Repletion of Copper-Deficient Rats with Dietary Copper Restores Duodenal Hephaestin Protein and Iron Absorption

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Copper (Cu) deficiency in rats reduces the relative concentration of duodenal hephaestin (Hp), reduces iron (Fe) absorption, and causes anemia. An experiment was conducted to determine whether these effects could be reversed by dietary Cu repletion. Five groups of eight weanling male rats each were used. Group 1 was fed a Cu-adequate diet (5.0 mg Cu/kg; CuA) and Group 2 was fed a Cu-deficient diet (0.25 mg Cu/kg; CuD) for 28 days. The rats were fed 1.0 g each of their respective diets labeled with ^{59}Fe (37 kBq/g), and the amount of label retained was measured one week later by whole-body-counting (WBC). Group 3 was fed a CuA diet and Groups 4 and 5 were fed a CuD diet for 28 days. Group 5 was then fed the CuA diet for another week while Groups 3 and 4 continued on their previous regimens. Rats in Groups 3, 4, and 5 were fed 1.0 g of diet labeled with ^{59}Fe, and the amount of label retained was measured by WBC one week later. Rats were killed and duodenal enterocytes isolated for Hp protein analysis, whole blood was analyzed for hematological parameters, and various organs for ^{59}Fe content. CuD rats absorbed less ($P<0.05$) Fe than CuA rats, the relative amount of duodenal Hp was less ($P<0.05$) in CuD rats, and the CuD rats developed anemia. After the CuD rats had been repleted with Cu for one week, Fe retention rose to values even higher ($P<0.05$) than those in CuA rats. After two weeks, the relative amount of duodenal Hp was higher ($P<0.05$) than normal, and most signs of anemia were reversed. Liver ^{59}Fe was elevated in CuD rats, but was restored to normal upon Cu repletion. These findings suggest a strong association between duodenal Hp abundance and Fe absorption in the CuD rat, and that reduced Fe absorption is an important factor in the cause of anemia. Exp Biol Med 230:320–325, 2005

Key words: ceruloplasmin; copper deficiency; hephaestin; iron absorption; rats

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Copper (Cu) is required for the efficient utilization of iron (Fe) in mammalian systems (1). Signs of Fe-deficiency anemia appear quickly in rats fed a Cu-deficient (CuD) diet and include low blood hemoglobin, low hematocrit, and low red blood cell (RBC) count (2–4). Humans with Cu deficiency resulting from malabsorption syndrome develop anemia, neutropenia, and myelodysplasia (5, 6). It has been shown that CuD rats absorb only 50% as much Fe as Cu-adequate (CuA) rats (3); thus, the manifestations of Cu deficiency could result from reduced Fe absorption alone. Reeves *et al.* (7) found reduced amounts of the Cu-dependent ferroxidase protein hephaestin (Hp) in the duodenal enterocytes of CuD rats and suggested that this was the cause of reduced Fe absorption in these rats (7). This hypothesis is supported by the observation that the *sla* mouse, which has a mutated and inactive Hp protein, cannot efficiently absorb Fe and becomes Fe deficient (8, 9). The apical supranuclear region of the enterocyte is the primary location of Hp, but it is also present in the lateral and basolateral membranes (10, 11). There is evidence that the basolateral Fe transporter ferroportin 1 (Fpn1) is the primary conduit of dietary Fe from the enterocyte into the blood (12, 13), but it is not known whether there is a direct connection between this transporter and Hp for the efflux of Fe. Previous studies have shown that signs of Cu-deficiency anemia can be reversed by repleting CuD with dietary Cu (14). The following experiment was designed to determine whether Cu repletion restores duodenal enterocyte Hp protein and Fe absorption.

Materials and Methods

This study was approved by the Animal Use Committee of the USDA-ARS, Grand Forks Human Nutrition Research Center (Grand Forks, ND). The procedures followed the guidelines of the National Institutes of Health for the experimental use of laboratory animals (15).

