water) until all blood was removed. The spleen was collected without perfusion. All tissues were lyophilized and dry ashed at 475°C for 12 h. The ash was further digested for 12 h in 10 ml of concentrated nitric acid and 2.0 ml of 30% hydrogen peroxide and allowed to come to dryness. Then the samples were ashed again as described, and the dry

residue was diluted appropriately in HCl (1.0 mol/L) for the determination of Fe and Cu content by inductively coupled argon plasma analysis.

2.2. Experiment 2

Forty-eight weanling male rats similar to those in Experiment 1 were divided into six groups of eight rats each and fed CuA and CuD diets similar to those described.

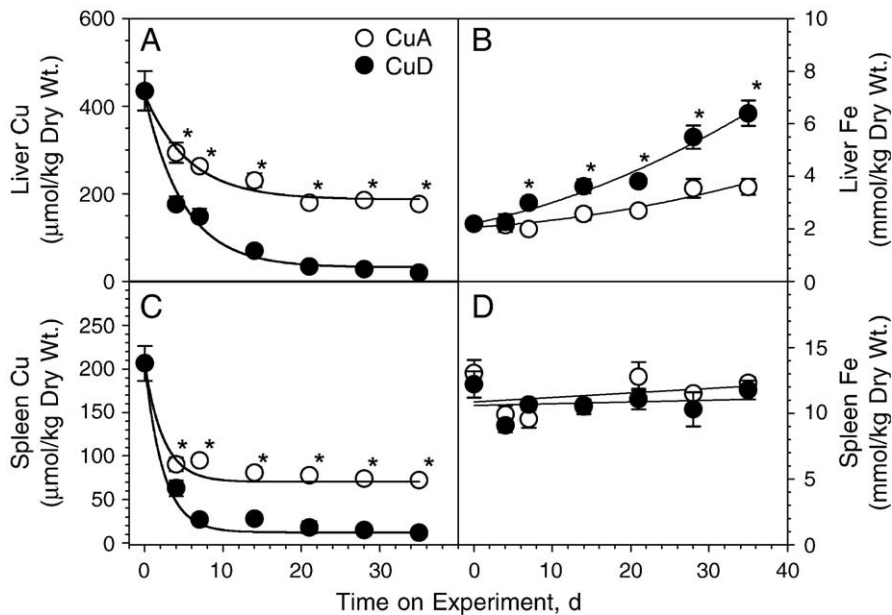


Fig. 2. Over time, Cu deficiency (CuD) reduced Cu concentrations in rat liver (A) and spleen (C) but elevated Fe in liver (B) compared with that in CuA rats. Copper deficiency had no effect on spleen Fe (D). Values are means±S.E.M., *n*=5. An asterisks (*) indicates a significant (*P*<.05) difference between CuA and CuD groups on a particular day.

On day 14, Fe supplementation regimens were begun. The dietary Fe supplementation regimen consisted of feeding eight rats in each dietary group three times the normal amount of Fe (105 mg Fe/kg). The injected regimen consisted of daily intraperitoneal injections of Fe in eight rats per group while they consumed their original normal Fe diet (35 mg/kg). The injection consisted of 100 μ l of normal saline per rat, which contained 400 μ g of Fe as ferric citrate. By analysis, no Cu was found in this solution. A third set included eight rats from each group that did not receive supplemental Fe but received 100 μ l of normal saline. On

