Hepatic iron accumulation in copper-deficient rats

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1. Studies of anaemia and tissue iron distribution were carried out in copper-deficient rats and pair-fed control animals given Fe orally or parenterally in varying doses.

2. The anaemia of Cu deficiency was partially but incompletely corrected by oral Fe supplementation of one- to five-fold normal dietary levels or by intramuscular Fe supplementation.

3. Serum Fe increased in Cu-deficient animals as the dose of supplemental Fe was increased.

4. Hepatic Fe accumulation occurred in Cu-deficient rats which were administered with either oral Fe in two- to five-fold excess or low doses of intramuscular Fe. This difference was not seen in animals receiving high doses of intramuscular Fe, but similar relative differences were seen in Cu-deficient and Cu-replete rats which had been given no Fe supplementation.

5. Duodenal Fe was not increased in Cu deficiency. Bone marrow Fe was present in Cu-deficient animals receiving either parenteral or oral Fe supplementation.

6. Present studies suggest that a decrease in caeruloplasmin (*EC* 1.16.3.1) activity does not wholly explain the anaemia of Cu deficiency. Fe accumulation may be restricted to the liver, suggesting that Cu may be required for normal intracellular Fe metabolism.

Copper deficiency results in microcytic hypochromic anaemia in association with impaired iron utilization even when adequate Fe is provided by parenteral administration (Lee *et al*. 1976). Such findings have provided the basis for the concept that Cu is required for the normal movement of Fe. In swine, evidence has been presented that gastrointestinal mucosal Fe absorption, hepatic Fe release and reticuloendothelial Fe release are impaired and that all these factors contribute to the anaemia and abnormal Fe distribution (Lee *et al*. 1968). A unifying hypothesis has been sought to explain these abnormalities. The prevailing explanation holds that defective Fe release from these various sites results from reduced levels of the plasma cuproprotein, caeruloplasmin. This protein is a potent ferroxidase. Presumably, caeruloplasmin acts at cell surfaces to oxidize Fe so that ferric iron can be bound to the transport protein, transferrin (Roeser *et al*. 1970).

However, there is evidence which suggests that this hypothesis may not completely explain the abnormalities of Fe metabolism which are seen in Cu deficiency. This evidence includes the observations: (1) that there is a defect of Fe metabolism within the Cu-deficient reticulocyte (Williams *et al*. 1976) and (2) that Fe administered intraperitoneally can be identified in the bone marrow of Cu-deficient rats (Marston *et al*. 1971). Recent studies have shown that when Fe is supplied in two- to four-fold excess to Cu-deficient rats, anaemia may be prevented (Weisenberg *et al*. 1980). These studies are in contrast to numerous earlier publications which have shown that Fe replacement at conventional doses will only partially alleviate the anaemia of Cu deficiency.

The purpose of this report was to describe the influence of the route and dosage of Fe administration on the anaemia of Cu deficiency in rats and to determine the nature of tissue Fe distribution in these animals.

MATERIALS AND METHODS
Animals and diet

Male Sprague-Dawley rats were weaned at 21 d of age and paired, by weight, to within 0·5 g. Rats were placed in individual hanging stainless-steel cages and fed by matched-pair technique with a diet of unsupplemented condensed milk. This diet has previously been used in a series of studies extending back over 30 years (Chase *et al.* 1952; Williams *et al.* 1974, 1981). By assay, the diet was found to contain (mg/kg): Cu 0·12, Fe 0·44. Cu-replete animals received 1·0 mg Cu/d in the form of copper sulphate added to the diet. Animals were weighed weekly during the course of the experiments. In all experiments, weight gain of matched pairs was similar until the last 2 weeks of the experiment when Cu-deficient animals failed to keep up with matched pair-fed, Cu-replete animals. Growth curves were comparable to those reported by others (Mills & Murray, 1960). Control rats were fed *ad lib.* from weaning on a commercial diet (Ralston-Purina) which was found by analysis to contain (mg/kg): Cu 10·7, Fe 300.