Animals and Treatment. The experimental design consisted of five groups of eight weanling male Sprague-Dawley rats each (strain: SAS:VAF[SD]; Table 1). Group 1 was fed a CuA diet (5.0 mg Cu/kg) and Group 2 was fed a CuD diet (0.25 mg Cu/kg) for 28 days. Rats were then fed 1.0 g each of their respective diets that had been labeled

Table 1. Experimental Design[a]

Group	Diet	Days 1–7	Days 8–14	Days 15–21	Days 28–35	Days 36–42	Day 42
1	CuA				Feed ^{59}Fe: Day 28 Killed: Day 35	—	—
2	CuD				Feed ^{59}Fe: Day 28 Killed: Day 35	—	—
3	CuA					Feed ^{59}Fe: Day 36	Killed
4	CuD					Feed ^{59}Fe: Day 36	Killed
5	CuD				Switch to CuA diet: Day 28	Feed ^{59}Fe: Day 36	Killed

[a] CuA, Cu adequate; Fe, iron; CuD, Cu deficient.

with ^{59}Fe (37 kBq/g) and the amount of label absorbed and retained was measured 1 week later by whole-body counting (WBC) (3). Group 3 was fed a CuA diet and Groups 4 and 5 were fed CuD diets for 28 days. Group 5 rats were then repleted with Cu by being fed the CuA diet for another week. Groups 3 and 4 continued on their previous regimens. Rats in Groups 3, 4, and 5 were then fed 1.0 g of their respective diets labeled with ^{59}Fe and the amount of label absorbed and retained was measured by WBC 1 week later. After WBC, the rats were killed and duodenal enterocytes were isolated for Hp protein analysis, whole blood was analyzed for hematologic parameters, and various organs were analyzed for ^{59}Fe content. The diets were based on the AIN-93G formulation (16, 17) and contained 35 mg Fe/kg as ferric citrate. The rats were housed in hanging, stainless steel cages with wire-mesh bottoms in an atmosphere of 50% relative humidity at 22°C and with a 12:12-hr light:dark cycle with light beginning at 0600 hrs. Food was offered *ad libitum* in glass containers with stainless steel screw caps that had holes to 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.

Nutritional Assessment of Fe and Cu. At the end of the WBC period, the rats were anesthetized without having been fasted. Each rat was anesthetized ip with a 1.37:1 mixture of ketamine to xylazine (1µl/g body weight). Whole blood was collected from the abdominal aorta into EDTA-coated tubes and immediately analyzed with a Cell-Dyn 3500 automated hematology cell counter (Abbott Laboratories, Abbott Park, IL). To assess the effects of Cu deficiency on Fe status, erythrocyte (i.e., RBC) count, hemoglobin (Hgb) concentration, hematocrit (Hct), mean corpuscular volume (MCV), red cell distribution width (RDW), and platelet count were measured. To assess Cu status, a second sample of blood was collected from each rat and serum was separated for the analysis of ceruloplasmin amine oxidase (CpAO) activity (18), Cu concentration, and superoxide dismutase 3 (SOD3) activity (19, 20).

Duodenal Enterocyte Isolation and Western Blot Analysis of Hp Protein. Enterocytes were isolated from the proximal duodenum, beginning at the pylorus, as follows (21). Ten centimeters of intestine were removed and rinsed inside and out with ice-cold saline. This segment was inverted onto two wooden skewers, each 3 mm in diameter, and rinsed again by placing it into a 15-ml conical centrifuge tube filled with ice-cold phosphate-buffered saline (PBS). The intestinal segment and a skewer with nothing on it were placed in a tube containing ice-cold PBS and 1.5 m*M* EDTA. The segment and empty skewer were gently rotated together inside the tube for 10 mins to release the enterocytes. The enterocytes were pelleted by centrifugation (750 *g* for 5 mins at 4°C), and the cells were washed twice by suspending them in PBS and centrifuging at 4°C. The cells were lysed in three volumes of 1.5% Triton-X in PBS with a cocktail of protease inhibitors (#8340; Sigma Chemical Co., St. Louis, MO). The cell lysate was incubated for 30 mins on ice with occasional vortexing. The cells were also sheared by passing them through a 22-gauge needle, and the cellular debris was pelleted by centrifugation at 15,000 *g* for 5 mins at 4°C. The supernatant was snap frozen in liquid nitrogen and stored at –80°C until analyzed for Hp protein by Western-blotting techniques as previously described by Reeves *et al.* (7). The antibody to an 18-amino-acid fragment (i.e., aa1140–aa1157) of the mouse Hp protein (9) and the antigen itself were purchased from Alpha Diagnostic International, Inc. (San Antonio, TX).

