COPPER METABOLISM AND ITS INTERACTIONS WITH DIETARY IRON, ZINC, TIN AND SELENIUM IN RATS

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Bijzonder hoogleraar voeding van laboratoriumdieren

vanwege de Vereniging Utrechts Universiteitsfonds

COPPER METABOLISM AND ITS INTERACTIONS WITH DIETARY IRON, ZINC, TIN AND SELENIUM IN RATS

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Proefschrift

ter verkrijging van de graad van

doctor in de landbouw- en milieuwetenschappen

op gezag van de rector magnificus,

Prof. dr. C.M. Karssen,

in het openbaar te verdedigen

op maandag 20 december 1993

des namiddags te vier uur in de Aula

van de Landbouwuniversiteit te Wageningen

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Yu, Shiguang

Copper metabolism and its interactions with dietary iron, zinc, tin and selenium in rats / Shiguang Yu. - [S.l.: s.n.].- Ill.

Thesis Wageningen. - With ref. - With summary in Dutch and Chinese.

ISBN 90-5485-189-9

Subject headings: copper metabolism; rats.

Cover Design : Ernst van Cleef

Printing : Grafisch Service Centrum

PROPOSITIONS

(Attached to the thesis entitled "Copper metabolism and its interactions with dietary iron, zinc, tin and selenium in rats")

1. High dietary iron or tin concentrations reduce copper status of rats through inhibition of copper absorption in the gastrointestinal tract (this thesis).

2. A greater increase in absorption and greater decrease in biliary excretion of copper lead to hepatic copper accumulation after copper loading in mutant rats with conjugated hyperbilirubinemia (this thesis).

3. Plasma albumin is not crucial in copper transport from the intestine to the liver (this thesis).

4. The ratio of copper to selenium in the diet determines the effect of dietary copper on selenium metabolism in rats (this thesis).

5. The combined effect of extra dietary iron and zinc on plasma copper concentration in rats is additive (this thesis).

6. Biliary copper concentration is positively correlated with hepatic coper concentration (this thesis).

7. Elements with similar physical and chemical properties act antagonistically in biological systems.

8. Toxicological testing cannot guarantee safety.

9. Carcinogens occur everywhere, so that cancer can only be minimized by prevention while effective treatment will always be needed.

10. If relationships in the nature cannot be described by a formula they are not sufficiently known.

11. Dissatisfaction makes one creative and satisfaction makes one happy.

Wageningen, The Netherlands, 20 December, 1993. Shiguang Yu

To those who have helped me in one way or another

Contents

Copper metabolism and its interactions with dietary iron, zinc, tin and selenium in rats

Ph.D. Thesis, Department of Human Nutrition, Wageningen Agricultural University, Wageningen, The Netherlands. 20 December 1993. **Shiguang Yu**

Abstract: This thesis describes various studies on copper metabolism and its interactions with selected dietary trace elements in rats. The rats were fed purified diets throughout. High intakes of iron or tin reduced copper concentrations in plasma, liver and kidneys. The dietary treatments also reduced biliary copper excretion through inhibition of intestinal copper absorption. When rats were fed on diets with moderately elevated iron and/or zinc concentrations, only copper concentrations in plasma were lowered. In essence, the combined effects of iron and zinc on plasma copper metabolism were additive. The ratio of copper:selenium in the diet determines the effect of copper intake on selenium metabolism. High intakes of copper decreased apparent selenium absorption and increased urinary selenium excretion in rats fed either low or normal amounts of selenium, but not in rats fed high-selenium diets. Raised dietary copper concentrations elevated selenium contents of liver and kidneys but slightly lowered that of spleen in rats fed a normal amount of selenium. Jaundiced rats with hereditary conjugated hyperbilirubinemia displayed a greater copper accumulation in liver after dietary copper challenge than did their normal counterparts. The aberrant response of the jaundiced rats was due to greater rates of intestinal copper absorption and lesser rise in biliary copper excretion when given a high-copper diet. Rats with hereditary analbuminemia had higher iron and copper concentrations in liver, kidneys and plasma when compared with their normal counterparts. Despite the absence of plasma albumin, the analbuminemic rats could maintain a relatively normal copper metabolism even after dietary copper or iron loading, suggesting that albumin in not crucial in copper transport from the intestine to the liver.

Chapter 1

GENERAL INTRODUCTION

General introduction

Copper is a well-characterized element essential for life. It is a component of many metalloenzymes, such as ceruloplasmin, cytochrome C oxidase, superoxide dismutase and tyrosinase. Copper plays an important role in a number of physiological functions including erythropoiesis, leucopoiesis, connective tissue synthesis, myelin formation and immune function. Both under- and overfeeding with copper can cause abnormalities in animals as well as humans. Two inherited diseases of abnormal copper metabolism, Wilson's disease and Menkes' syndrome are known. Many review articles have dealt with copper chemistry, copper functions and metabolism in living organisms and diseases related to copper (McArdle, 1992; Linder & Goode, 1991; Kies, 1989; Johnson, 1989a; Johnson, 1989b; Howell & Gawthorne, 1989; Bremner, 1987; Cousins, 1985; Evans, 1973; Owen, 1982a,b,c). Here, the properties of copper as an essential element, copper metabolism and influencing factors are briefly reviewed. Finally, the objectives and scope of this thesis are described.

Copper as an essential element for life

Long before the establishment of copper as an essential element for living organisms, its presence in plant and animal tissues had been described (Linder & Goode, 1991). In 1847, copper was identified in the blood of snails and shown to be associated with blood proteins. Haemocyanin, a copper-containing pigment in the blood of the octopus, and turacin, a copper-containing porphyrin in the feathers of turaco, were subsequently discovered (Underwood, 1962). The evidence that copper is essential to plants was obtained by McHargue (1926, 1925) who demonstrated that various plants grew poorly in copper-deficient soils and that the administration of copper improved growth. The conclusive evidence leading to the establishment of copper as an essential dietary component for animals was provided by Hart et al. (1928). In a series of experiments on haemoglobin regeneration in rats suffering from anaemia induced by feeding milk as sole source of nutrition, copper was demonstrated to be

essential for haematopoiesis. In the 1930s it was found that various cattle and sheep diseases, such as 'salt-sickness' (Neal et al., 1931), 'lechsucht' (Sjollema, 1938, 1933) and enzootic neonatal ataxia (Bennetts & Chapman, 1937), were caused by copper deficiency and could be cured by copper supplements. Copper deficiency in humans was first observed by Cordano et al. (1966, 1964) who reported on copper deficiency in 25 marasmic infants recovering from malnutrition after receiving a copper-poor, milk diet. A case of copper deficiency in an infant on total parenteral nutrition was reported by Karpel and Peden (1972). It is now well established that copper is essential to most if not all plants and animals and to man.

Copper metabolism

Copper is absorbed primarily in the small intestine (Van Campen, 1971; Crampton, 1965; Owen, 1964a). Copper may also be absorbed in the stomach of rats (Van Campen & Mitchell, 1965) and possibly also in that of humans (Bearn & Kunkel, 1955; Bush et al., 1955). The absorption of copper in the intestinal tract involves two steps: uptake by the mucosal cell and transport through the basolateral cell membrane (Kirchgessner, 1973). Energy is required for transport of copper into the serosal fluid, but it is probably not required for uptake across the mucosal wall (Crampton et al., 1965). Once in the cell cytosol, part of the copper becomes bound to metallothionein (Hall et al., 1979), a low molecular weight sulfhydryl-rich protein. Other portions of the copper either find their way into cell organelles or cross the basolateral cell membrane into the blood and interstitial fluid by an energy-dependent, saturable process.

The transport of copper from the serosal side of the gut mucosa to the liver is believed to be associated with serum albumin (Gordon et al., 1987; Mason, 1979). Albumin has a specific binding site involving three amino acids at the amino terminal (Bradshaw & Peters, 1969; Peters & Blumenstock, 1967; Breslow, 1964). The histidine residue in position 3 plays a key

role in binding copper with high affinity. However, the copper transporting function of albumin has been questioned (Laurie & Pratt, 1986). Besides albumin, a protein named transcuprein (Weiss & Linder, 1985) may also transport copper in plasma, but this view is not unanimous (Gordon et al., 1987). In plasma a portion of copper is bound to low molecular weight substances (Linder et al., 1985), including amino acids (Zeumann & Sass-Kortsak, 1967; Sarkar & Kruck, 1966). The amino acids-bound fraction of copper in plasma may be important in the transport of copper (Sarkar & Kruck, 1966).

Absorbed copper moves to the liver where it is incorporated into ceruloplasmin, secreted as such from the hepatic cells (Evans et al., 1970; Fujii, 1969a,b; Bowland et al., 1961) and carried in the plasma to other tissues and organs (Owen, 1971; Marceau & Aspin, 1972; Owen, 1965, 1964b). Ceruloplasmin may not be the direct or sole source of copper for extrahepatic tissues (Sternlieb et al., 1961; Scheinberg & Morell, 1957; Bush et al., 1956). At least part of the absorbed copper may reach the kidneys directly without mediation of the liver.

Copper is removed from the body mainly via the bile (Owen, 1965). Reabsorption of copper excreted with bile is poor (Farrer & Mistilis, 1967; Owen, 1964a). A small portion of copper is discharged from the body through the wall of lumen, and with the urine (Owen, 1964a; Cartwright & Wintrobe, 1964).