Fe supplementation

In some experiments, rats received parenteral Fe as iron dextran (Imferon; Merrill, 50 mg Fe/ml) given intramuscularly (IM) in a daily dose of 5 mg Fe with a total dose of 0, 5, 10, 25 or 50 mg. In other experiments, rats received oral Fe supplementation in the form of ferrous ammonium sulphate added to the experimental diet in the final concentrations of 100, 200 and 500 mg Fe/kg. These levels approximated to one, two and five times the levels of Fe recommended in other synthetic diets (Mills & Murray, 1960).

Analytical methods

After 6 weeks on the experimental diet, animals were anaesthetized by carbon dioxide narcosis and killed by exsanguination from the inferior vena cava. Haemoglobin, packed cell volume and erythrocyte counts were determined by automated methods (Pinkerton *et al.* 1970). Bone marrow samples were obtained from the femoral shaft, stained for Fe by the Prussian blue reaction (Cartwright, 1968), examined for Fe by a single observer without knowledge of the sample source, and scored 0 to 4+ for the presence of reticuloendothelial Fe stores. Other tissues were harvested, rinsed in saline (9 g sodium chloride/l), blotted dry and weighed.

Serum Fe and Cu were measured by previously published methods and tissue Fe and Cu were measured by atomic absorption spectroscopy following acid digestion (Williams *et al.* 1976). Caeruloplasmin (*EC* 1.16.3.1) was measured by its capacity to oxidize *p*-phenylenediamine (*p*PD; Ravin, 1961).

RESULTS
Effect of Fe supplementation

In animals receiving no Fe supplementation, haemoglobin levels were decreased in both Cu-replete and Cu-deficient animals (Table 1). The mean level of haemoglobin was less in Cu-deficient than in the Cu-replete groups. When Fe supplementation was provided either orally or parenterally at levels estimated to be near basal requirement, haemoglobin levels of Cu-replete animals were similar to those of animals receiving the commercial diet. Haemoglobin levels of Cu-deficient animals remained significantly lower than those of controls given the commercial diet, but were greater than those of Cu-replete animals which received no Fe supplementation.

Microcytosis was a feature in both groups receiving no Fe supplementation. Erythrocyte size increased with either oral or parenteral Fe supplementation, but the cells remained

Table 1. *Effect of iron supplementation on haematologic values and tissue copper content of Cu-deficient rats*

(Mean values with their standard errors)

Fe source	Cu status	n	Body-wt (g)		Haemoglobin (g/100 ml)		Mean corpuscle volume (fl)		Serum Cu (μg/100 ml)		Serum caeruloplasmin*		Liver Cu (μg/g fresh wt)	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Commercial diet	–	6	332	15.4	16.8	0.20	56.5	0.29	110	3.7	0.550	0.0225	2.5	0.24
Experimental diet	Replete	12	138	9.8	7.1[C]	0.75	43.0[C]	0.40	161[C]	11.5	0.525	0.0173	11.9[C]	1.82
(no Fe supplement)	Deficient	12	114	6.0	5.1[Ca]	0.49	39.3[Ce]	0.35	23[C]	3.5	0.021[Ce]	0.0023	0.4[Ce]	0.06
Experimental diet	Replete	12	160	5.4	16.1	0.35	49.7[C]	1.21	150[B]	9.8	0.502	0.0309	15.6[C]	2.91
(IM Fe, 5 mg)	Deficient	12	117	6.7	11.8[Ccγ]	0.29	47.9[Cγ]	0.40	23[Cc]	2.0	0.026[Cc]	0.0040	1.0[Ccγ]	0.03
Experimental diet	Replete	8	162	5.6	17.3	0.35	54.9[A]	0.60	144[B]	8.5	0.547	0.0544	5.3[C]	1.17
(oral Fe, 100 mg/kg)	Deficient	8	118	5.1	12.3[Ccγ]	0.67	48.3[Ccγ]	0.71	15[Cc]	2.5	0.032[Ce]	0.0106	0.6[Ccα]	0.07

* Change in absorbance at 530 nm per 0.1 ml serum.

IM, intramuscular injection.

Value significantly different from that for animals given a commercial diet: [A] $P < 0.05$, [B] $P < 0.01$, [C] $P < 0.001$.

Differences between groups of matched paired animals were significant: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Value different from that for Cu-deficient animals receiving no Fe supplement: [α] $P < 0.05$, [β] $P < 0.01$, [γ] $P < 0.001$.