Mineral Analysis. Liver, kidney, and spleen were collected and analyzed for ^{59}Fe by using a Packard Cobra gamma counter (7). A 1.0-g piece of saline-perfused liver, one kidney, and whole spleen were assayed for ^{59}Fe. Kidney and spleen were not perfused. Serum proteins were precipitated with a mixture of 10% trichloroacetic acid and 0.1% hydrochloride, and the supernatant was analyzed for Fe and Cu contents by inductively coupled plasma-mass spectroscopy.

Statistical Analysis. For data collected on Day 35, the Student's *t* test was used to determine significance between the two means. For data collected on Day 42, a one-way ANOVA was used and followed by the Tukey-Kramer multiple-comparison test to verify differences between means. Significance was set at $P \leq 0.05$.

Results

Two rats in Group 4 and one rat in Group 5 died because of complications from Cu deficiency. Death in each case was caused by a dissecting aneurysm in the thoracic aorta. Rats fed the CuD diet were Cu deficient as indicated by a number of factors. Rats that were Cu deficient and killed on either Day 35 or Day 42 had gained significantly

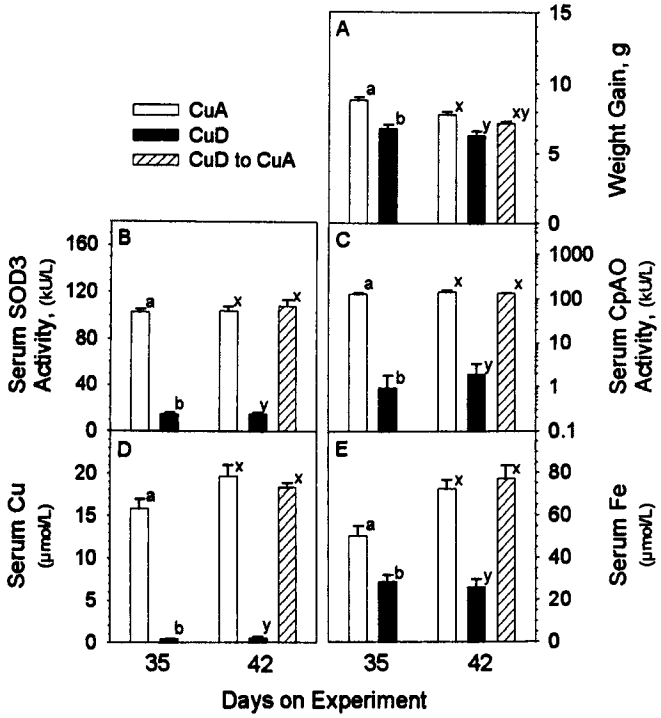

Figure 1. Signs of copper (Cu) deficiency such as reduced weight gain (Panel A), reduced serum superoxide dismutase 3 (SOD3) activity (Panel B), reduced serum ceruloplasmin amine oxidase (CpAO) activity (Panel C), reduced serum copper concentration (Panel D), and reduced serum iron (Panel E) were all reversed in copper-deficient (CuD) rats that were re-fed copper. Bars represent the mean ± SEM for 6 to 8 replicates per group. Means with different superscripts within a specific day were significantly different ($P \leq 0.05$). CuA, Cu adequate.