Factors influencing copper metabolism

Copper metabolism can be influenced by many factors which can be classified into two groups: endogenous and exogenous factors. Endogenous factors are hormones, metallothionein, tumours etc. (Linder & Goode, 1991; Evans, 1973). The exogenous factors are usually ingested with food. They consist of fibres, phytate, amino acids, ascorbic acid, protein, phosphate, iron, zinc (Linder & Goode, 1991; Johnson, 1989a), cadmium (Hill et al., 1963), arsenic (Elsenhans et al., 1987), nickel (Schroeder & Nason, 1974), molybdenum (Humphries et al., 1983; Nederbragt,

1980), selenium (Awad et al., 1973) and tin (Pekelharing et al., 1993). The influences of iron, zinc, selenium and tin are briefly reviewed here.

High intakes of iron reduce copper status as indicated by depressed copper concentrations or activity of copper containing enzymes in tissues and organs of ruminants (Humphries et al., 1983; Standish et al., 1969), guinea pigs (Smith & Bidlack, 1980) and rats (Johnson & Hove, 1986; Bremner & Young, 1981). High dietary iron concentrations impaired copper absorption in one experiment (Gipp et al., 1974) but not in others (Johnson & Murphy, 1988; Johnson & Hove, 1986).

High intakes of zinc also impair copper status of rodents, pigs, sheep, cattle, and poultry (Hambidge et al., 1986). The effect is believed to be mediated through intestinal metallothionein induction by extra zinc in the diet. Zinc is an effective inducer of metallothionein synthesis in liver, kidneys and intestine (Bremner, 1987), but copper binds metallothionein with greater affinity than zinc (Hall et al., 1979). After dietary zinc loading copper will be trapped by metallothionein in the mucosa and lost in the faeces with the sloughing of mucosa cells. As a result, copper absorption is reduced.

The results of studies on interactions between dietary copper and selenium are conflicting. Both copper loading and the feeding of diets deficient in copper can have similar effects on selenium metabolism. The retention of orally administered ^{75}Se by the liver was depressed in rats fed a high-copper diet (Rahim et al., 1986). In rats with copper deficiency hepatic retention of intraperitoneally administered ^{75}Se was depressed too (Jenkinson et al., 1982). The toxic effect of high intakes of selenium in chicks, as evidenced by growth retardation and increased mortality, could be partially alleviated by raising the copper content of the diet (Hill, 1974), but a dietary copper challenge by itself resulted in a considerable accumulation of selenium in the liver (Jensen, 1975). A significant increase in liver selenium concentration has been found in copper-loaded, adult

sheep (Gooneratne & Howell, 1982), but there was no effect of supplemental copper on selenium status of ewes and lambs (White et al., 1989). The inconsistency of the literature data may be due to differences in duration of dietary treatment, the forms of dietary copper and selenium, and the animal species used.

There are only few studies concerning the effects of dietary tin on copper metabolism. High intakes of tin reduced plasma, liver and kidney copper concentrations in rats (Pekelharing et al., 1993; Greger & Johnson, 1981).

The objectives of this thesis

The objectives of the experiments described in this thesis are as follows.

1. To study the mechanisms underlying the reduced copper status as induced by high intakes of iron or tin.

2. To study the altered copper metabolism in jaundiced rats with hereditary conjugated hyperbilirubinemia, with particular reference to their response of liver copper concentration to dietary copper challenge.

3. To study copper metabolism in rats with hereditary analbuminemia, with special reference to the response of copper metabolism to dietary copper and iron loading.

4. To study the effects of dietary copper on selenium metabolism.

5. To study copper status in rats fed diets with moderately elevated concentrations of iron or zinc.

Organization of the thesis

In this chapter, a brief review is given on the essentiality and metabolism of copper. The mechanisms underlying the adverse effects of high intakes of iron or tin on the copper status of rats are discussed in chapters 2 and 3. Copper and zinc metabolism in jaundiced rats and their control counterparts are presented in chapter 4. Chapters 5 and 6 describe iron and copper metabolism in analbuminemic and control rats. In chapters 7 and

8, the interactions of copper with selenium and the combined effects of moderately elevated iron and zinc intakes on copper metabolism are presented. A general discussion of the results obtained is given in chapter 9.

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Chapter 2

INCREASING INTAKES OF IRON REDUCE STATUS, ABSORPTION, AND BILIARY EXCRETION OF COPPER IN RATS

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Br. J. Nutr. (*in press*)

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

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

INTRODUCTION

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

High intakes of iron have been shown to adversely affect copper 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 iron alters plasma and tissue copper concentrations in animals. Bremner & Young (1981) suggested that intake of excess iron stimulates excretion of stored copper. If copper balance is attained after iron feeding, the suggestion of Bremner & Young (1981) implies that increased intake of dietary iron, at least as a secondary effect, would enhance copper absorption. In feeding trials with rats given copper-adequate diets for up to 20 days, dietary iron concentrations ranging from about 35 to 500 mg/kg have been shown not to affect apparent copper absorption (Johnson & Hove, 1986; Johnson & Murhpy, 1988; Reichlmayer-Lais & Kirchgessner, 1992).

Copper balance in rats is determined essentially by the efficiency of copper absorption and by the faecal loss of endogenous copper, essentially representing the unabsorbed fraction of copper excreted with bile (Van den Berg & Beynen, 1992). We hypothesized that the impaired copper status seen after iron loading is due either to diminished copper absorption followed by a decrease in biliary copper excretion or to enhanced biliary copper excretion with an increase in copper absorption as secondary feature. The two possibilities were checked in a 6-week trial using rats given copper-adequate diets containing

either low, normal or high amounts of iron.

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 (60x21x19 cm) with wire mesh bases and given ad libitum the purified diet with 40 mg Fe/kg (Table 1) and

Table 1. Composition of the experimental diets used

	Low Fe	Normal Fe	High Fe
Ingredients (mg or g/kg diet)			
Constant components* (g)	290.6	290.6	290.6
Glucose (g)	709.4	709.2	707.7
$FeSO_4.7H_2O$ (mg)	0	174	1740
Chemical analysis (mg/kg)			
Fe	6.8	40.2	388.8
Cu	8.1	8.1	8.1

*The constant components consisted of (g): casein, 151; maize oil, 25; coconut oil, 25; cellulose, 30; $CaCO_3$, 12.4; $NaH_2PO_4.2H_2O$, 15.1; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; mineral premix, 10; and vitamin premix, 12. The iron free mineral premix consisted of (mg) MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2 and maize meal, 9853.2. The vitamin premix consisted of (mg) thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D, L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (1200 retinol equivalents); cholecalciferol, 0.025; maize meal, 9828.125.

demineralized water for 10 days. Then (day 0 of the experiment), the rats were divided into three groups of 15 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 copper (8 mg/kg). One group remained on the diet with 40 mg iron/kg, and the other groups were transferred to the diets containing either 7 or 389 mg iron/kg. Table 1 shows the ingredient composition of the diets which only differed with regard to iron concentration. Iron was added to the diets in the form of $FeSO_4.7H_2O$. The diet with 40 mg iron/kg was formulated according to the recommended nutrient requirements of rats (National Research Council, 1978). The diets were stored at 4°C 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 (24x17x17 cm) in a room with controlled lighting (light on: 06.00-18.00), temperature (19-21°C) 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 four days of the experiment.

At the end of the experiment on week 6, bile was collected by common bile duct cannulation with polyethylene tubing (inner diameter 0.28 mm, outer diameter 0.61 mm, INTRAMEDIC, Clay Adams, Parsippary, N.J., U.S.A.). The abdomen was opened while the rats were under anaesthesia induced by a combination of ketamine (6 mg/100 g body weight) administered intramuscularly and xylazine (0.8 mg/ 100 g 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°C). 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°C 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°C until analysis.

Analytical methods

Haemoglobin concentration and haematocrit of fresh, heparinized blood samples were measured by using the Sysmex K-1000D (Sysmex-TOA, TOA Medical Electronics Co, LTD, Kobe, Japan). The concentration of iron and copper in organs, faeces and feed was determined by flame atomic absorption spectrometry (Varian AA-475, Varian Techtron, Springvale, Australia). For the determination of copper and iron in organs and feed, samples were dried in a vacuum dryer for 48 hours and digested in 1.0 ml of 14 mol/L nitric acid (Suprapur, Merk, Darmstadt, Germany) at 80°C for 2 hours. Samples of faeces were first dried, ashed at 500°C for 17 hours in a muffle furnace and then dissolved in 6 mol/L HCl. Copper in plasma was measured directly.

Iron and total iron-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 copper and iron 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, U.S.A)

was used to assess bias of iron and copper analysis. Analyzed iron and copper concentrations were 105.3% (SE 4.03, n=6) and 98.7% (SE 4.61, n=6) of the target values. Ceruloplasmin (EC 1.16.3.1) in plasma was assayed as p-phenylenediamine oxidase 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 iron and copper concentrations were analyzed using two-way analysis of variance. The level of significance was preset at $p < 0.05$. All data were analyzed using a computer program (SPSS Inc., 1988).

RESULTS

Feed intake, body and organ weights

The concentration of iron 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-iron group were significantly lower than those in the high-iron group. Liver weight of the low-iron group was also lower than that of normal-iron group.