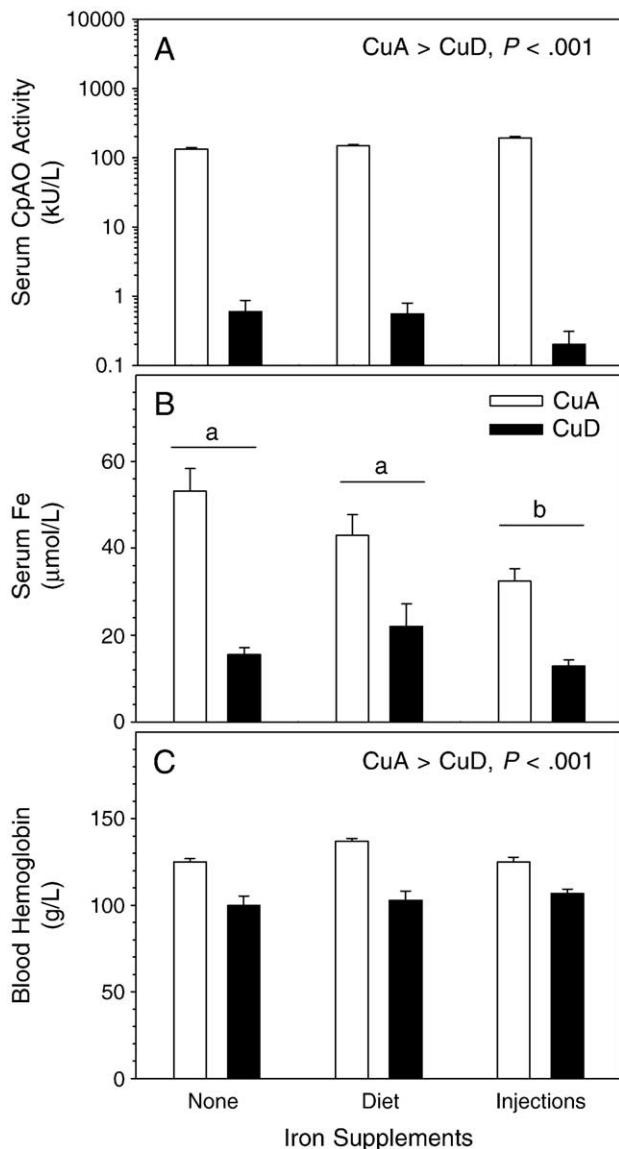


Fig. 3. Copper deficiency (CuD) significantly reduced serum CpAO activity (A), serum Fe (B), and blood Hgb (C) regardless of Fe treatment relative to the CuA rats. Independent of dietary Cu concentration, serum Fe (B) was significantly ($P < .013$) lower in the Fe-injected group than in the groups receiving Fe by diet or no extra Fe. This is indicated by different letters above the Fe treatment groups. There was no interaction between diet Cu and Fe supplements. Values are means \pm S.E.M., $n = 8$.

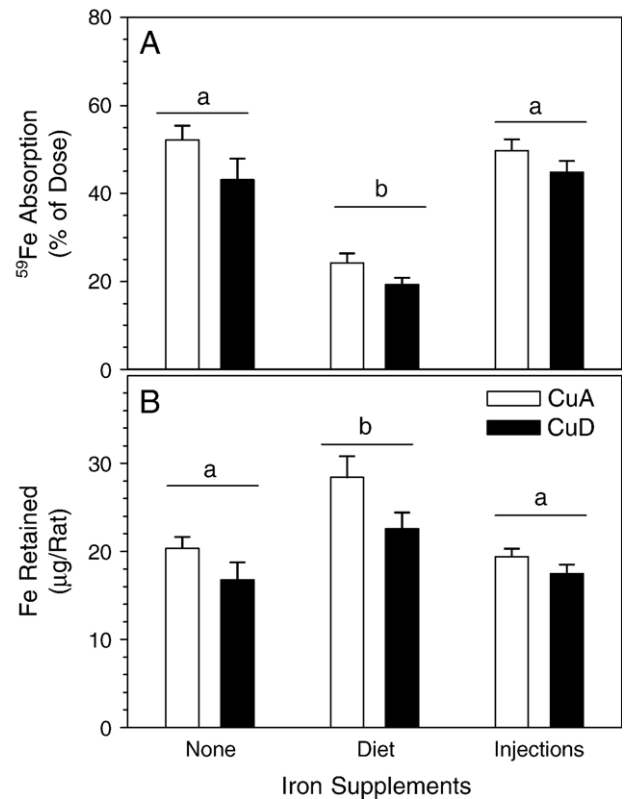


Fig. 4. Both the percent absorption of dietary Fe (A) and the amount of dose retained per rat (B) were significantly ($P < .014$ and $P < .009$, respectively) reduced in CuD rats compared with CuA rats. The main effect of Fe administration on absorption was that rats usually given Fe by diet absorbed significantly ($P < .001$) less than those usually given Fe by injections or not given extra Fe. This is indicated by different letters above the Fe treatment groups. On the other hand, feeding extra Fe significantly ($P < .001$) elevated the amount of Fe retained from the dose (B). Values are means \pm S.E.M., $n = 8$.