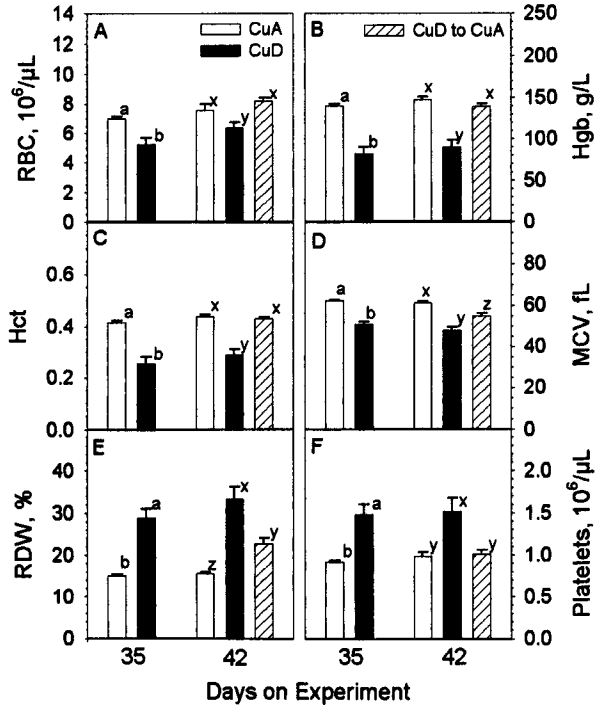

Figure 2. Signs of copper-deficient (CuD) anemia were reversed in rats that were re-fed copper (Cu). Bars represent the mean ± SEM for 6 to 8 replicates per group. Means with different superscripts within a specific day were significantly different ($P \leq 0.05$). CuA, Cu adequate; RBC, red blood cells; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; RDW, red cell distribution width.

less weight than CuA rats ($P < 0.05$; Fig. 1, panel A). However, when CuD rats were replenished with Cu for 14 days and killed on Day 42, their differences in gain from the CuA rats were no longer significant ($P > 0.05$). Serum SOD3 activity in CuD rats was also significantly reduced at both Day 35 and Day 42 compared with CuA rats ($P < 0.05$); however, SOD3 activity of CuD rats replenished with Cu for 14 days returned to normal (Fig. 1, panel B). Likewise, CpAO activity was reduced by Cu deficiency, but returned to normal on refeeding Cu (Fig. 1, panel C; note that these data are plotted on a $\log_{10}$ scale). Serum Cu concentrations followed those of SOD3 and CpAO activities (Fig. 1, panel D).

Rats fed the CuD diet also were Fe deficient as indicated by low serum Fe (Fig. 1, panel E) and a change in blood parameters (Fig. 2). In CuD rats, RBC number, Hgb, Hct, and MCV were all lower than in CuA rats (Fig. 2, panels A–D). However, when CuD rats were replenished with dietary Cu for 14 days, all of these parameters except MCV (Panel D) rebounded to normal levels. The RDW of CuD rats was elevated; however, it began returning to control values in Cu-repleted rats, but did not reach complete normality by Day 14 (Fig. 2, panel E). As seen in other studies (4), platelet count was elevated in CuD rats (Fig. 2, panel F). Replenishing these rats with dietary Cu reversed this effect.

As we observed in previous studies (7), the amount of whole-body ^{59}Fe retained from the labeled diet was significantly less in CuD rats than in CuA rats ($P < 0.05$; Fig. 3, panel A). However, when CuD rats were replenished with dietary Cu, the level of ^{59}Fe retention was significantly greater than normal ($P < 0.05$; crosshatched bar). Likewise, the relative amount of Cu-dependent Hp protein in the isolated duodenal enterocytes was significantly reduced in CuD rats compared with CuA rats ($P < 0.05$; Fig. 3, panel B). Again, when CuD rats were replenished with dietary Cu, the relative amount of Hp protein rebounded to a level three times higher than that in CuA rats ($P < 0.05$; panel B).

