

## Increasing intakes of iron reduce status, absorption and biliary excretion of copper in rats

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(Received 17 May 1993 – Revised 25 August 1993 – Accepted 23 September 1993)

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High intakes of Fe may impair Cu status, but the underlying mechanism is not known. Male rats, aged 7 weeks, were given purified diets adequate in Cu (8 mg Cu/kg) and containing either 7, 40 or 389 mg Fe/kg. After 6 weeks the concentrations of Fe in liver and spleen were positively related with dietary Fe level and those of Cu were negatively related with dietary Fe level. Increasing Fe intakes reduced apparent absorption and biliary excretion of Cu in a dose-dependent fashion. In individual rats, biliary Cu excretion showed a significant, positive correlation with liver Cu concentration. It is concluded that increased Fe intakes depress Cu absorption which produces a decrease in plasma and organ Cu concentrations. As a result, biliary Cu excretion is lowered which contributes to achieving Cu balance at high Fe intakes. Because the concentrations of Cu in plasma and bile, and also plasma ceruloplasmin (EC 1.16.3.1) activities, showed much greater percentage reductions with increasing Fe intake than did the concentrations of Cu in organs, it is possible that increased Fe status interferes with the mobilization of Cu stores.

**Iron: Copper: Absorption: Biliary excretion**

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Iron-deficiency anaemia is a global nutritional problem in children and pregnant women. One measure to fight this condition is Fe fortification of foods. In livestock production the feeding of supplemental Fe is common practice. The benefit of Fe supplements in preventing anaemia is well recognized, but the potential negative effects, if any, have been less well studied.

High intakes of Fe have been shown adversely to affect Cu status in ruminants (Standish *et al.* 1969; Humphries *et al.* 1983), guinea-pigs (Smith & Bidlack, 1980) and rats (Bremner & Young, 1981; Bremner *et al.* 1982; Bremner & Price, 1985; Johnson & Hove, 1986). It is not yet clear how extra dietary Fe alters plasma and tissue Cu concentrations in animals. Bremner & Young (1981) suggested that intake of excess Fe stimulates excretion of stored Cu. If Cu balance is attained after Fe feeding, the suggestion of Bremner & Young (1981) implies that increased intake of dietary Fe, at least as a secondary effect, would enhance Cu absorption. In feeding trials with rats given Cu-adequate diets for up to 20 d, dietary Fe concentrations ranging from about 35 to 500 mg/kg have been shown not to affect apparent Cu absorption (Johnson & Hove, 1986; Johnson & Murphy, 1988; Reichlmayer-Lais & Kirchgessner, 1992).

Cu balance in rats is determined essentially by the efficiency of Cu absorption and by the faecal loss of endogenous Cu, essentially representing the unabsorbed fraction of Cu excreted with bile (Van den Berg & Beynen, 1992). We hypothesized that the impaired Cu

\* For reprints.

status seen after Fe loading is due either to diminished Cu absorption followed by a decrease in biliary Cu excretion or to enhanced biliary Cu excretion with an increase in Cu absorption as a secondary feature. The two possibilities were examined in a 6-week trial using rats given Cu-adequate diets containing either low, normal or high amounts of Fe.

#### MATERIALS AND METHODS

The protocol of the experiment was approved and its conduct supervised by the Animal Welfare Officer of the Wageningen Agricultural University.

##### *Animals and diets*

Male Wistar rats (Hsd/Cpb:WU) aged about 7 weeks were used. On arrival they were housed in groups of five in stainless steel cages (600 × 210 × 190 mm) with wire mesh bases and given the purified diet containing 40 mg Fe/kg (Table 1) and demineralized water *ad lib.* for 10 d. Then (day 0 of the experiment) the rats were divided into three groups of fifteen rats each, stratified for body weight and blood haemoglobin concentration. The groups were randomly allocated to one of the experimental diets, each of which contained an adequate amount of Cu (8 mg/kg). One group remained on the diet containing 40 mg Fe/kg, and the other groups were transferred to the diets containing either 7 or 389 mg Fe/kg. Table 1 shows the ingredient composition of the diets which only differed with regard to Fe concentration. Fe was added to the diets in the form of FeSO<sub>4</sub> · 7H<sub>2</sub>O. The diet with 40 mg Fe/kg was formulated according to the recommended nutrient requirements of rats (National Research Council, 1978). The diets were stored at 4° until feeding. The rats had free access to the experimental diets and demineralized water. As from day 0 of the experiment the rats were housed individually in stainless steel cages (240 × 170 × 170 mm) in a room with controlled lighting (light on: 06.00–18.00 hours), temperature (19–21°) and relative humidity (50–60%). Feed intake and body weight were recorded.