Table 2. *Effect of iron supplementation on tissue Fe content in copper-deficient rats*

(Mean values with their standard errors)

Fe source	Cu status	n	Serum Fe (μg/100 ml)		Bone marrow Fe score (0 to 4+)*		Tissue Fe (μg/g fresh wt)							
							Liver		Heart		Spleen		Kidney	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Commercial diet	Replete	6	276	12·7	1·0	0·24	30	2·4	42	3·7	80	6·1	29	3·3
Experimental diet (no Fe supplement)	Replete	12	84^C	8·4	0^C	—	10^C	0·6	24^C	1·2	46^C	5·5	11^C	0·9
	Deficient	12	81^C	13·6	0^C	—	21^{Ac}	2·3	26^C	1·2	64^a	6·6	17^{Bb}	1·7
Experimental diet (IM Fe, 5 kg)	Replete	12	351	34·4	2·5^A	0·64	19^A	3·5	42	4·0	138^C	13·3	25	3·5
	Deficient	12	151^{Ccγ}	10·4	1·4^γ	0·29	75^{Ccγ}	8·9	48^γ	4·0	214^{Cbγ}	19·6	28^γ	2·6
Experimental diet (oral Fe, 100 mg/kg)	Replete	8	410^B	29·7	2·9^B	0·42	93^C	14·1	53	6·4	207^{Cc}	9·9	59^B	8·8
	Deficient	8	173^{Ccγ}	12·0	1·6^{aγ}	0·39	173^{Cbγ}	13·8	58^γ	6·4	202^{Cc}	18·4	46^{Aγ}	6·7

* Score for the presence of reticuloendothelial Fe stores.

IM, intramuscular injection.

Value significantly different from animals given a commercial diet: ^{A} $P < 0.05$, ^{B} $P < 0.01$, ^{C} $P < 0.001$.

Difference between groups of matched paired animals were significant: ^{a} $P < 0.05$, ^{b} $P < 0.01$, ^{c} $P < 0.001$.

Value different from that for Cu-deficient animals receiving no Fe supplement: ^{α} $P < 0.05$, ^{β} $P < 0.01$, ^{γ} $P < 0.001$.

statistically significantly smaller than those of control animals, and the cells of Cu-deficient animals were smaller than those of their Cu-replete counterparts.

The serum Cu levels of all Cu-replete animals were significantly higher than those of animals given a commercial diet, while levels in Cu-deficient groups were decreased. However, serum caeruloplasmin of each of the Cu-replete groups was not appreciably different from control animals receiving commercial diets. In Cu-deficient animals caeruloplasmin activity, using pPD as substrate, was 4·0, 5·2 and 5·9% of respective values in matched counterparts in groups receiving no Fe supplementation, parenteral Fe supplementation and oral Fe supplementation. Liver Cu content reflected the status of Cu repletion.

Serum Fe was significantly decreased in all animals receiving no Fe supplement, regardless of Cu status (Table 2). In other experimental groups, the serum Fe was lower in Cu-deficient rats than in either Cu-replete counterparts or control animals given the commercial diet.

Bone marrow Fe was absent by histochemical analysis in rats receiving no Fe supplementation, but in other groups, regardless of the route of Fe supplementation, bone marrow Fe stores were greater in experimental animals than in control rats given a commercial diet. In animals receiving oral Fe supplementation, bone marrow Fe was significantly greater in Cu-replete than in Cu-deficient animals.

Liver Fe concentration was consistently greater in Cu-deficient than in Cu-replete counterparts, regardless of the route of Fe supplementation. Splenic iron was greater in Cu-deficient animals only when Fe was administered parenterally. Kidney Fe was greater in Cu-deficient animals when there was no Fe supplementation of the diet.

Effect of varying dose of oral Fe

In these experiments (Table 3), the experimental diet was supplemented with Fe at two or five times (200 and 500 mg/kg) the level in the previous study (100 mg/kg). In both treatment groups, haemoglobin levels of Cu-replete animals were similar to those observed previously in rats given a commercial diet, but haemoglobin levels in Cu-deficient animals were significantly decreased. At both dosages of Fe the level of haemoglobin in both Cu-deficient and Cu-replete rats was higher than had been observed in animals receiving no Fe supplement.