Indicators of iron status

Fig. 1 shows that blood haemoglobin concentrations and haematocrit decreased in the rats given the low-iron diet, whereas in the other two groups there was a similar rise with time. After six weeks, iron concentrations in all organs and plasma were significantly lower in rats fed the low-iron diet compared with those fed the normal-iron diet (Table 3). Rats given the high-iron instead of the normal-iron diet displayed significantly higher values except for kidney, heart and plasma. Total iron-binding capacity was similar in rats fed the normal

Table 2. Feed intake and body and organ weights of rats fed the experimental diets* (Mean values for 15 rats per dietary group)

	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)				
wk 1	20.5	21.6	21.5	0.40
wk 6	19.9	20.9	20.7	0.34
Organ weight (g/100 g body weight)				
Liver	3.45^{a}	3.72^{b}	3.78^{b}	0.057
Spleen	0.23	0.21	0.22	0.008
Kidney	0.58^{a}	0.60^{ab}	0.63^{b}	0.015
Heart	0.34	0.33	0.34	0.009

*Means in the same row not sharing the same superscript letter are significantly different ($p<0.05$)

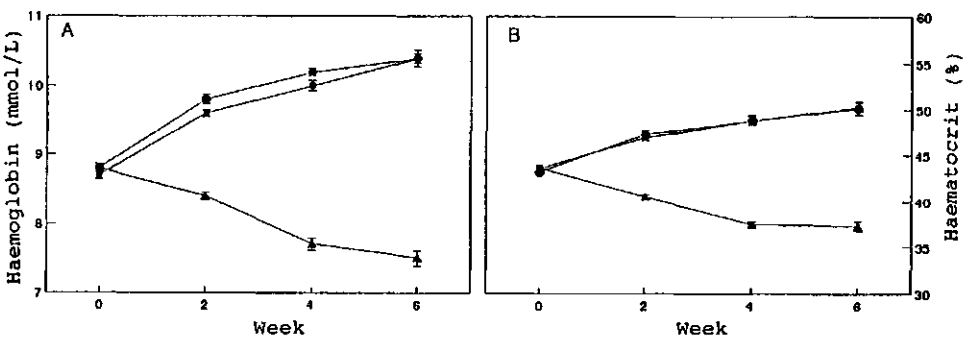

Fig. 1. Time course of blood haemoglobin concentrations (panel A) and haematocrit (panel B) in rats fed either the low (▲), normal (■) or high- (●) iron diet. There were significant effects of diet ($p<0.05$) for both haemoglobin (pooled SE 0.022 mmol/L) and haematocrit (pooled SE 0.767%).

Table 3. Organ and plasma iron concentrations in rats fed the experimental diets* (Mean values for 15 rats per dietary group)

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

*Means in the same row not sharing the same superscript letter are significantly different ($p<0.05$).
†On a dry weight basis.

and high-iron diets (1.6 and 1.5 mmol/L), but was significantly raised ($p < 0.05$) in their counterparts given the low-iron diet (2.1 mmol/L, pooled SE 0.03 mmol/L).

Indicators of copper status

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

Apparent copper absorption

Increasing dietary iron concentrations significantly reduced apparent copper absorption (Fig. 2).

Biliary iron and copper excretion

Bile flow in the cannulated rats decreased with time, but there was no effect of dietary iron concentration (Table 5). The concentration of iron in bile was slightly, but significantly,

Table 4. Organ and plasma copper concentrations and plasma ceruloplasmin concentrations in rats fed the experimental diets* (Mean values for 15 rats per dietary group)

Measure	Low Fe	Normal Fe	High Fe	Pooled SE
Organ copper (mmol/kg)†				
Liver	0.28[a]	0.26[b]	0.23[b]	0.008
Spleen	0.17[a]	0.15[ab]	0.12[b]	0.011
Kidney	0.40[a]	0.43[a]	0.31[b]	0.014
Heart	0.42[a]	0.41[a]	0.39[b]	0.058
Tibia	0.08	0.08	0.08	0.046
Plasma				
copper (μmol/L)	23.6[a]	22.0[a]	15.7[b]	1.10
ceruloplasmin (Δabsorption/L.min.)	144.1[a]	159.9[a]	85.9[b]	10.05

*Means in the same row not sharing the same superscript letter are significantly different ($p<0.05$).
†On a dry weight basis.

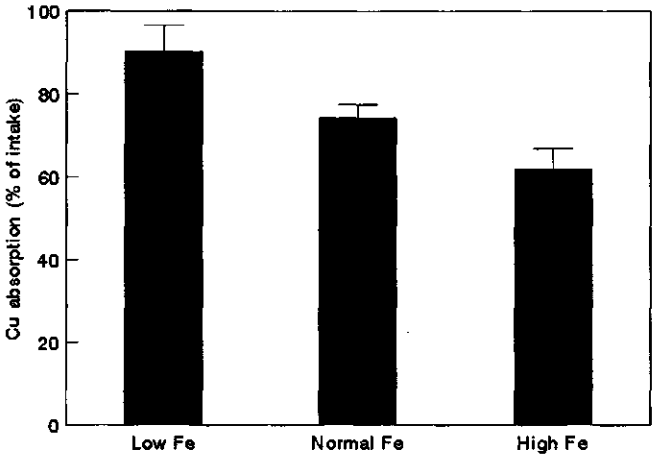

Fig. 2. Influence of the experimental diets on apparent copper absorption. Results are expressed as means and SE (n=15) and were significantly different ($p<0.05$) between dietary groups.

reduced in rats fed the low-iron diet. With time there was an increase in biliary iron concentration. The concentration of copper was not determined in the initial 15 min sample of bile collected because the quantity of fluid obtained was insufficient. For the other two periods, there was a significant decrease in biliary copper concentration with increasing iron intake (Table 5).

Table 5. Bile flow and biliary iron and copper concentrations in rats fed the experimental diets*

(Mean values for 15 rats per dietary group)

Measure and period of collection	Low Fe	Normal Fe	High Fe	Pooled SE	ANOVA†
Bile flow (ml/100 g body weight.hour)					
0-15 min	0.27	0.30	0.29		
15-45 min	0.26	0.27	0.26		
45-75 min	0.26	0.25	0.24	0.014	P
Biliary Fe concentration (μmol/L)					
0-15 min	22	25	24		
15-45 min	25	29	30		
45-75 min	28	34	34	1.9	Fe; P
Biliary Cu concentration (μmol/L)					
15-45 min	19.4[a]	12.3[b]	1.9[c]		
45-75 min	19.5[a]	13.1[b]	1.6[c]	1.45	Fe

*Means in the same row not sharing the same superscript letter are significant different ($p<0.05$).

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

Fig. 3 illustrates the absolute amounts of iron and copper excreted in bile by the rats fed the diets differing in iron concentration. Iron excretion was lower in rats given the low-iron diet than in rats given either the normal or high-iron diet. Increased concentrations of iron in the diet were associated with

a marked drop in biliary copper excretion.

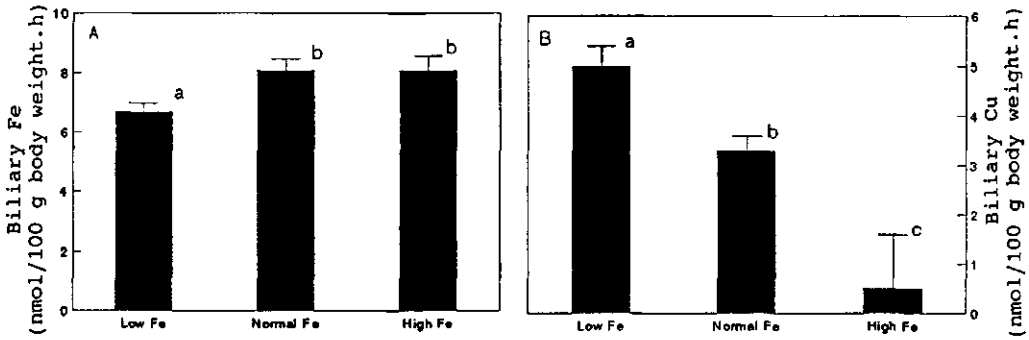

Fig. 3. Biliary excretion of iron (panel A) and copper (panel B) by rats fed the experimental diets for a period of six weeks. Bile was collected quantitatively from anaesthetized, cannulated rats for a period of 75 min immediately after cannulation. Iron and copper excretions refer to the 15-75 min collection period and are expressed as nmol/100 g body weight.hour. Results are expressed as means and SE (n=15); bars within a panel not sharing a common letter are significantly different ($p<0.05$).

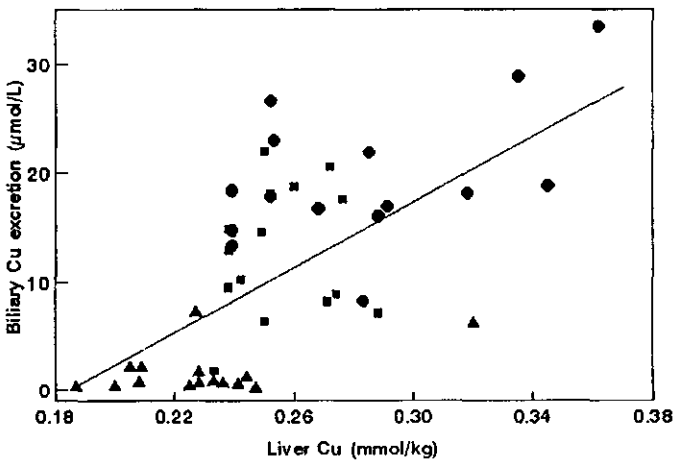

Fig. 4. Relationship between biliary copper excretion and hepatic copper concentration in individual rats fed either the low (▲), normal (■) or high- (●) iron diet. The regression equation is $y = 150 x - 28$ ($r=0.63$, $n=45$, $p<0.05$).