day 21, all rats were fasted from 0800 to 1500 h and then fed a 1.0-g meal of their respective diets labeled with 59 Fe as ferric chloride. After the rats had consumed their meal (about 1 h), the amount of 59 Fe in each rat was determined by WBC. On day 28, the amount of 59 Fe retained was determined by WBC. Throughout this test period, all rats continued to receive their initial dietary and injection regimens. The animals were killed without fasting as outlined in Experiment 1, and similar procedures were used to collect blood and tissues, except that duodenal enterocytes instead of mucosal scrapings were isolated by the procedure of Chen et al. [28]. The interval between the last Fe injection and sample collection was 16 h.

A two-way ANOVA was used to verify differences between means for all parameters measured. For Experiment 1, the two variables were Cu treatment and time, and for Experiment 2, the variables were Cu treatment and Fe supplementation. For all pairwise multiple comparisons, the procedure of Tukey [29] was used. Significance was set at $P \leq .05$ (two-tailed tests).

3. Results

3.1. Experiment 1

Parts of the data collected from this experiment were presented in a previous publication [2] and showed that rats fed the CuD diet became CuD and anemic very quickly compared with the CuA group. After only 4 days, serum CpAO activity was reduced ($P<.001$) to only 3% of that in the CuA group, and by day 7, to only 0.8% of the control group. By day 14, anemia had developed in the CuD group as shown by significant ($P<.001$) reductions in Hgb, RBC number, and MCV compared with those values in the CuA group [2]. The differences seem to result from a rise over time for each of these parameters in the CuA group, but no change in values in the CuD group.

Over the course of the experiment, rats in the CuD group gained about 10% less body weight than those in the CuA group, but this did not result in a significant change in the organ weights (data not shown). Mucosal Cu concentrations were significantly ($P<.001$) lower in CuD rats at day 4 compared with those in CuA rats (Fig. 1A). Mucosal Fe, on the other hand, was significantly ($P<.001$) higher in the CuD group by day 7, compared with that in the CuA group (Fig. 1B). A curious observation was that mucosal Cu in the CuD group began to increase by the end of the experiment,