At both dosages of Fe, serum Cu levels were decreased in Cu-deficient animals and serum Fe was significantly lower in Cu-deficient groups. Serum Fe in Cu-deficient animals increased with increasing Fe dose so that at an oral Fe dosage level of 500 mg/kg Fe, serum Fe in Cu-deficient animals was 79% of that observed in rats given a commercial diet. Even at that level of serum Fe, haemoglobin levels of Cu-deficient animals were significantly less than in matched groups.

Liver iron was significantly increased in Cu-deficient rats compared with their matched controls at both levels of Fe supplementation while spleen and kidney Fe were significantly decreased. No difference was observed in cardiac Fe.

Effect of varying dose of parenteral Fe

In these studies (Table 4), both Cu-deficient and Cu-replete rats were given graduated doses of iron dextran by intramuscular injection. Increasing Fe supplementation again resulted in incomplete correction of anaemia in Cu-deficient animals. Indeed, as the dosage of Fe was increased, increased mortality was observed, and there was a progressive decline of haemoglobin levels in Cu-replete animals. Serum Cu levels were significantly decreased in Cu-deficient animals of each study group but, as serum Fe increased with increased Fe dosages, anaemia remained uncorrected in the Cu-deficient rats. Liver Fe was significantly

Table 3. *Effect of varying oral iron supplementation on haemoglobin and tissue Fe in copper-deficient rats*

(Mean values with their standard errors)

Oral Fe supplement (mg/kg)	Cu status	n	Body-wt (g)		Haemoglobin (g/100 ml)		Serum Cu (μg/100 ml)		Serum Fe (μg/100 ml)		Tissue Fe content (μg/g fresh wt)									
											Liver		Heart		Duodenum		Spleen		Kidney	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
200	Replete	8	196	6·3	16·7	0·31	117	12·7	314	23·7	79	4·2	48	3·5	32	3·9	191	23·0	42	4·2
	Deficient	8	158	10·7	12·9^c	0·53	14^c	2·1	175^c	23·3	174^c	19·4	40	2·1	17^b	0·7	112^b	10·3	25^b	2·1
500	Replete	7	195	9·0	17·0	0·23	99	7·6	457	55·9	102	8·3	42	4·5	65	10·2	212	23·0	52	6·4
	Deficient	7	191	13·1	12·1^c	0·83	22^c	3·0	218^b	15·9	169^a	20·4	35	2·6	25^b	2·6	99^c	7·6	25^b	0·8

Differences between groups of matched paired animals were significant: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Table 4. *Effect of varying intramuscular iron supplementation on haemoglobin and tissue Fe in copper-deficient rats*

(Mean values with their standard errors)

Intramuscular Fe supplement (mg)	Cu status	n	Body-wt (g)		Haemoglobin (g/100 ml)		Serum Cu (μg/100 ml)		Serum Fe (μg/100 ml)		Tissue Fe content (μg/g fresh wt)							
											Liver		Heart		Spleen		Kidney	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
10	Replete	12	161	6·8	17·1	0·06	138	2·3	184	3·2	293	5·2	ND	—	ND	—	ND	—
	Deficient	12	121	6·2	10·1^c	0·20	12^c	0·6	52^c	1·7	491^c	4·3	ND	—	ND	—	ND	—
25	Replete	6	156	5·8	15·4	0·45	188	4·5	462	22·0	1560	204·9	213	14·3	2180	498·1	237	39·2
	Deficient	6	133	18·0	9·7^c	1·02	17^c	1·6	144^c	12·2	2060	326·6	218	30·2	1930	416·4	215	26·5
50	Replete	4	149	21·6	13·4	0·65	177	14·5	740	26·5	1800	285·0	288	46·0	1510	375·0	235	31·0
	Deficient	4	140	6·7	10·1	1·95	8^c	1·5	294^c	4·0	2400	335·0	227	27·5	3150^a	430·0	197	20·0

Differences between groups of matched paired animals were significant: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.
ND, not determined.

increased in Cu-deficient animals only at a level of Fe supplementation of 10 mg Fe. At higher doses of Fe, mean liver Fe was higher in Cu-deficient animals, but considerable variability was observed so that no significant difference was observed between groups of animals. Unfortunately, Fe concentrations of other organs were not measured in animals receiving 10 mg Fe, but splenic Fe was significantly increased in Cu-deficient animals receiving 50 mg Fe.