##### *Collection of samples*

Blood samples were taken at weeks 0, 2, 4 and 6. For the first three time points the rats, while in the fed state, were subjected to orbital puncture while they were under light diethyl-ether anaesthesia, and blood samples were collected in heparinized tubes. Faeces were collected quantitatively during the last 4 d of the experiment.

At the end of the experiment in week 6, bile was collected by common bile duct cannulation with polyethylene tubing (0.28 mm i.d., 0.61 mm o.d.; Intramedic; Clay Adams, Parsippany, NJ, USA). The abdomen was opened while the rats were under anaesthesia induced by a combination of ketamine (60 mg/kg body weight) administered intramuscularly and xylazine (8 mg/kg body weight) administered subcutaneously. This combination of the two drugs was used since it has been shown not to influence bile flow in rats (Fleck & Barth, 1990). After the cannula was inserted into the common bile duct and secured with suture thread, the rats were kept on a heating pad (36–38°). Bile was collected into pre-weighed vials for three consecutive periods of 15, 30 and 30 min, and the volume of bile was calculated from the weight and specific gravity of bile. Bile samples were stored at –20° until analysis.

Following bile collection, blood samples were taken from the anaesthetized rats by abdominal aorta puncture. The rats were then killed by decapitation and liver, spleen, kidneys, heart and left tibia were removed, weighed and stored at –20° until analysis.

##### *Analytical methods*

Haemoglobin concentration and packed cell volume of fresh, heparinized blood samples were measured by using the Sysmex K-1000D (Sysmex-TOA; TOA Medical Electronics

Table 1. *Composition of the experimental diets*

Diet...	Low-Fe	Normal-Fe	High-Fe
Ingredients			
Constant components* (g)	290.6	290.6	290.6
Glucose (g)	709.4	709.2	707.7
FeSO <sub>4</sub> ·7H <sub>2</sub> O (mg)	0	174	1740
Chemical analysis			
Fe (mg)	6.8	40.2	388.8
Cu (mg)	8.1	8.1	8.1

\* The constant components consisted of (g/kg diet): casein 151, maize oil 25, coconut oil 25, cellulose 30, CaCO<sub>3</sub> 12.4, NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O 15.1, MgCO<sub>3</sub> 1.4, KCl 1.0, KHCO<sub>3</sub> 7.7, mineral premix 10, and vitamin premix 12. The Fe-free mineral premix consisted of (mg/kg feed): MnO<sub>2</sub> 79, ZnSO<sub>4</sub>·H<sub>2</sub>O 33, NiSO<sub>4</sub>·6H<sub>2</sub>O 13, NaF 2, KI 0.2, CuSO<sub>4</sub>·5H<sub>2</sub>O 15.7, Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O 0.3, CrCl<sub>3</sub>·6H<sub>2</sub>O 1.5, SnCl<sub>2</sub>·2H<sub>2</sub>O 1.9, NH<sub>4</sub>VO<sub>3</sub> 0.2 and maize meal 9853.2. The vitamin premix consisted of (mg/kg feed): thiamin 4, riboflavin 3, nicotinamide 20, DL-calcium pantothenate 17.8, pyridoxine 6, cyanocobalamin 50, choline chloride 2000, pteroylmonoglutamic acid 1, biotin 2, menadione 0.05, DL- $\alpha$ -tocopheryl acetate 60, retinyl acetate and retinyl palmitate 8 (1200 retinol equivalents), cholecalciferol 0.025, maize meal 9828.125.