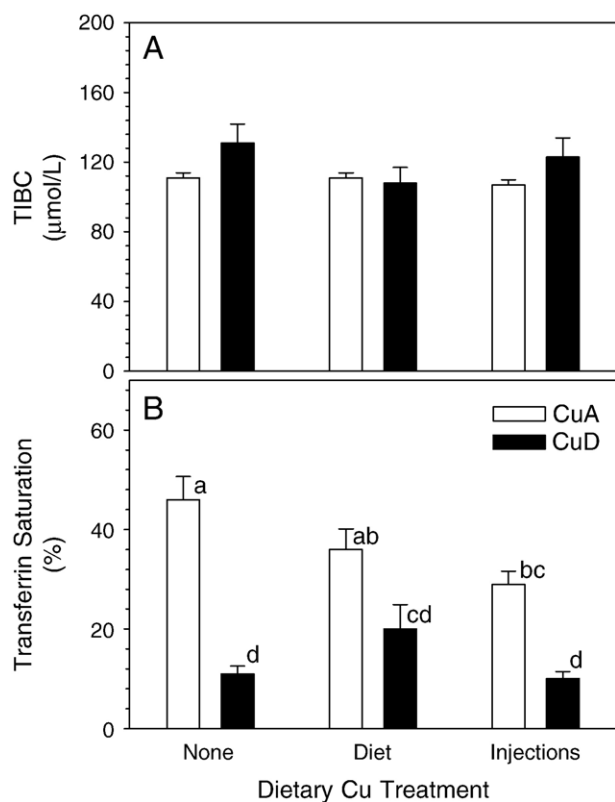


Fig. 5. (A) Serum TIBC was not affected by Fe supplementation or Cu deficiency (CuD). (B) Percent Tf saturation followed the same pattern as that for serum Fe (Fig. 3B), but the difference among groups was more distinct. Bars with different letters are significantly different, $P<.010$. Values are means \pm S.E.M., $n=8$.

Table 1

Experiment 2: copper concentrations in enterocytes, liver, and spleen were reduced by Cu deficiency in rats but were lowered by supplemental Fe only in spleen

Treatment	Enterocytes ($\mu\text{mol/kg}$ dry weight)	Liver	Spleen
<i>CuA Fe supplement</i>			
None	177.0 \pm 6.8	184.5 \pm 5.0	71.8 \pm 2.4 ^b
Diet	180.2 \pm 6.8	200.2 \pm 10.0	74.9 \pm 3.2 ^b
Injection	173.1 \pm 7.8	184.9 \pm 12.1	85.2 \pm 2.1 ^a
<i>CuD Fe supplement</i>			
None	52.4 \pm 5.0	21.5 \pm 2.5	10.4 \pm 1.3 ^c
Diet	55.5 \pm 1.2	21.8 \pm 2.5	7.8 \pm 1.0 ^c
Injection	48.6 \pm 6.2	34.4 \pm 3.8	7.7 \pm 1.1 ^c
Statistics	<i>P</i> values		
Diet Cu	<.001	<.001	<.001
Fe supplement	NS	NS	<.021
D x F	NS	NS	<.002

Data are expressed as mean \pm S.E.M., $n=8$. Means with different superscript are significantly different, $P<.002$. NS=not significant.

whereas mucosal Fe began to decrease. Serum Cu in the CuD group followed a similar pattern as Cu in the mucosa (Fig. 1C). On the other hand, serum Fe concentrations were opposite that in the mucosa (Fig. 1D).

The concentrations of liver Cu were significantly ($P<.001$) reduced with time on experiment in both treatment groups; however, values for the CuD group were significantly ($P<.001$) lower than those in the CuA group (Fig. 2A). On the other hand, liver nonheme Fe was significantly ($P<.001$) increased in both groups with time, but it was significantly ($P<.001$) higher overall in the CuD group than in the CuA group (Fig. 2B).

The concentration of spleen Cu also was significantly ($P<.001$) reduced with time on experiment in both treatment groups; however, the values for the CuD group were significantly ($P<.001$) lower than those in the CuA group (Fig. 2C). Spleen Fe was not significantly ($P=.50$) changed with time in either group, and there were no differences between CuA and CuD groups ($P=.14$) (Fig. 2D).

3.2. Experiment 2

Regardless of Fe treatment, the activity of serum CpAO was significantly ($P<.001$) lower in CuD than CuA rats (Fig. 3A). Iron treatment had no effect on serum CpAO activity. Serum Fe concentrations were significantly ($P<.001$) lower in CuD rats than in CuA rats in all Fe treatment groups (Fig. 3B). Serum Fe was significantly lower in rats treated with Fe injections than in rats not receiving extra iron or extra Fe by diet regardless of Cu status (Fig. 3B). Blood Hgb was significantly lower ($P<.001$) in CuD rats than in CuA rats, but there was no effect of Fe supplements on this parameter (Fig. 3C).