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

DISCUSSION

The experimental diets containing different amounts of iron predictably affected the selected indicators of iron status without differently influencing feed intake or body weight gain. Thus, the low-iron diet which contained about one fifth of the recommended dietary iron concentration for rats (35 mg iron/kg diet; National Research Council, 1978) lowered blood haemoglobin concentration and haematocrit and also plasma and organ iron concentrations. This agrees with earlier work (Sørensen, 1965; Brouwer et al., 1993). On the other hand, iron loading with a dietary concentration more than ten times the recommended concentration did not alter haemoglobin and haematocrit, but raised iron concentrations in liver and spleen. These data also support previous studies (Dhur et al., 1989; Kreuzer & Kirchgessner, 1991).

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

The major objective of this study was to determine the influence of dietary iron concentration on copper metabolism. It is clear that increasing intakes of iron caused impairment of

copper status as based on the lowering of copper concentrations in plasma and organs. The important finding here is that the antagonistic effect of dietary iron on copper status can now be explained by the decrease in apparent copper absorption. There was an inverse relation between iron intake and copper 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 iron-induced decrease in copper absorption must be associated with a decrease in copper excretion so that copper balance can be attained. Indeed, biliary excretion of copper was found to be depressed with higher intakes of iron (Fig. 3 B). The rats given the low-iron diet had an apparent absorption of about 2.28 μmol copper per day (0.90 x intake), and for the rats given the high-iron diet apparent copper absorption equalled 1.64 μmol/day (0.62 x intake). Based on the data in Fig. 3 B, biliary copper excretion in rats given the low-iron diet was in the order of 0.43 μmol/day, and in rats given the high-iron diet it was 0.04 μmol/day. Thus these calculations, which should be interpreted cautiously, suggest that the iron-induced reduction in biliary copper excretion may not fully compensate for the decrease in copper absorption. This notion is reinforced by the fact that true copper absorption will be greater than the calculated values for apparent copper absorption. In addition, part of the copper excreted with bile will be re-absorbed so that the net loss of copper with bile may be lesser than that calculated. Excretion of copper in bile is probably regulated by copper concentration in liver. At least for rats given the normal or high-iron diet, the output of copper with bile was directly related to liver copper concentration (Fig. 4).

Liver copper concentrations were only marginally reduced with increasing iron intake while plasma and biliary copper concentrations, and also plasma ceruloplasmin activities, showed much greater percentage reductions. This could be interpreted in that dietary iron interferes with copper metabolism not only at

the absorption but also at the post-absorptive level. Perhaps an increased iron status affects the mobilization of copper stores in the liver resulting in depressed incorporation of copper into ceruloplasmin and bile fluid.

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

ACKNOWLEDGEMENTS

Shiguang Yu 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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Chapter 3

HIGH TIN INTAKE REDUCES COPPER STATUS IN RATS THROUGH INHIBITION OF COPPER ABSORPTION

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(submitted for publication)

High tin intake reduces copper status in rats through inhibition of copper absorption

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Abstract: The mechanism underlying the reduced copper status in rats fed a high-tin diet was investigated. Male rats aged 4 weeks were fed ad libitum purified diets containing either 1 or 100 mg Sn/kg and demineralized water for a period of 4 weeks. The high-tin diet had no effect on feed intake, body-weight gain and weight of liver and kidney but significantly reduced copper concentrations in plasma, liver and kidney. Biliary copper excretion was decreased significantly in rats fed the high-tin diet. Apparent copper absorption (Cu intake - faecal Cu) was not affected by the high-tin diet, but the estimation of true copper absorption (Cu intake - (faecal Cu - biliary Cu)) was significantly reduced. We conclude that high tin intake reduces copper status in rats through inhibition of copper absorption which is reflected as a secondary feature by a decrease in biliary copper excretion.

INTRODUCTION

High intakes of tin in the form of stannous chloride reduce plasma, liver and kidney copper concentrations in rats (Greger & Johnson, 1981; Pekelharing et al., 1993). The mechanism underlying the adverse effect of high dietary tin concentrations on copper status is not known. We hypothesized that high tin

intake inhibits intestinal copper absorption followed by a depressed biliary copper excretion in order to achieve copper balance or, alternatively, that high tin intake primarily stimulates biliary copper excretion with enhanced copper absorption as secondary feature. Both mechanisms would lead to the observed impaired copper status in rats fed diets high in tin. Our hypothesis was tested in the present experiment.

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; Harlan/CPB, Zeist, The Netherlands), aged about four weeks, were used. On arrival, they were housed in groups of five in stainless steel cages (60 x 21 x 19 cm) with wire mesh bases and given ad libitum a commercial, pelleted diet (RMH-B, Hope Farms, Woerden, The Netherlands) and tap water. After three days, the purified control diet (Table 1) and demineralized water were given. The control diet was formulated according to the recommended nutrient requirements of rats (National Research Council, 1978). After four days (d -4), the rats were divided randomly into two groups of 12 each and stratified for body weight. After another four days (day 0), one group was randomly allocated to the purified, high-tin diet containing 100 mg added tin/kg (Table 1), and the other group remained on the control diet. Extra tin was added to the test diet in the form of stannous chloride. The control and test diets were balanced for calcium and chlorine (Table 1). Both groups had free access to the diets, which were in powdered form, and to demineralized water. Feed intake and body weight were recorded regularly. As from day -4, the rats were housed individually in metabolism cages (314 cm^2 x 12 cm) in a room with controlled

lighting (light on: 06.00-18.00 h), temperature (19-21°C) and relative humidity (50-60%).

Table 1. Composition of the experimental diets

	Control	High Sn
Ingredients (g/kg diet)		
Constant components*	278.2	278.2
Glucose	709.3	709.1
$CaCO_3$	12.0	12.1
$CaCl_2$	0.467	0.375
$SnCl_2.2H_2O$	0	0.190

*The constant components consisted of (g): casein, 151; maize oil, 25; coconut fat, 25; cellulose, 30; $NaH_2PO_4.2H_2O$, 15.1; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; mineral premix, 10; and vitamin premix, 12. The mineral premix consisted of (mg): $FeSO_4.7H_2O$, 174; MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2 and maize meal, 9679.2. The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D, L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (1200 retinol equivalents); cholecalciferol, 0.025; maize meal, 9828.125.

Collection of samples

Faeces and urine were collected separately and quantitatively during days -4 to 0, 0 to 4, 7 to 11 and 24 to 28. At the end of the experiment (day 28), bile was collected by common bile duct cannulation with polyethylene tubing (inner diameter 0.28 mm, outer diameter 0.61 mm, INTRAMEDIC, Clay Adams, Parsippary, N.J., U.S.A.). The abdomen was opened while the rats were under anaesthesia as induced by a combination of ketamine (6 mg/100 g body weight) administered intramuscularly and xylazine (0.8 mg/ 100 g 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°C). Bile was collected into pre-weighed vials for one hour and the volume of bile was calculated from the weight and specific gravity of the bile. One rat in the control group died immediately after induction of the anaesthesia. Following bile collection, blood samples were taken from the anaesthetized rats by abdominal aorta puncture into heparinized tubes. The rats were then killed and liver and left kidney were removed and weighed. All samples collected were stored at -20°C until analysis.

Analytical methods

The concentrations of copper in organs, faeces, urine and feed samples were determined by flame atomic absorption spectrometry (PERKIN-ELMER 2380, Perkin-Elmer Coorporation, Norwalk, CT, U.S.A.). For the determination of copper in organs, samples were dried in a vacuum dryer for 48 hours and digested in 1.0 ml of 14 mol/L nitric acid at 80°C for 2 hours. Samples of faeces, but not feed samples, were also dried in the vacuum dryer before ashing. Samples of feed and dried faeces were ashed at 500°C for 17 hours in a muffle furnace and then dissolved in 6 mol/L HCl. The determination of copper in bile and plasma was carried out using flameless atomic absorption spectrometry (Varian AA-300, Varian Techtron Pty. Ltd., Springvale, Australia) after proper dilution of the samples with demineralized water. An external control in the form of a bovine liver sample (NBS 1577b, National Institute of Standards Technology, Gaithersburg, MD, U.S.A) was used to assess bias of copper analysis. Analyzed copper concentration was 103.8% (SE 1.73, n=4) of the NBS certified value.

Statistical analyses

The data of the control and test group were subjected to Student's t-test to identify statistically significant

differences. Mann-Whitney U test was used to evaluate copper concentrations in liver and plasma because the variances were not homogeneous (F test). Copper absorption and urinary copper excretion were evaluated using MANOVA repeated measurements test. The level of significance was pre-set at $p < 0.05$. All data were processed using a computer programme (SPSS Inc., 1988).

RESULTS

Feed consumption, body and organ weights

The high-tin diet had no effect on feed consumption and body weight of the rats (Table 2). Likewise, there was no tin effect on the weights of liver and kidney.