Co. Ltd, Kobe, Japan). The concentration of Fe and Cu in organs, faeces and feed was determined by flame atomic absorption spectrometry (Varian AA-475; Varian Techtron, Springvale, Australia). For the determination of Cu and Fe in organs and feed, samples were dried in a vacuum dryer for 48 h and digested in 1.0 ml 14 M-HNO<sub>3</sub> (Suprapur; Merck, Darmstadt, Germany) at 80° for 2 h. Samples of faeces were first dried, ashed at 500° for 17 h in a muffle furnace and then dissolved in 6 M-HCl. Cu in plasma was measured directly.

Fe and total Fe-binding capacity in plasma were determined using a commercial reagent kit (Iron FZ Test, Roche; Roche Diagnostics, Basel, Switzerland) and a Cobas-Bio autoanalyser (Hoffmann-La Roche BV, Mijdrecht, The Netherlands). The determination of Cu and Fe in bile was carried out using flameless atomic absorption spectrometry (Varian AA-300) after dilution of the samples with demineralized water. An external control in the form of a bovine liver sample (NBS 1577a; National Institute of Standards Technology, Gaithersburg, MD, USA) was used to assess bias of Fe and Cu analysis. Analysed Fe and Cu concentrations were 105.3 (SE 4.03)% (*n* 6) and 98.7 (SE 4.61)% (*n* 6) of the target values respectively. Ceruloplasmin (EC 1.16.3.1) in plasma was assayed as *p*-phenylenediamine oxidase (EC 1.16.3.1) activity as described by Sunderman & Nomoto (1970).

#### Statistical analyses

The data were subjected to one-way analysis of variance and a multiple comparison test (Tukey test). The data for bile flow and biliary Fe and Cu concentrations were analysed using two-way analysis of variance. The level of significance was pre-set at *P* < 0.05. All data were analysed using a computer program (SPSS Inc., 1988).

## RESULTS

### Feed intake, body and organ weights

The concentration of Fe in the diet had no significant effect on feed intake or body weight of the rats (Table 2). Likewise, there were no group differences in the weights of spleen and heart. Liver and kidney weights in the low-Fe group were significantly lower than those in

Table 2. Feed intake and body and organ weights of rats fed on experimental diets containing low, normal or high amounts of iron\*

(Mean values for fifteen rats per dietary group)

Diet...	Low-Fe	Normal-Fe	High-Fe	Pooled se
Body weight (g)				
Initial	192.4	191.4	192.7	3.96
Final	353.9	360.1	352.8	6.43
Feed intake (g/d)				
Week 1	20.5	21.6	21.5	0.40
Week 6	19.9	20.9	20.7	0.34
Organ wt (g/kg body wt)				
Liver	34.5 <sup>a</sup>	37.2 <sup>b</sup>	37.8 <sup>b</sup>	0.57
Spleen	2.3	2.1	2.2	0.08
Kidney	5.8 <sup>a</sup>	6.0 <sup>ab</sup>	6.3 <sup>b</sup>	0.15
Heart	3.4	3.3	3.4	0.09

<sup>a, b</sup> Means in the same row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* For details of diets and procedures, see Table 1 and pp. 888-889.

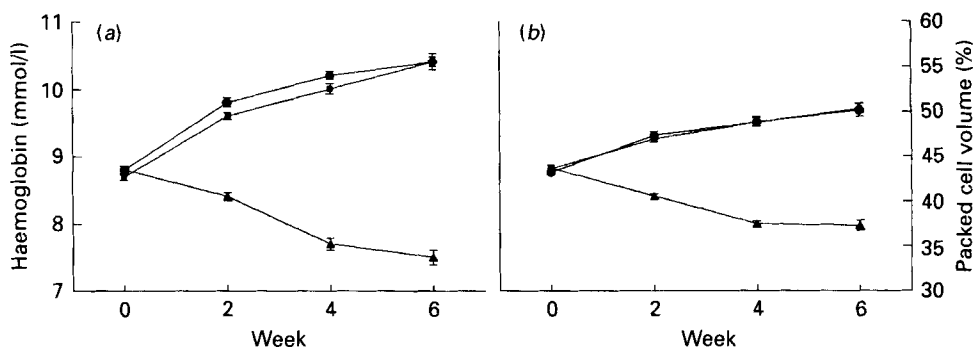


Fig. 1. Time course of blood haemoglobin concentrations (a) and packed cell volume (b) in rats fed on either a low- (▲), normal- (■) or high- (●) Fe diet. There were significant effects of diet ( $P < 0.05$ ) for both haemoglobin (pooled SE 0.022 mmol/l) and packed cell volume (pooled SE 0.767%). For details of diets and procedures, see Table 1 and pp. 888-889.

the high-Fe group. Liver weight of the low-Fe group was also lower than that of the normal-Fe group.