The percent absorption of ^{59}Fe was significantly lower ($P=.014$) in CuD rats than in CuA rats (Fig. 4A). Rats that received dietary supplements of Fe also had reduced absorption of ^{59}Fe regardless of their Cu status; however,

rats that received Fe injections absorbed similar amounts of Fe as rats receiving no supplemental Fe. On the other hand, when the amount of Fe retained was calculated based on the specific activity (becquerel ^{59}Fe per milligram Fe) of ^{59}Fe in the initial 1.0-g diet dose, the total amount of Fe retained by CuD rats was only about 80% ($P=.009$) of that in CuA rats (Fig. 4B). When excess Fe was administered by diet, the

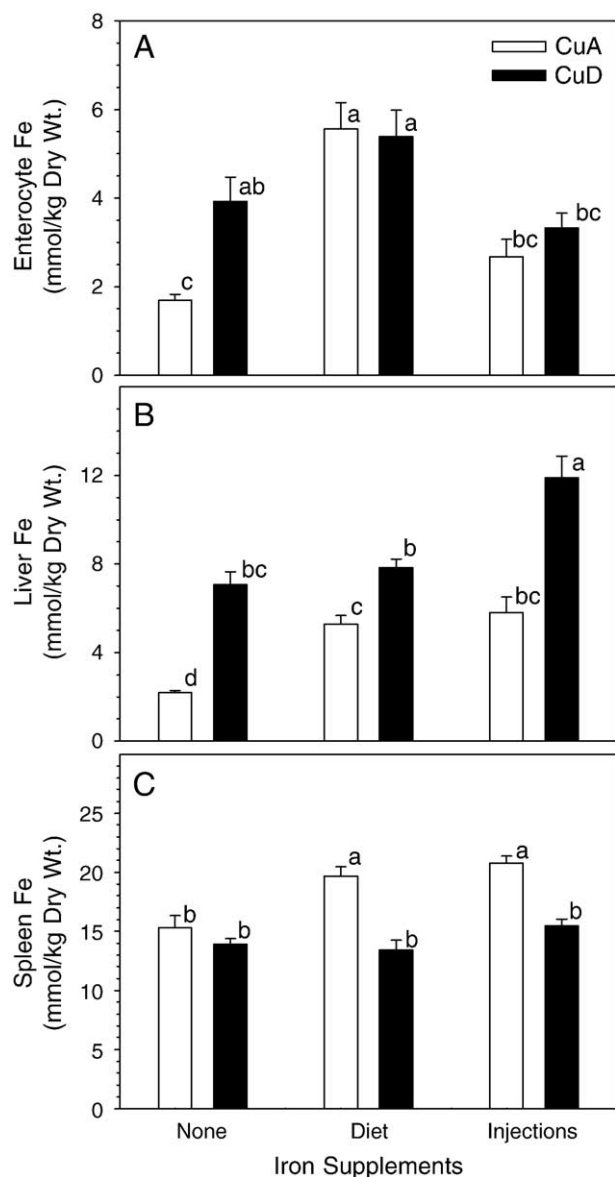


Fig. 6. The concentration of enterocyte Fe in CuA rats fed extra Fe was increased threefold over that in rats not receiving extra Fe or those injected with excess Fe (A). Enterocyte Fe in CuD rats was higher in those receiving no extra dietary Fe or injected Fe when compared with similar groups in the CuA rats. However, feeding high dietary Fe to CuD rats caused no additive increase in enterocyte Fe (A, column 5). Liver Fe concentration was elevated in CuA rats receiving extra Fe (B), and CuD rats had significantly ($P<.01$) more liver Fe than the CuA rats. Injecting Fe into CuD rats further increased liver Fe compared with the other routes of administration (B, column 6). Values are means \pm S.E.M., $n=8$. Bars with different letters for Panel A are significantly different at $P<.001$, different letters for Panel B are significantly different at $P<.004$, and different letters for Panel C are significantly different at $P<.001$.

amount of Fe absorbed from the dose per rat was significantly ($P<.001$) higher than that from injections or from no additional Fe (Fig. 4B).