The distribution of absorbed ^{59}Fe in blood and various organs was affected by CuD diets and Cu repletion (Fig. 4). The amount of ^{59}Fe in whole blood of CuD rats, expressed as a percentage of whole-body ^{59}Fe, was about 50% of that in CuA rats (Fig. 4, panel A). However, during dietary Cu repletion, blood ^{59}Fe returned to normal (crosshatched bar). Liver ^{59}Fe was 4 to 5 times higher in CuD rats than in CuA rats (Fig. 4, panel B). During Cu repletion of CuD rats, liver ^{59}Fe quickly returned to values that were significantly below normal ($P < 0.05$). The distribution of ^{59}Fe to spleen was not affected by CuD (Panel C). At Day 35, Cu deficiency had no effect on kidney ^{59}Fe. At Day 42, kidney ^{59}Fe was

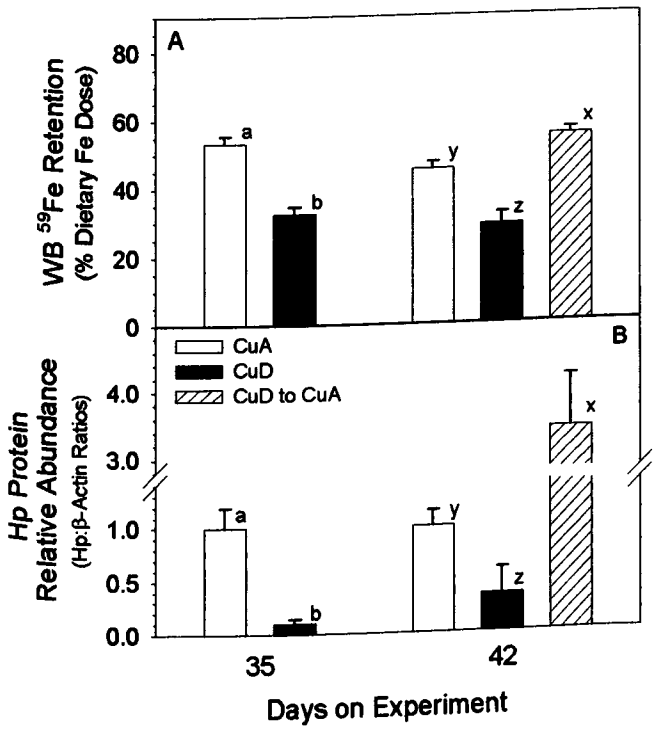

Figure 3. Whole-body (WB) retention of ^{59}Fe and the relative amount of duodenal enterocyte hephaestin (Hp) protein were reduced in copper (Cu) deficiency, but were restored when copper-deficient (CuD) rats were re-fed Cu. Bars represent the mean ± SEM for 6 to 8 replicates per group. Means with different superscripts within a specific day were significantly different ($P \leq 0.05$). CuA, Cu adequate.