#### Indicators of iron status

Fig. 1 shows that blood haemoglobin concentrations and packed cell volume decreased in the rats given the low-Fe diet, whereas in the other two groups there was a similar rise with time. After 6 weeks, Fe concentrations in all organs and plasma were significantly lower in rats fed on the low-Fe diet compared with those fed on the normal-Fe diet (Table 3). Rats given the high-Fe instead of the normal-Fe diet displayed significantly higher values except for kidney, heart and plasma. Total Fe-binding capacity was similar in rats fed on the normal and high-Fe diets (1.6 and 1.5 mmol/l), but was significantly raised ( $P < 0.05$ ) in their counterparts given the low-Fe diet (2.1 mmol/l, pooled SE 0.03 mmol/l).

Table 3. *Organ and plasma iron concentrations in rats fed on experimental diets containing low, normal or high amounts of iron\**

(Mean values for fifteen rats per dietary group)

Diet ...	Low-Fe	Normal-Fe	High-Fe	Pooled SE
Organ Fe (mmol/kg)†				
Liver	3.3 <sup>a</sup>	7.6 <sup>b</sup>	9.8 <sup>c</sup>	0.32
Spleen	19.1 <sup>a</sup>	46.3 <sup>b</sup>	55.9 <sup>c</sup>	1.72
Kidney	3.4 <sup>a</sup>	5.2 <sup>b</sup>	5.4 <sup>b</sup>	0.17
Heart	4.8 <sup>a</sup>	6.3 <sup>b</sup>	6.4 <sup>b</sup>	0.15
Tibia	0.75 <sup>a</sup>	1.57 <sup>b</sup>	1.77 <sup>c</sup>	0.052
Plasma Fe (mmol/l)	0.19 <sup>a</sup>	0.55 <sup>b</sup>	0.56 <sup>b</sup>	0.019

<sup>a, b, c</sup> Means in the same row with unlike superscript letters were significantly different ( $P < 0.05$ ).

† On a dry-weight basis.

\* For details of diets and procedures, see Table 1 and pp. 888–889.

Table 4. *Organ and plasma copper concentrations and plasma ceruloplasmin (EC 1.16.3.1) concentrations in rats fed on experimental diets containing low, normal or high concentrations of iron\**

(Mean values for fifteen rats per dietary group)

Diet ...	Low-Fe	Normal-Fe	High-Fe	Pooled SE
Organ Cu (mmol/kg)†				
Liver	0.28 <sup>a</sup>	0.26 <sup>b</sup>	0.23 <sup>b</sup>	0.008
Spleen	0.17 <sup>a</sup>	0.15 <sup>ab</sup>	0.12 <sup>b</sup>	0.011
Kidney	0.40 <sup>a</sup>	0.43 <sup>a</sup>	0.31 <sup>b</sup>	0.014
Heart	0.42 <sup>a</sup>	0.41 <sup>a</sup>	0.39 <sup>b</sup>	0.058
Tibia	0.08	0.08	0.08	0.046
Plasma				
Cu ( $\mu$ mol/l)	23.6 <sup>a</sup>	22.0 <sup>a</sup>	15.7 <sup>b</sup>	1.10
Ceruloplasmin ( $\Delta$ absorption/l per min)	144.1 <sup>a</sup>	159.9 <sup>a</sup>	85.9 <sup>b</sup>	10.05

<sup>a, b</sup> Means in the same row with unlike superscript letters were significantly different ( $P < 0.05$ ).

† On a dry-weight basis.

\* For details of diets and procedures, see Table 1 and pp. 888–889.