Although there seemed to be a small increase in total iron binding capacity (TIBC) of the serum in the CuD rats compared with CuA rats, the difference was not statistically significant (Fig. 5A). There was an interaction ($P<.03$) between Cu treatment and Fe supplementation with regard to percent Tf saturation. Overall, percent saturation was lower in the CuD rats than in the CuA rats; however, Fe supplements did not affect this parameter in CuD rats, whereas in the CuA rats, it was significantly lower in rats receiving Fe injections than in those receiving no extra Fe. Percent Tf saturation was not different between rats receiving extra Fe in the diet and injected Fe, or between those receiving no extra Fe and those that received extra Fe from the diet.

Copper concentrations in enterocytes, liver, and spleen were significantly ($P<.001$) lower in CuD rats than in CuA rats (Table 1). The administration of supplemental Fe only affected spleen Cu concentration in the CuA group. Rats receiving Fe injections had higher spleen Cu than the other two groups.

Supplemental Fe affected organ Fe concentration (Fig. 6). There was an interaction showing that enterocyte Fe in rats not receiving supplemental Fe was significantly ($P<.001$) higher in CuD rats than in CuA rats (Fig. 6A). When rats were fed or injected with extra Fe, Cu deficiency had no effect on Fe concentration in the enterocytes. However, those rats receiving injected Fe had lower enterocyte Fe than those fed extra Fe.

There also was an interaction between treatment groups for nonheme Fe in the liver (Fig. 6B). Copper deficiency significantly elevated liver Fe in all Fe supplemental groups compared with that in the CuA group. However, the degree to which liver Fe was elevated in those rats receiving no extra Fe and those receiving injected Fe was much greater than that in rats receiving extra dietary Fe.

The concentration of Fe in spleen was affected by dietary Cu treatment, but only in those rats receiving additional Fe by diet or injections (Fig. 6C). Spleen Fe was not affected by Fe supplementation in the CuD group, but was elevated by Fe supplementation in the CuA group.

4. Discussion

The objective of Experiment 1 was to observe the time course of biochemical changes during the development of Cu deficiency in the rat. Other investigators [7,8] have pursued this course of investigation in the past, but for the most part, they concentrated on changes in events that affected Cu or that were affected specifically by Cu. Here, we also observed changes in those events that affected Fe and those that were affected by changes in Fe status during Cu deficiency. These included Cu and Fe concentrations in serum and tissues.

Signs of Fe deficiency developed rather quickly in the CuD rats where serum Fe in the CuA group increased with time, whereas that of the CuD group remained rather low and constant. However, blood parameters related to Fe deficiency such as Hgb and RBCs were not significantly lowered until at least day 14, as shown in this study and as reported in Ref. [2]. These data correlated well with the proposed half-life of RBCs in rats of ~14 days [30].

In most CuD studies in rats, Fe concentrations in duodenal enterocyte are elevated, and this most likely is caused by the effect of CuD on Hp protein concentration and activity. Reeves et al. [5,6] showed that Hp protein concentrations were reduced in CuD rat enterocytes, with a concurrent rise in enterocyte Fe, which probably inhibited the efflux of Fe to the circulation. This in turn provided the basis for Fe accumulation in the enterocyte cytosol. Indeed, on refeeding Cu to CuD rats, Hp protein was elevated and enterocyte Fe was reduced [6].

In the current study, it was shown that liver Fe was significantly elevated by day 14 and continued to climb throughout the study. Evans and Abraham [7] showed similar results with liver Fe, but unfortunately, their control diets contained 35 mg Cu/kg and the rats only gained about 1.9 g/day, which is about one third the normal growth rate for male rats. This suggests that high dietary Cu caused a toxic reaction and inhibited adequate growth rates. Johnson and Saari [8] used a more moderate diet, but with sucrose as the carbohydrate source, and found very low liver Cu 7 days after starting the diet. However, liver Fe did not begin to rise until week 5. They did not explain the seemingly long period for liver Fe to respond to the onset of Cu deficiency.

In our experiment, mucosal and serum Cu concentrations were depressed at a very rapid rate shortly after rats were fed CuD diets. After only 4 days, mucosal Cu in CuD rats was only 30% of that in the controls. This suggests that mucosal Cu was below normal even at an earlier stage, perhaps within 24 h. Similar rapid reductions in liver and spleen Cu were observed within 7 days after initiating the deficiency. Other studies in our laboratory have shown a reduction in spleen Fe in CuD rats [5]; however, in the current one, there was no effect.