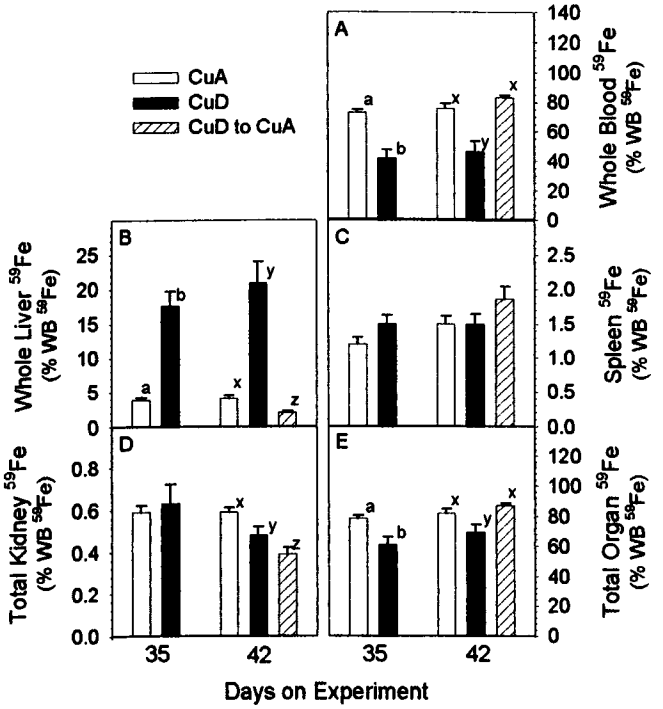

Figure 4. Whole-blood and organ distributions of absorbed ^{59}Fe were affected by copper (Cu) deficiency in rats. Bars represent the mean ± SEM for 6 to 8 replicates per group. Means with different superscripts within a specific day were significantly different ($P \leq 0.05$). CuA, Cu adequate; CuD, Cu deficient; WB, whole body.

lower in CuD rats than in CuA rats ($P < 0.05$; panel D). However, on repletion of dietary Cu, kidney ^{59}Fe moved even lower (crosshatched bar). Total blood and organ ^{59}Fe followed the same pattern as whole blood, but the differences between treatments were not as great (Fig. 4, panel E).

Discussion

Earlier studies on the metabolic interaction between Cu and Fe in swine and rats pointed to the possibility that Cu deficiency reduced Fe absorption and, thus, partially contributed to Cu-deficiency anemia (22–24). However, studies by Thomas and Oates (25) cast doubt on whether Cu deficiency reduced Fe absorption when they showed that CuD female rats actually absorbed more Fe than CuA rats. Even so, recent findings in our laboratory offer strong evidence that both male and female CuD rats absorb significantly less Fe than CuA rats (3, 7).

A probable mechanism for reduced Fe absorption in Cu deficiency is its effect on Hp protein and activity. It was found that the sex-linked anemic (i.e., *sla*) mouse (8) became Fe deficient because of inefficient Fe absorption caused by the absence of a functional Hp protein (9). We found a similar relationship in our studies where both duodenal enterocytes Hp protein and intestinal Fe absorption were significantly reduced in CuD rats (7). The main transporter of Fe out of the enterocyte is Fpn1, but a direct connection between Fpn1 and Hp has not been firmly established. However, in yeast, there is a link between the membrane Fe transporter Ftr1p and a ferroxidase Fet3p for the cellular uptake of Fe (26, 27). Kuo *et al.* (10) showed that both Hp and Fpn1 are located in the lateral and basolateral membranes of mouse enterocytes. In addition, Nittis and Gitlin (11) showed that Hp was located in the basolateral membrane of differentiated T84 colon carcinoma cells and that the induction of Cu deficiency in these cells resulted in a rapid loss of Hp protein. These studies established a close association of Fpn1 and Hp and suggested that the two proteins have similar arrangements in the enterocyte for Fe release as there is in yeast for Fe uptake. Of interest in this connection was the observation that Cu supplementation of cultured macrophages enhanced the expression of Fpn1, which was associated with an increased efflux of Fe (28). However, these investigators found no effect of Cu deficiency on Fpn1 expression in mice (29).

We entered the current study with the question of whether the reductions in Fe absorption and Hp enterocyte protein and the signs of anemia in CuD rats could be reversed by short-term repletion of dietary Cu. The results strongly suggest that they can. Rats fed a CuD diet absorbed less Fe than those fed a CuA diet, and the relative amount of duodenal enterocyte Hp protein was strongly associated with reduced Fe absorption. Within 1 week after replenishing the rats with Cu by feeding them CuA diets, Fe absorption rebounded to values higher than normal. Within

2 weeks after Cu repletion, Hp protein also was higher than normal. Although an overshoot such as this in response to a stimulus is common (30), the fact that it lasted for such an extended period was unexpected. Nonetheless, these data suggest a strong link between changes in the relative abundance of enterocyte Hp protein and Fe absorption. In a preliminary experiment where the dietary Cu concentration of CuD rats was raised from 0.25 mg/kg to 1.0 mg/kg and fed for 7 days, Fe absorption increased by 40% over the CuA rats (data not shown). This suggests that the Fe absorption mechanisms in the gut are very sensitive to small amounts of dietary Cu.