*Indicators of copper status*

The concentration of Cu in kidneys, heart and plasma as well as the ceruloplasmin activity was lower in the high-Fe group compared with the normal-Fe group while the Cu concentrations in liver were raised significantly in the low-Fe group (Table 4).

*Apparent copper absorption*

Increasing dietary Fe concentrations significantly reduced apparent Cu absorption (Fig. 2).

*Biliary iron and copper excretion*

Bile flow in the cannulated rats decreased with time, but there was no effect of dietary Fe concentration (Table 5). The concentration of Fe in bile was slightly, but significantly, reduced in rats fed on the low-Fe diet. With time there was an increase in biliary Fe concentration. The concentration of Cu was not determined in the initial 15 min sample of bile collected because the quantity of fluid obtained was insufficient. For the other two

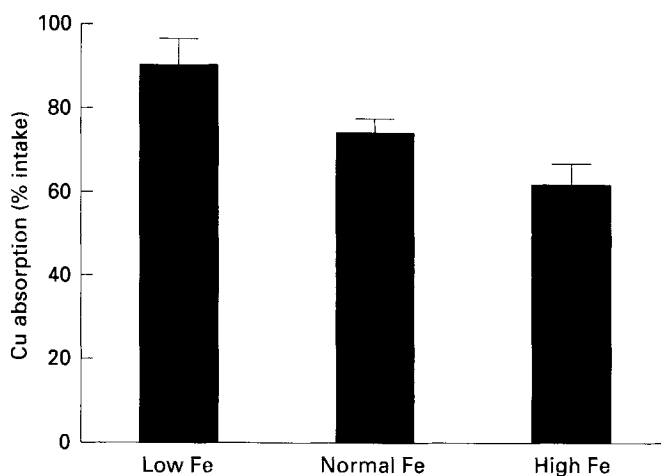


Fig. 2. Influence of experimental diets containing low, normal or high amounts of Fe on apparent Cu absorption in rats. Results are expressed as means and SE ( $n$  15) and were significantly different ( $P < 0.05$ ) between dietary groups. For details of diets and procedures, see Table 1 and pp. 888–889.

Table 5. *Bile flow and biliary iron and copper concentrations in rats fed on experimental diets containing low, normal or high amounts of iron\**  
(Mean values for fifteen rats per dietary group)

Diet...	Low-Fe	Normal-Fe	High-Fe	Pooled SE	Statistical significance of effects of:†
<b>Bile flow</b>					
(ml/kg body wt per h)					
0–15 min	2.7	3.0	2.9		
15–45 min	2.6	2.7	2.6		
45–75 min	2.6	2.5	2.4	0.14	P
<b>Biliary Fe concentration</b>					
( $\mu\text{mol/l}$ )					
0–15 min	22	25	24		
15–45 min	25	29	30		
45–75 min	28	34	34	1.9	Fe; P
<b>Biliary Cu concentration</b>					
( $\mu\text{mol/l}$ )					
15–45 min	19.4 <sup>a</sup>	12.3 <sup>b</sup>	1.9 <sup>c</sup>		
45–75 min	19.5 <sup>a</sup>	13.1 <sup>b</sup>	1.6 <sup>c</sup>	1.45	Fe

<sup>a, b, c</sup> Means in the same row with unlike superscript letters were significantly different ( $P < 0.05$ ).

† Two-way analysis of variance with level of dietary iron (Fe) and period of bile collection (P) as main effects; significant effects ( $P < 0.05$ ).

\* For details of diets and procedures, see Table 1 and pp. 888–889.

periods there was a significant decrease in biliary Cu concentration with increasing Fe intake (Table 5).

Fig. 3 illustrates the absolute amounts of Fe and Cu excreted in bile by the rats fed on the diets differing in Fe concentration. Fe excretion was lower in rats given the low-Fe diet than in rats given either the normal or high-Fe diet. Increased concentrations of Fe in the diet were associated with a marked drop in biliary Cu excretion.