It was shown earlier [1,2,21] that Cu deficiency in the rat quickly leads to reduced Fe absorption from the diet. This alone might have been enough to lower serum Fe. However, because of low Cp activity, Fe cannot be efficiently released from the liver, and it accumulates there because the senescent RBCs are destroyed by the spleen and the iron from heme is recycled. Both of these conditions could lead to low serum Fe. It is believed that serum Cp ferroxidase activity keeps Fe in the oxidized state to facilitate Fe binding to Tf [31]. Thus, Cu deficiency resulted in low Tf saturation. Because the erythroid cells depend upon an adequate level of diferric Tf to acquire Fe [14,31], heme synthesis was depressed and anemia ensued. There were speculations in the past that Cu deficiency directly affected heme and/or Hgb synthesis [12,13,32]; however, recent

studies have shown that Cu is not involved in the regulation of heme O or heme A synthesis in yeast and bacteria [10]. Whether this is true for mammalian heme in erythroid cells is yet to be determined.

The major objective of Experiment 2 was to determine whether the administration of excess Fe as Cu-free ferric citrate in the diet or as intraperitoneal injections would cure the anemia of Cu deficiency. The data clearly showed that the anemia of Cu deficiency was not reversed in rats receiving extra Fe from the diet, even though they were absorbing more Fe than controls. Likewise, CuD rats receiving even more Fe by injection than by diet were not cured of anemia. Over the years, there has been some controversy on this issue. Some reports provide evidence that Fe administered by injections to some CuD species, such as pigs [18,19], does not reverse the signs of anemia; however, other studies with mice and rats [20,22] showed that the anemic conditions were reversed by the administration of extra Fe. In addition, extensive studies by Cohen et al. [21,33] found no effect of CuD on anemia in rats fed as much as 3.5 times the Fe requirement, whereas mild anemia was present in rats fed marginal levels of Fe (25 mg/kg). No explanations have been found for the discrepancies among these studies, except that the animal's diet is a very important factor in nutrition studies of this nature. On close examination, some of the diets used in earlier studies revealed rather unusual formulations compared with the more nutritionally balanced diets used today. Some were based solely on condensed milk [1], and most others were based on sucrose as the major carbohydrate source, which is known to have a negative effect on Cu absorption and metabolism [34,35].

It was observed that CuA rats injected chronically with ferric citrate (400 μ g Fe daily for 2 weeks) and killed 16 h after the last dose did not have elevated serum Fe. In fact, the values were significantly lower than the control group that received no extra Fe. Perhaps the mere act of injecting the rats with ferric citrate caused an acute phase response that included transfer of Fe from the serum into the liver. Liver Fe in the CuA groups administered extra Fe was indeed higher than the control group. When the animals were CuD, liver Fe was even higher. The latter was most likely caused by very low activity of Cp, which is instrumental in Fe release from the liver [15].

Based on this study, it has been confirmed in at least two species of animals (pigs and rats) that the anemia of Cu deficiency does not respond to supplemental Fe, by diet or by intraperitoneal injections. The primary effects on Fe metabolism in the CuD animal centers around two Cu-dependent ferroxidases, Hp in the intestinal mucosa and Cp in the liver. Reduced activity of Hp in the intestine of CuD animals inhibits Fe absorption, and reduced activity of Cp in the liver and blood inhibits Fe release from liver, each contributing to low serum Fe³⁺. This in turn diminishes the amount of Fe³⁺-loaded Tf, which leads to reduced uptake of Fe by the erythroid cells, consequently lowering heme synthesis. The

eventual outcome is anemia. Injecting Fe intraperitoneal could bypass the low Hp activity in the intestine, but it cannot overcome the low Cp activity in the serum. Injected Fe still cannot be oxidized and bound to Tf; thus, erythroid cells receive too little Fe for efficient heme synthesis.

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