Ganzoni *et al.* (31) showed that the half-life of RBC in rats was between 14 and 18 days. In the current study, the period of Cu repletion was 14 days and, during this period, most parameters associated with Cu deficiency–induced anemia had recovered. These included RBC number, Hgb concentration, and Hct. However, two other parameters usually affected by Cu deficiency had not recovered completely during the repletion period. These parameters included MCV where the values were only partially restored, suggesting that a period exceeding 14 days would be required to establish full recovery of RBC size and shape. The other parameter not fully restored was RDW, which could be explained by the fact that RDW is a function of MCV. However, populations of both large and small cells may well exist together at Day 14, which could result in a larger RDW.

Copper-deficiency anemia could result solely from reduced Fe absorption; however, because the anemia is not cured in some animal models with parenteral administration of Fe (32), there must be other factors involved. Recently, Cherukuri *et al.* (33) observed that aceruloplasminemic mice had a 50% reduction in serum Fe, but they had only mild anemia compared with control mice. The authors proposed that the absence of Cp was inhibiting Fe binding to apo-transferrin and reducing transferrin saturation (33). Plasma transferrin is a major source of Fe for erythropoiesis. Because CuD rats have very low Cp activity, low serum Fe, and low transferrin saturation (34), this may be a part of the reason for anemia in these animals. However, Cu-deficiency anemia in rats can be rather severe, as shown in the current study. Also, it has been shown that the anemia of Cu deficiency in mice (35) and rats (36) can be reversed by parenteral administration of Fe. The Cu status of the rats in the latter study was elevated after Fe administration, suggesting that the parenteral solution was contaminated with Cu. However, Williams *et al.* (37) gave im injections of Fe and showed partial recovery of anemia in CuD rats without affecting Cu status.

Other factors affecting anemia in Cu deficiency might include the inefficiency of blood cell formation. Results of various studies suggest that Cu has an essential role in blood cell development and/or heme biosynthesis. Erythrocytes are smaller, and the maturation of neutrophils and granulocytes is impaired in Cu deficiency (4, 5, 38, 39). Williams *et al.* (40, 41) also showed that heme synthesis was reduced in liver mitochondria of both CuD pigs and CuD rats; however, these observations have not been made in erythrocyte precursors of bone marrow. On the other hand, Lee *et al.* (42) reported that Cu deficiency had no effect on heme synthesis in whole blood reticulocytes of swine and concluded that globin synthesis might be impaired.

Evans and Abraham (14) found that low hematocrit and hemoglobin in rats that were Cu deficient for 42 days were restored to near-normal levels within 14 days after dietary Cu repletion. These data are similar to those found in the current experiment. Similarly, Evans and Abraham (14) observed that plasma CpAO activity was restored to 80% of normal within two days after Cu repletion and to 100% of normal by 5 days after Cu repletion. Apparently, the release of Fe from the liver is dependent on the ferroxidase activity of Cp (43) and, in CuD rats, liver Fe is elevated. In the Evans and Abraham (14) study, liver Fe was reduced to normal within 5 days after Cu repletion, and it was below normal by Day 7. In the current study, serum CpAO activity was completely restored within 14 days of Cu repletion and ^{59}Fe in liver was reduced to below normal, even after the 14 days of repletion. We did not measure total liver Fe in this experiment, but it is likely to follow a similar pattern as the radioactive tracer. These data suggest that Cu deficiency–induced Fe loading of the liver is very much dependent on Cp activity, and when this activity is restored by repleting Cu stores, liver Fe concentrations are quickly reduced.

In summary, the main observations in this study were that reductions of duodenal Hp protein and Fe absorption in CuD rats were completely restored with Cu repletion. Signs of anemia induced by Cu deficiency and closely associated with Hgb production were also fully restored. These findings suggest that a strong association exists between duodenal Hp abundance and Fe absorption in the CuD rat and that reduced Fe absorption in these rats is an important factor in the cause of anemia.

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