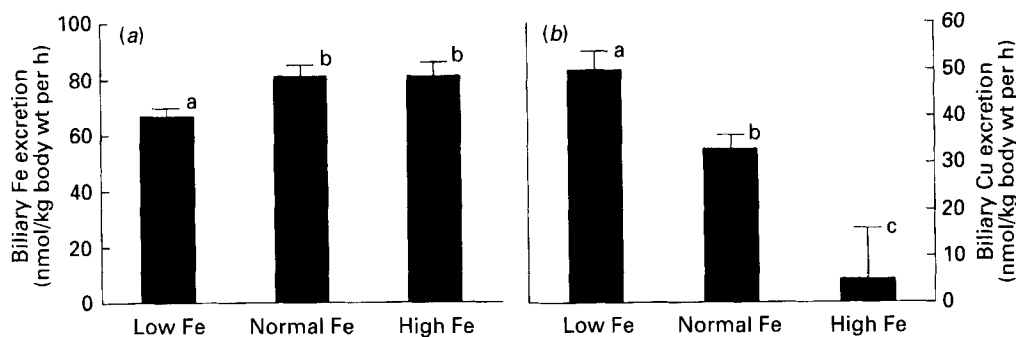


Fig. 3. Biliary excretion of Fe (a) and Cu (b) by rats fed on experimental diets containing low, normal or high amounts of Fe for a period of 6 weeks. Bile was collected quantitatively from anaesthetized, cannulated rats for a period of 75 min immediately after cannulation. Fe and Cu excretions refer to the 15–75 min collection period and are expressed as nmol/kg body weight per h. Results are expressed as means and SE ( $n$  15); bars within a panel not sharing a common letter were significantly different ( $P < 0.05$ ). For details of diets and procedures see Table 1 and pp. 888–889.

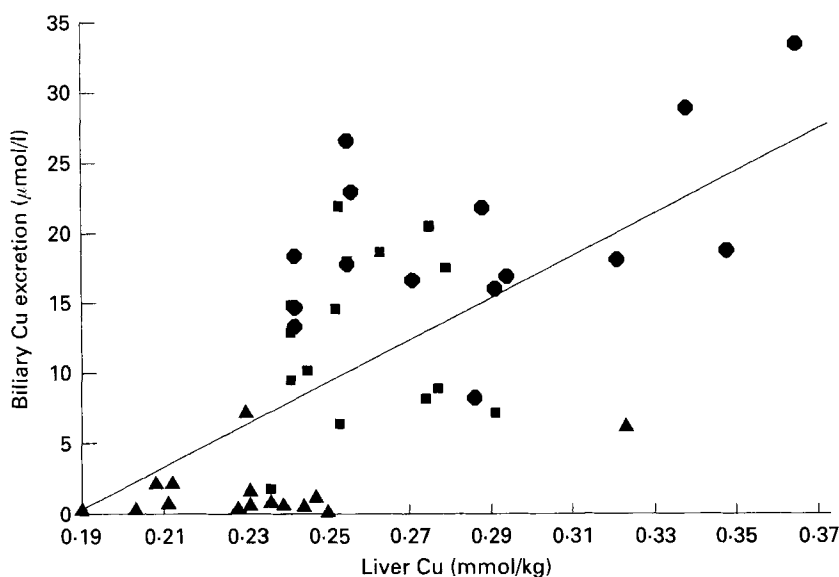


Fig. 4. Relationship between biliary Cu excretion and hepatic Cu concentration in individual rats fed on a low- (▲), normal- (■) or high- (●) Fe diet. The regression equation is  $y = 150x - 28$  ( $r$  0.63,  $n$  45,  $P < 0.05$ ).

For individual rats there was a significant, positive relationship between the concentration of Cu in liver and that in bile (Fig. 4).

#### DISCUSSION

The experimental diets containing different amounts of Fe predictably affected the selected indicators of Fe status without differently influencing feed intake or body-weight gain. Thus, the low-Fe diet which contained about one fifth of the recommended dietary Fe concentration for rats (35 mg Fe/kg diet; National Research Council, 1978) lowered blood haemoglobin concentration and packed cell volume and also plasma and organ Fe concentrations. This agrees with earlier work (Sørensen, 1965; Brouwer *et al.* 1993). On the

other hand, Fe loading with a dietary concentration more than ten times the recommended concentration did not alter haemoglobin and packed cell volume, but raised Fe concentrations in liver and spleen. These findings also support previous studies (Dhur *et al.* 1989; Kreuzer & Kirchgessner, 1991).