Table 2. Feed intake and body and organ weights of rats fed either the control or high-tin diet
(Mean values for 12 rats per dietary group)

	Control		High Sn	
	Mean	SE	Mean	SE
Body weight (g)				
Initial	95.8	2.91	94.6	2.11
Final	266.2	9.13	269.4	6.25
Feed intake (g/d)				
Days 0 - 7	12.7	0.49	12.5	0.38
Days 21 - 28	21.6	0.71	21.9	0.53
Organ weight (g/100 g body weight)				
Liver	3.87	0.076	3.86	0.042
Kidney	0.33	0.008	0.33	0.005

Indicators of copper status

Fig. 1 shows the copper concentrations in selected organs

and plasma. The high-tin diet produced significantly reduced copper concentrations in plasma, liver and kidney.

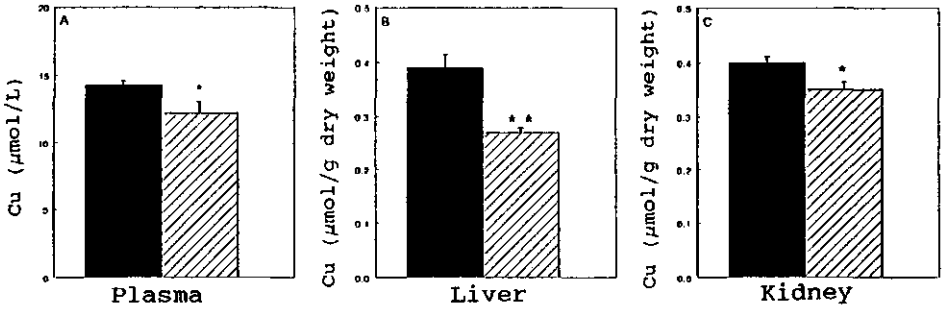

Fig. 1. Copper concentrations in plasma (panel A), liver (panel B) and kidney (panel C) of rats fed either the control (solid bars) or high-tin (hatched bars) diet. Results are expressed as means and SE (n=12; n=11 for plasma values of the control group) and differ significantly (*: $p<0.05$; **: $p<0.01$) between the dietary groups.

Apparent copper absorption

Analyzed copper concentrations of both the control and test diet were found to be 5 mg/kg. Apparent copper absorption was calculated as copper intake minus faecal copper excretion. The high-tin diet did not affect apparent copper absorption (Fig. 2).

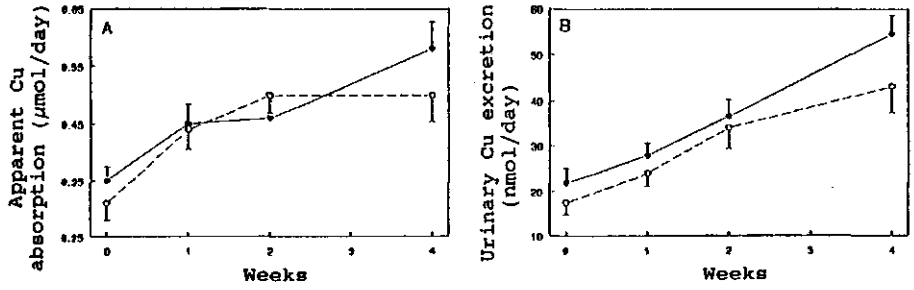

Fig. 2. Time course of apparent copper absorption (panel A) and urinary copper excretion (panel B) of rats fed either the control (solid line) or high-tin (dotted line) diet. Results are expressed as means for 12 rats with vertical bars as SE. The high-tin diet did not significantly influence apparent copper absorption and urinary copper excretion.

During the course of the experiment absolute copper absorption rose in both groups (Fig. 2) because feed intake increased (Table 2). Apparent copper absorption expressed as percentage of copper intake dropped with time (not shown). The high-tin diet systematically lowered group means of urinary copper excretion (Fig. 2), but the effect failed to reach statistical significance ($p = 0.118$).

Biliary copper excretion

Bile flow and biliary copper excretion are illustrated in Fig. 3. The high-tin diet had no effect on bile flow, but significantly reduced the absolute amount of copper excreted in bile. Biliary copper concentration was in direct correlation to hepatic copper concentration (Fig. 4).

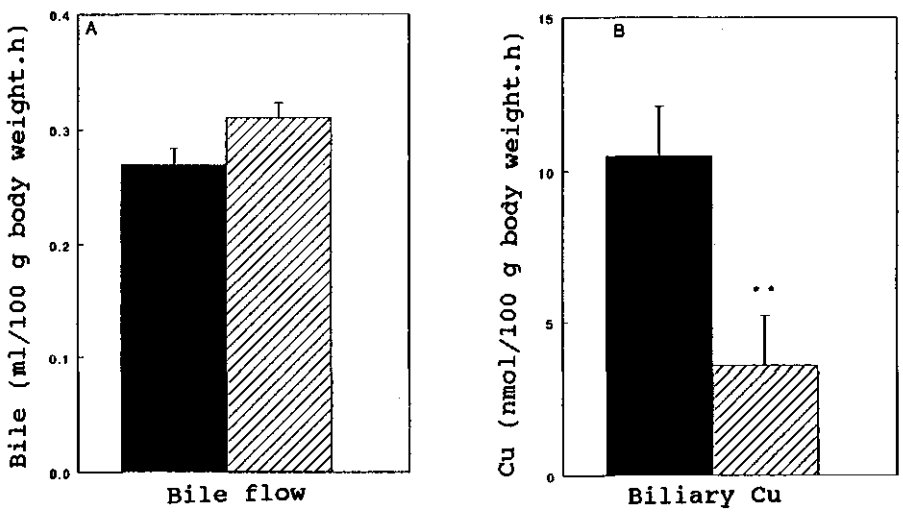

Fig. 3. Bile flow (panel A) and biliary excretion of copper (panel B) in rats fed either the control (solid bars) or high-tin (hatched bars) diet for 28 days. Bile was collected quantitatively from anaesthetized, cannulated rats for a period of one hour immediately after cannulation. Results are expressed as means and SE (n=11 for control group and n=12 for high-tin group). The effect of tin on biliary copper excretion was statistically significant (**: $p<0.01$).

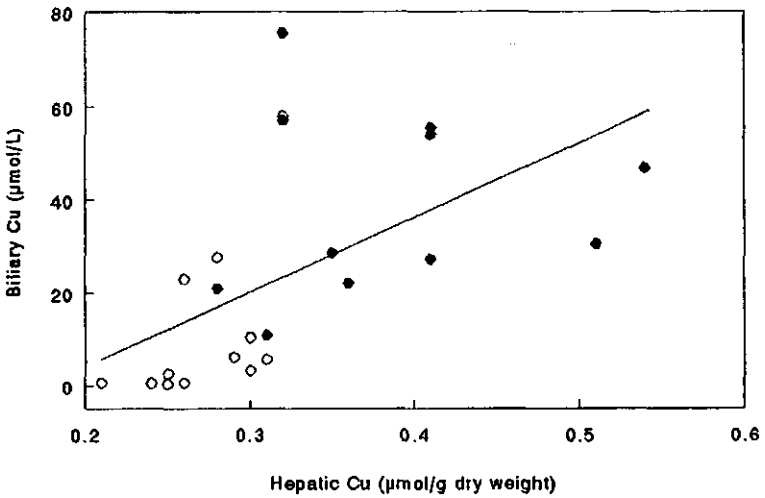

Fig. 4. Relationship between biliary copper concentration and hepatic copper concentration in individual rats fed either the control (●) or high-tin (o) diet for 28 days. The regression equation is $y = 150\ x - 24$ ($r=0.54$, $n=23$, $p < 0.01$).

DISCUSSION

The observed lowering effects of the high-tin diet on copper concentrations in plasma, liver and kidney agree well with previous findings (Greger & Johnson, 1981; Pekelharing et al., 1993). The challenge with 100 mg Sn/kg diet did not affect feed consumption and weight gain of the rats. Pekelharing et al., (1993) found that feed intake was significantly reduced in rats fed a diet containing as much as 200 mg Sn/kg.

High tin intake had no effect on bile flow but significantly reduced the amount of copper excreted in bile. The decrease in biliary copper excretion may be a compensatory response to the reduced copper status, which is supported by the direct correlation between hepatic and biliary copper concentrations (Fig. 4). It is likely that biliary copper excretion is determined by the concentration of copper in the liver. The tendency towards a diminished urinary copper excretion in the

rats fed the high-tin diet may also be secondary to the reduced copper status.