DISCUSSION

The results of this study show that under the reported experimental conditions, anaemia persisted in Cu-deficient rats regardless of the Fe dose or route of administration. Thus, even when oral Fe intake was increased five-fold, the haemoglobin level in Cu-deficient animals was less than that of Cu-replete animals but greater than that of Fe-deficient, Cu-deficient animals. These observations are at odds with recent reports that oral Fe given in two- to four-fold excess to Cu-deficient rats results in the alleviation of anaemia (Weisenberg *et al.* 1980). The source of this discrepancy is unclear, but Fe doses used in our study were likely to be as great as those in the report by Weisenberg *et al.* (1980). Direct comparisons are difficult because, in that report, animals received a solid diet containing 40–80 mg Fe/kg supplemented with drinking water containing 20 mg/l. The techniques of metal determinations are also different, but the relative extent of Cu deficiency and, in some experiments, Fe deficiency appear to be similar.

Our findings are similar to numerous earlier reports which showed that Fe supplementation incompletely corrected the anaemia of Cu deficiency (Smith & Medlicott, 1944; Roeser *et al.* 1970; Lee *et al.* 1968, 1976). We also observed that Fe accumulated in the livers of all Cu-deficient animals, regardless of dosage or route of Fe supplementation. Such an increase was not observed by Weisenberg *et al.* (1980), but similar elevations have been seen in Cu-deficient rats (Evans & Abraham, 1973; Owen, 1973) and swine (Lee *et al.* 1968, 1976; Roeser *et al.* 1970) receiving Fe orally or parenterally. It has been proposed that this hepatic Fe accumulation is due to deficiency of caeruloplasmin which catalyzes the release of Fe from the isolated, perfused liver (Osaki & Johnson, 1969). Recent studies have suggested that under conditions which simulate physiologic oxygen tension, caeruloplasmin may not be required for hepatic Fe release (Baker *et al.* 1980). Other blood-borne substances have also been shown to possess ferroxidase activity (Lee *et al.* 1969; Topham *et al.* 1980; Prohaska, 1981) raising still further objections to the caeruloplasmin ferroxidase hypothesis. The absence of anaemia or evidence of impaired Fe metabolism in Wilson's disease (O'Reilly *et al.* 1968) or Menkes' syndrome (Danks *et al.* 1972) pose additional questions about the role of Cu and of caeruloplasmin in Fe metabolism.

In the studies which we report, the pattern of Fe accumulation in other Fe storage organs does not parallel that seen in the liver. The spleen and bone marrow are major sources of Fe for erythroid haem synthesis (Jacobs & Worwood, 1978). However, in Cu-deficient animals receiving oral Fe supplementation, splenic and marrow Fe are both less than in Cu-replete animals while liver Fe is greater. If Fe metabolism in these tissues were dependent upon caeruloplasmin, it would be anticipated that the Fe content in these tissues, like the liver, would be increased. If, on the other hand, the reduced Fe content would be attributed to decreased mucosal absorption of Fe, then Fe accumulation within the liver should also be decreased. Our studies do not permit a direct comment on the nature of Fe absorption in Cu-deficient rats. We observed that duodenal Fe content of Cu-deficient animals was less than that of Cu-replete controls, but Lee *et al.* (1968) have shown that Fe accumulates in the mucosa and submucosa of Cu-deficient swine. We did not attempt to identify such microscopic mucosal and submucosal Fe accumulation.

Previous studies from our laboratory have shown that intracellular Fe metabolism and

haem synthesis is impaired in Cu-deficient reticulocytes (Williams *et al.* 1976) and that haem catabolism may be accelerated in Cu-deficient hepatocytes (Williams *et al.* 1981). Either mechanism may contribute to hepatic Fe accumulation without invoking the caeruloplasmin ferroxidase hypothesis. Further investigation of hepatic Fe accumulation may provide greater insight into the defect of Fe metabolism associated with Cu deficiency.

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