Modulation of Fe excretion in bile did not appear to be a compensatory mechanism for dealing with Fe loading. Despite the modest rise in liver Fe concentration, the high-Fe diet did not raise biliary Fe excretion, supporting the view that Fe homeostasis essentially depends on regulating Fe absorption (McCance & Widdowson, 1937). However, the low-Fe diet induced lower rates of biliary Fe excretion than did the other two diets (Fig. 3(a)). This may relate to the decreased hepatic Fe concentrations seen in the rats given the low-Fe diet. In any event, our results indicate that decreasing biliary Fe excretion contributes to maintenance of Fe balance after consumption of low amounts of Fe.

The major objective of the present study was to determine the influence of dietary Fe concentration on Cu metabolism. It is clear that increasing intakes of Fe caused impairment of Cu status as based on the lowering of Cu concentrations in plasma and organs. The important finding here is that the antagonistic effect of dietary Fe on Cu status can now be explained by the decrease in apparent Cu absorption. There was an inverse relationship between Fe intake and Cu absorption (Fig. 2). Other investigators were not able to demonstrate such an effect in rats (Bremner & Young, 1981; Johnson & Hove, 1986). The reason for this discrepancy is not known.

Theoretically, the observed Fe-induced decrease in Cu absorption must be associated with a decrease in Cu excretion so that Cu balance can be attained. Indeed, biliary excretion of Cu was found to be depressed with higher intakes of Fe (Fig. 3(b)). The rats given the low-Fe diet had an apparent absorption of about  $2.28 \mu\text{mol Cu/d}$  ( $0.90 \times$  intake), and for the rats given the high-Fe diet apparent Cu absorption equalled  $1.64 \mu\text{mol/d}$  ( $0.62 \times$  intake). Based on the data in Fig. 3(b), biliary Cu excretion in rats given the low-Fe diet was in the order of  $0.43 \mu\text{mol/d}$ , and in rats given the high-Fe diet it was  $0.04 \mu\text{mol/d}$ . Thus these calculations, which should be interpreted cautiously, suggest that the Fe-induced reduction in biliary Cu excretion may not compensate fully for the decrease in Cu absorption. This notion is reinforced by the fact that true Cu absorption will be greater than the calculated values for apparent Cu absorption. In addition, part of the Cu excreted with bile will be re-absorbed so that the net loss of Cu with bile may be less than that calculated. Excretion of Cu in bile is probably regulated by Cu concentration in liver. At least for rats given the normal or high-Fe diet, the output of Cu with bile was directly related to liver Cu concentration (Fig. 4).

Liver Cu concentrations were only marginally reduced with increasing Fe intake while plasma and biliary Cu concentrations, and also plasma ceruloplasmin activities, showed much greater percentage reductions. This could be interpreted to mean that dietary Fe interferes with Cu metabolism not only at the absorptive but also at the post-absorptive level. Perhaps an increased Fe status affects the mobilization of Cu stores in the liver resulting in depressed incorporation of Cu into ceruloplasmin and bile fluid.

In summary, increasing Fe intakes impaired Cu status in rats. This was probably caused by inhibition of Cu absorption followed by a decrease in biliary Cu excretion, but the initial effect of increased Fe intake on Cu absorption is not known. In addition, an increased Fe status could interfere with the mobilization of Cu stores. The adverse effect of dietary Fe on Cu metabolism might be important in man under the extreme condition of a very high Fe intake combined with a low Cu intake. Ingestion of Fe supplements and/or Fe-fortified foods occurs frequently in humans (Ashworth & March, 1973; Rios *et al.* 1975; Li *et al.* 1988), while Cu intake in humans is considered to be often marginal or even deficient (Guthrie & Robinson, 1977; Holden *et al.* 1979).



S. Y. was the recipient of a fellowship provided by the Cultural Exchange Program between the State Education Commission of The People's Republic of China and The Ministry of Education and Science of The Netherlands, and was supported by The Netherlands Foundation for Nutrition and Health Research.

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