Thus, high tin intake may impair copper status which in turn dampens biliary and urinary copper excretion. In order to maintain whole-body copper balance, copper absorption should be depressed in rats fed the high-tin diet. However, apparent copper absorption was not systematically influenced by tin loading. Copper is discharged from the body mainly via bile (Cartwright & Wintrobe, 1964) while biliary copper is poorly reabsorbed (Owen, 1964, Farrer & Mistilis, 1967). Assuming that the diurnal rate of biliary copper excretion is constant and that copper

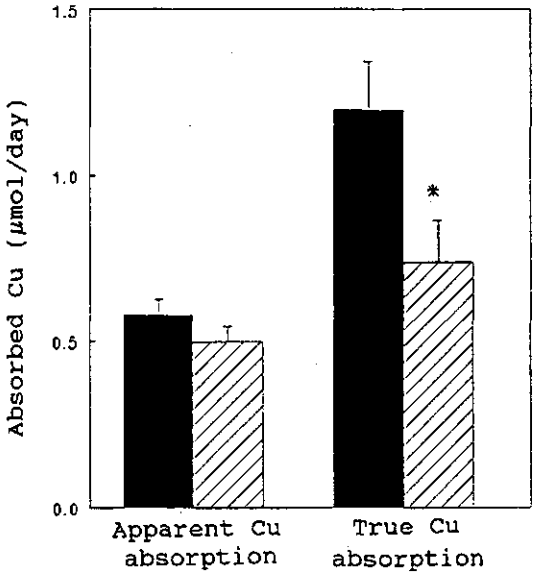

Fig. 5. Apparent and true copper absorption in rats fed either the control (solid bars) or high-tin (hatched bars) diet for 28 days. Apparent copper absorption was calculated as Cu intake - faecal Cu and true copper absorption as Cu intake - (faecal Cu - biliary Cu). Results are expressed as means and SE (n=11 for the control group; n=12 for the high-tin group). True copper absorption was significantly depressed (*: $p<0.05$) in the high-tin group.

excreted with bile is not reabsorbed, true copper absorption can be calculated as Cu intake - (faecal Cu - biliary Cu). In the rats fed the control and high-tin diets, biliary copper excretion (day 28) was 0.62 (SE 0.12) and 0.24 (SE 0.10) μmol/day, and faecal copper excretion (day 24 to 28) was 1.12 (SE 0.05) and 1.21 (SE 0.04) μmol/day, respectively. Copper intake during days 24 to 28 was 1.7 μmol/day for both groups. Thus, unlike apparent copper absorption, true copper absorption was decreased significantly in rats fed the high-tin diet (Fig. 5). We conclude that high tin intake reduces copper status in rats through inhibition of copper absorption which is reflected by a decrease in biliary copper excretion.

ACKNOWLEDGEMENTS

Shiguang Yu was supported by The Netherlands Foundation for Nutrition and Health Research. Thanks are due to Gerrit van Tintelen for taking care of the rats.

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Chapter 4

EXCESSIVE HEPATIC COPPER ACCUMULATION IN JAUNDICED RATS FED A HIGH-COPPER DIET

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(*submitted for publication*)

Excessive hepatic copper accumulation in jaundiced rats fed a high-copper diet

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Abstract: The response of copper metabolism to dietary copper challenge was investigated in jaundiced rats with elevated plasma concentrations of conjugated bilirubin. Control and jaundiced rats were fed purified diets with either normal copper and zinc or high concentrations of copper and/or zinc. Copper loading produced a greater increase in hepatic copper concentrations in the jaundiced than in control rats. The rise in hepatic copper tended to be partly counteracted by simultaneous zinc loading. The greater dietary-copper-induced increase in hepatic copper in the jaundiced rats can be explained by a smaller rise in biliary copper excretion and a somewhat greater efficiency of dietary copper absorption. In individual rats there were positive relationships between hepatic copper concentrations and either biliary copper concentrations or absolute biliary copper excretion. These relationships did not differ clearly between control and jaundiced rats, and it is suggested that not the transport of copper from liver cells to bile but rather that from plasma to bile is diminished in the jaundiced rats. The elevated plasma copper concentrations in the jaundiced rats may support this suggestion.

INTRODUCTION

Jansen et al. (1985) have described a mutant rat with hereditary conjugated hyperbilirubinemia. The autosomal recessive defect in this jaundiced rat, and also in the identical, so-called Groningen-Yellow rat (Kuipers et al., 1988), is impaired canicular transport of organic anions such as bilirubin glucuronides, glutathione conjugates and sulfated bile acids (Jansen et al., 1985; Kuipers et al., 1988; Oude Elferink et al., 1989). Houwen et al. (1990) reported that this mutant jaundiced rat also has an altered metabolism of copper: after intracardiac administration of massive copper doses, anesthetized and bile-duct cannulated jaundiced rats excreted less copper with bile than did their non-jaundiced counterparts. We thus hypothesized that after feeding a high-copper diet the jaundiced rats would accumulate more copper in their livers than normal rats. In an attempt to explain any difference in hepatic copper accumulation between jaundiced and normal rats, biliary copper excretion and apparent copper absorption were determined. To assess the specificity of the response to high copper intake in the jaundiced rats, we also studied the response to high zinc intake.

MATERIALS AND METHODS

The experimental protocols were approved and their conduct supervised by the animal welfare officer of the Wageningen Agricultural University.

Animals, housing and diets

We used male Wistar (Cpb:WU) rats and progeny of their jaundiced mutant (Beynen et al., 1989) which were both bred in the colony of the Laboratory Animals Center, Wageningen Agricultural University. Cross-breeding experiments had demonstrated that our jaundiced rats are identical to the Groningen-Yellow rats (unpublished). The normal and jaundiced

rats, aged 3 (experiment 1) or 14 (experiment 2) weeks, were housed in groups of 4 or 5 animals in stainless steel cages (60 x 20 x 19 cm) with wire mesh bases. All rats were given free access to the purified control diet (Table 1) and demineralized water for the pre-experimental period of 10 (experiment 1) or 14 (experiment 2) days.

Table 1. Composition of the experimental diets

	Control[1]	High Cu	High Zn	High Cu+Zn
Ingredients				
Constant components,[2] g	290.6	290.6	290.6	290.6
Glucose, g	709.35	709.21	709.05	708.91
$CuSO_4.5H_2O$, mg	16	160	16	160
$ZnSO_4.H_2O$, mg	33	33	330	330
Chemical analysis[3]				
Copper, μmol/kg	113/69	745/825	114	782
Zinc, μmol/kg	344	315	2927	3020

[1]The control diet also served as pre-experimental diet.

[2]The constant components consisted of (g): casein, 151; corn oil, 25; coconut oil, 25; cellulose, 30; $CaCO_3$, 12.4; $NaH_2PO_4.2H_2O$, 15.1; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; mineral premix, 10; and vitamin premix, 12. The mineral premix consisted of (mg) FeSO4.$7H_2O$, 174; MnO_2, 79; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2 and corn meal, 9727.9. The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (1200 retinol equivalents); cholecalciferol, 0.025; corn meal, 9828.125.

[3]Figures before slash: experiment 1; after slash: experiment 2.

The control diet was formulated according to the recommended nutrient requirements of rats (National Research Council, 1978). The high-copper and high-zinc experimental diets contained copper

and zinc supplements that were about ten-fold and sixteen-fold more, respectively, than the recommended amounts. Separate batches of diet were made for experiment 1 and 2. The diets, which were in powdered form, were stored at 4°C until used for feeding. During the experimental period the rats had free access to food and demineralized water. Feed intake and body weight were recorded at regular intervals.

During the experimental period, which lasted 28 days (experiment 1) or 14 (experiment 2) days, the rats were housed individually in metabolism cages (314 cm^2 x 12 cm) in a room with controlled temperature (20-22°C), relative humidity (55-75%) and lighting (light on: 06.00-18.00 h).

Experiment 1. On d 0 of the experiment, at the end of the run-in period, the rats of each strain were divided into four groups of six rats each as based on stratification for body weight. Within each strain, the groups were randomly allocated to one of the four experimental diets (Table 1).

One of the jaundiced rats fed the control diet began to loose body weight during the last week of the experiment. It appeared that this animal had developed abnormal teeth and was not able to eat properly. The animal was culled and its data were excluded.

Experiment 2. One week after the beginning of the pre-experimental period six jaundiced and five normal rats were transferred to metabolism cages for another 7 days. At the end of the two-week pre-experimental period (d 0), the remaining 12 jaundiced and 11 normal rats were all switched to the high-copper diet.

Collection of samples

Feces and urine of each rat were collected quantitatively during d 21-25 (experiment 1) and during d -3-0, 3-7 and 11-14 (experiment 2) of the experiments. At the end of experiment 1 (d 28) and at the end of each balance period (experiment 2), bile was collected by common bile duct cannulation with the use of

polyethylene tubing (i.d., 0.28 mm; o.d., 0.61 mm, Clay Adams, Parsippany, NJ, U.S.A.). The abdomen of the rats was opened under anesthesia using a combination of ketamine (6 mg/100 g body weight) administered intramuscularly and xylazine (0.8 mg/100 g body weight) administered subcutaneously. The combination of the two drugs does not affect 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°C) and bile was collected into pre-weighed vials for 150 min. Bile flow was calculated using weight and specific gravity of the bile collected. Following bile collection, blood samples were taken from the anesthetized rats by abdominal aorta puncture. Then, the rats were killed by decapitation and livers were removed and weighed. In experiment 1, tibias were also removed. All samples were stored at -20°C until analysis.

Analytical methods

Zinc (experiment 1) and copper in samples of liver, tibia (experiment 1), feces and diet were determined by flame atomic absorption spectrometry with the Varian AA-475 (Varian Techtron, Springvale, Australia). The organ samples were first dried at 60 °C for 48 h in a vacuum dryer, and then digested in 14 mol/L nitric acid at 80°C for 2 h. Feces and diet samples were ashed at 500°C for 17 h in a muffle furnace and subsequently dissolved in 6 mol/L HCl. Zinc (experiment 1) and copper in plasma and bile were measured directly using flameless atomic absorption spectrometry (Varian AA-300) after the samples were diluted properly with demineralized water. Conjugated and total bilirubin in plasma were determined using a test kit (Hoffmann-La Roche BV, Mijdrecht, The Netherlands) and the COBAS-BIO autoanalyser (Hoffmann-La Roche BV). Ceruloplasmin in plasma (experiment 1) was assayed as p-phenylenediamine oxidase activity and expressed as absorbance change at 530 nm due to the formation of the colored oxidation product of p-phenylenediamine (Sunderman and Nomoto, 1970). Reference bovine liver (NBS 1577a, National

Institute of Standards Technology, Gaithersburg, U.S.A.) was used to assess accuracy of copper and zinc analysis. Copper and zinc concentrations measured in the reference sample were found to be on average 99 and 118% (n=3) of the certified values.

Statistical analysis

When in experiment 1 the variances for a measure were homogeneous among the eight experimental groups (Bartlett's test), the data were subjected to three-way ANOVA with rat strain and amounts of dietary copper and zinc as main effects. The level of statistical significance was pre-set at $p<0.05$. The statistical significance of differences between two groups with one variable (jaundiced versus control rats; high versus normal dietary copper; high versus normal dietary zinc) were evaluated with two-sided Student's t-test. Mann-Whitney U test instead of Student's t-test was used if the variances of two groups were not homogeneous (F test). To take into account the increased probability of a type I error, the level of statistical significance used to indicate an effect was pre-set at $p<0.025$ instead of $p<0.05$ (Bonferroni's adaptation). Significant strain differences in experiment 2 were identified with the use of two-tailed Student's t-test with $p<0.05$ as level of statistical significance. Statistical analysis was carried out with a computer using SPSS software (SPSS Inc. 1988).

RESULTS

Characteristics of the jaundiced rats

As would be expected, the jaundiced rats had markedly raised plasma bilirubin concentrations of which about 60% was in the conjugated form (Table 2). This was also seen in experiment 2 (not shown). The jaundiced rats had lower feed intake and body weights than the control rats (Table 2). In experiment 2 average body weights of the jaundiced and normal rats were 321 $\pm$ 6 and 362 $\pm$ 5 g (mean $\pm$ SEM, n=18 or 15). Average feed intakes were

Table 2. Experiment 1: Growth performance, liver weights and plasma bilirubin concentrations in control (C) and jaundiced (J) rats fed the experimental diets[1]

Measure	Rats	Control	High Cu	High Zn	HighCu+Zn	SEM	ANOVA[2]
Feed intake, g/100 g body weight.d							
	C	12.2	12.2	12.0	12.0		
	J	12.0	11.4[S]	11.6	10.8[S]	0.25	S
Body weight, g							
Initial	C	89	88	90	88		
	J	80[S]	80[S]	81[S]	81	2.0	S
Final	C	249	246	245	247		
	J	211[S]	211[S]	207[S]	199[S]	5.7	S
Liver weight, g/100 g body weight (d 28)							
	C	3.86	3.80	3.79	3.87		
		(0.05)	(0.10)	(0.16)	(0.06)		
	J	4.48[S]	4.56[S]	4.39	4.55[S]		
		(0.06)	(0.09)	(0.02)	(0.12)		NP
Plasma bilirubin, μmol/L							
Conjugated	C[4]	ND	ND	ND	ND		
	J	7.4	12.8	11.1[z]	11.6		
		(0.5)	(1.6)	(0.9)	(1.7)		NP
Total	C	1.2	1.0	1.5	1.1		
		(0.2)	(0.3)	(0.1)	(0.2)		
	J	13.4[S]	20.3[S,c]	15.8[S]	17.4[S]		
		(1.3)	(2.0)	(1.2)	(1.3)		NP

[1]Values are means for six rats, except for the control group of jaundiced rats which consisted of five animals. Pooled SEMs (for homogeneous variances) or separate SEMs (in parentheses) are given.

[2]ANOVA significance ($p<0.05$); S = significant strain effect (control vs. jaundiced rats); NP = ANOVA not performed because variances were not homogeneous.

[3]Group comparisons: s = significant strain difference ($p<0.025$; jaundiced vs. control rats); c = significant effect of high-copper diet within strains and for diets with identical amount of zinc ($p<0.025$; high-copper vs. control diet or high-copper, high-zinc vs. high-zinc diet); z = significant effect of high-zinc diet within strains and for diets with identical amount of copper ($p<0.025$; high-zinc vs. control diet or high-zinc, high-copper vs. high-copper diet).

[4]Student's t-test was not performed because of the lack of variance in the control rats.

ND: not detectable.

5.3 ± 0.1 and 5.1 ± 0.1 g/100 g body weight.day (mean ± SEM, n=18 or 15) for the jaundiced and control rats. The jaundiced rats had significantly higher relative liver weights than the control rats (Table 2). In experiment 2, average relative liver weights of the jaundiced and control rats were 4.33 ± 0.06 and 3.47 ± 0.05 g/100 g body weight (mean ± SEM, n=18 or 15).

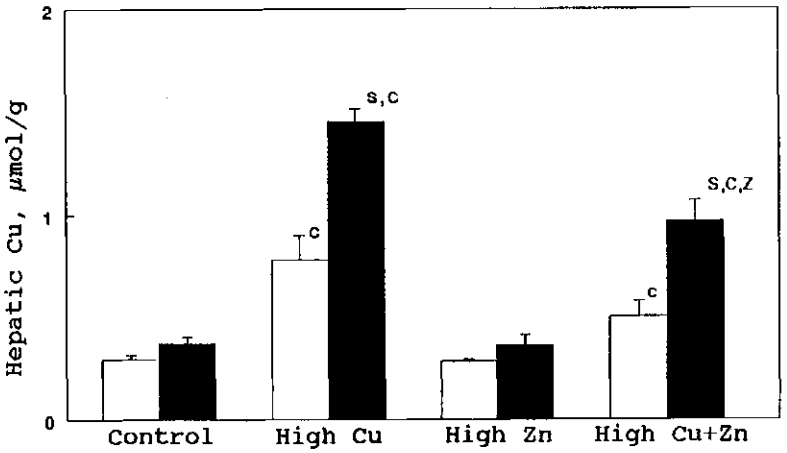

Fig. 1. Experiment 1: Hepatic copper concentrations in control and jaundiced rats fed the experimental diets for 28 days. Results are expressed as means and SEMs (vertical bars) for 6 rats per dietary group, except for the five jaundiced rats fed the control diet. Open bars: control rats; closed bars: jaundiced rats. Group comparisons: s = significant strain difference (control vs. jaundiced rats); c = significant effect of high-copper diet within strains and for diets with identical amount of zinc ($p<0.025$; high-copper vs. control diet or high-copper, high-zinc vs. high-zinc diet); z = significant effect of high-zinc diet within strains and for diets with identical amount of copper ($p<0.025$; high-zinc vs. control diet or high-zinc, high-copper vs. high-copper diet).

When fed the control diet, the jaundiced rats had hepatic copper concentrations that did not differ from those of the control rats (Fig. 1 and 2). Ceruloplasmin activities and copper concentrations in plasma of the jaundiced rats were generally

higher than those in control rats (Table 3). In experiment 2, baseline plasma copper concentrations of the jaundiced and control rats were 16.8 ± 1.0 and 13.6 ± 0.6 nmol/L (mean ± SEM, *n*=6 or 5).

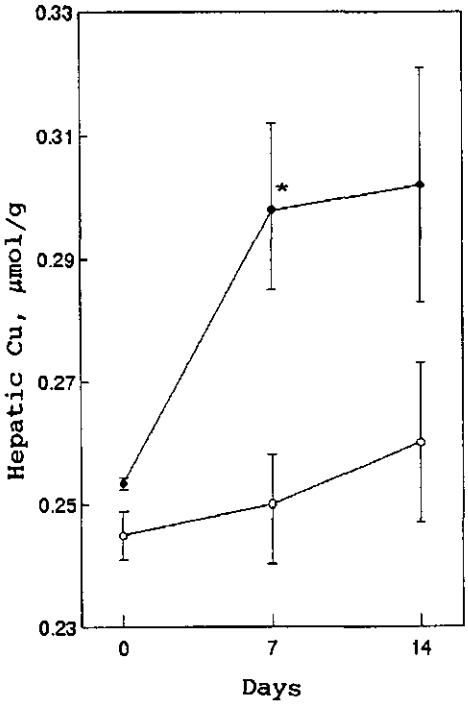

Fig. 2. Experiment 2: Hepatic copper concentrations in jaundiced (closed circles) and control (open circles) rats fed the high-copper diet from d 0. Each point represents the mean and SEM (vertical bar) for six jaundiced or five control rats. *: Significant strain difference ($p<0.05$).

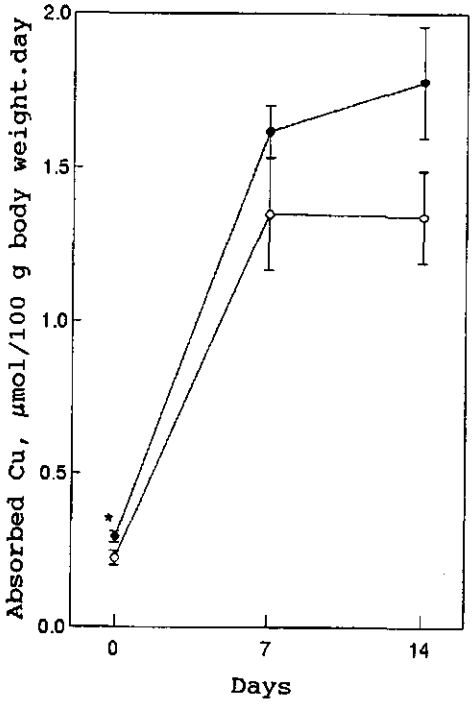

Fig. 3. Experiment 2: Absorbed amounts of copper in jaundiced (closed circles) and control (open circles) rats fed the high-copper diet from d 0. Each point represents mean and SEM (vertical bar) for six jaundiced or five control rats. *: Significant strain difference ($p<0.05$).

The bile flow in jaundiced rats was about 60% lower than that in control rats (Table 4). This was also found in experiment 2 (not shown). Biliary copper concentrations were systematically higher in jaundiced rats. Biliary zinc

concentration in the jaundiced rats did not differ significantly from that in the control rats, but absolute biliary zinc excretion was lower (Table 4). When fed the control diet, the rates of urinary copper and zinc excretion and apparent absorption of copper were similar in the two strains in experiment 1 (Table 5). In experiment 2, urinary copper excretion after feeding the control diet was higher in jaundiced than in control rats, the rates being 33.1 ± 3.0 and 25.2 ± 1.0 nmol/100 g body weight.day (mean ± SEM, $p<0.05$, n=6 or 5). Baseline apparent copper absorption was significantly higher in the jaundiced rats (Fig. 3). Apparent zinc absorption in the jaundiced rats was systematically higher than in the control rats (Table 5).

Responses to diets of body weight and feed intake

The experimental diets had no effect on feed intake and body weight of either strain of rats (Table 2). The experimental diets did not significantly influence liver weights. In experiment 2, the effects of the high-copper diet on body weight and feed intake could not be assessed because there were no groups fed the control diet concurrently.

Responses to diets of copper and zinc concentrations in organs

After feeding the diets with extra copper in experiment 1, the jaundiced rats accumulated more copper in their livers than did the control rats (Fig. 1). In experiment 2, hepatic copper concentrations rose in the jaundiced rats after they were transferred to the high-copper diet, but such an effect was not obvious in the control rats (Fig. 2). In both strains of rats, extra zinc in the diet alone did not affect hepatic copper concentration (Fig. 1). Extra zinc antagonized the copper accumulation seen after copper loading, but the jaundiced rats still had higher hepatic copper concentrations.

The high-copper diet, without or with supplemental zinc,

significantly raised copper concentrations in plasma (Table 3). In experiment 2, plasma copper concentrations did not significantly change in either strain after the rats had been transferred to the high-copper diet (not shown). Copper loading raised plasma ceruloplasmin activities in both strains of rats (Table 3).

Tabel 3. Experiment 1: Copper and zinc concentrations in liver and plasma in control (C) and jaundiced (J) rats fed the experimental diets[1]

Measure	Rats	Control	High Cu	High Zn	High Cu+Zn	SEM	ANOVA[2]
Plasma, μmol/L							
Cu	C	15.1	16.6	13.2	16.8		
	J	17.9	18.7	15.6	17.3	0.97	S;C
Zn	C	37.1	39.1	40.9	39.9		
		(0.8)	(2.3)	(2.0)	(0.8)		
	J	35.1	37.3	38.4[z]	39.4		
		(0.4)	(1.1)	(0.5)	(0.9)		NP
Plasma ceruloplasmin activity, absorbance change/L.min							
	C	188	208	145	178		
	J	203	209	176	208	9.6	S;C;Z
Liver, μmol/g dry wt							
Zn	C	1.60	1.62	1.67	1.65		
	J	1.56	1.55	1.50[s]	1.57	0.045	S

[1]Values are means for six rats, except for the control group of jaundiced rats which consisted of five animals. Pooled SEMs (for homogeneous variances) or separate SEMs (in parentheses) are given.

[2]ANOVA significance ($p<0.05$); S = significant strain effect (control vs. jaundiced rats); C = significant effect of dietary copper (high- vs. normal-copper diets); Z = significant effect of dietary zinc (high- vs. normal-zinc diets); NP = ANOVA not performed because variances were not homogeneous.

[3]Group comparisons: s = significant strain difference ($p<0.025$; jaundiced vs. control rats); z = significant effect of high-zinc diet within strains and for diets with identical amount of copper ($p<0.025$; high-zinc vs. control diet or high-zinc, high-copper vs. high-copper diet).

In experiment 1, Zinc loading slightly depressed plasma ceruloplasmin activities in the two strains (Table 3).

Supplemental zinc produced a slight increase in tibia zinc concentrations. The mean values after feeding the high-zinc and control diets were 3.26 and 3.08, and 3.26 and 3.07 μmol/g dry weight in control and jaundiced rats, respectively. The values after feeding the high-zinc, high-copper and high-zinc diets were 3.23 and 3.11, and 3.04 and 2.91 μmol/g dry weight in control and jaundiced rats, respectively. There was a significant effect of zinc ($p<0.05$, pooled SEM = 0.067, three-way ANOVA). Copper loading had no effect on hepatic zinc concentrations.(Table 3).

Responses to diets of biliary copper and zinc output

Dietary treatments had no effect on bile flow (Table 4). Fig. 4 shows that absolute biliary copper excretion in the jaundiced rats given the normal-copper diets without or with

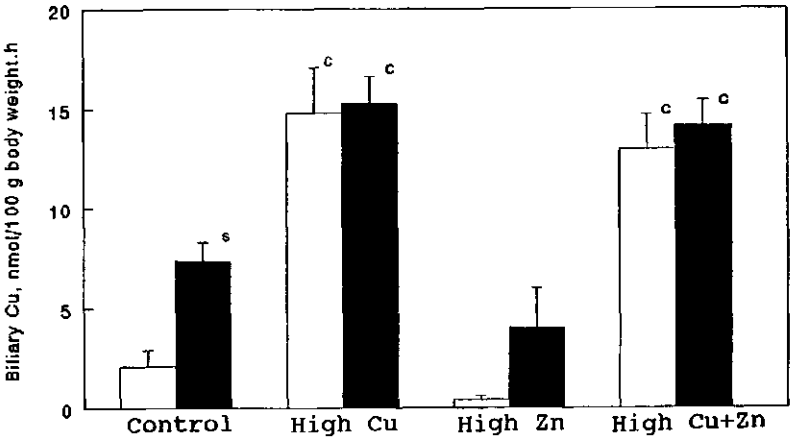

Fig. 4. Experiment 1: Biliary copper excretion in control and jaundiced rats fed the experimental diets for 28 days. Results are expressed as means and SEMs (vertical bars) for 6 rats per dietary group, except for the five jaundiced rats fed the control diet. Open bars: control rats; closed bars: jaundiced rats. Group comparisons: s = significant strain difference (control vs. jaundiced rats); c = significant effect of high-copper diet within strains and for diets with identical amount of zinc ($p<0.025$; high-copper vs. control diet or high-copper, high-zinc vs. high-zinc diet).

Table 4. Experiment 1: Bile flow and biliary output of copper and zinc in control (C) and jaundiced (J) rats fed the experimental diets[1]

Measure	Rats	Control	High Cu	High Zn	High Cu+Zn	SEM	ANOVA[2]
Bile flow, ml/100 g body weight.h							
	C	0.25	0.27	0.27	0.27		
		(0.01)	(0.02)	(0.01)	(0.02)		
	J	0.10^{S}	0.11^{S}	0.11^{S}	0.12^{S}		
		(0.01)	(0.01)	(0.01)	(0.01)		NP
Bile							
Cu, nmol/L	C	8.4	55.6^{C}	1.3^{Z}	48.2^{C}		
		(3.7)	(6.8)	(0.7)	(5.4)		
	J	72.0^{S}	$143.2^{S,C}$	$35.6^{S,Z}$	$133.5^{S,C}$		
		(8.1)	(9.7)	(17.5)	(17.1)		NP
Zn, μmol/L	C	1.9	2.4^{C}	1.9	2.6^{C}		
		(0.1)	(0.1)	(0.1)	(0.2)		
	J	2.4	2.9	2.7	3.1		
		(0.3)	(0.5)	(0.3)	(0.2)		NP
Zn, nmol/100 g body weight.h							
	C	0.48	0.64	0.51	0.68		
	J	0.26	0.32^{S}	0.29^{S}	0.34	0.052	S;C

[1]Values are means for six rats, except for the control group of jaundiced rats which consisted of five animals. Pooled SEMs (for homogeneous variances) or separate SEMs (in parentheses) are given.

[2]ANOVA significance ($p<0.05$); *S* = significant strain effect (control vs. jaundiced rats); C = significant effect of dietary copper (high- vs. normal-copper diets); NP = ANOVA not performed because variances were not homogeneous.

[3]Group comparisons: s = significant strain difference ($p<0.025$; jaundiced vs. control rats); c = significant effect of high-copper diet within strains and for diets with identical amount of zinc ($p<0.025$; high-copper vs. control diet or high-copper, high-zinc vs. high-zinc diet); z = significant effect of high-zinc diet within strains and for diets with identical amount of copper ($p<0.025$; high-zinc vs. control diet or high-zinc, high-copper vs. high-copper diet).

extra zinc was higher than in the control rats. However, after feeding the high-copper diets without or with extra zinc, biliary copper excretion rose to almost identical levels in the jaundiced

and control rats. Thus, the copper-induced stimulation of biliary copper excretion was less in the jaundiced than in the control rats. In experiment 2 (Fig. 5), this strain difference was clearer than in experiment 1. In the second experiment baseline biliary copper excretion was greater in the jaundiced rats and 14 days after transfer to the high-copper diet the enhanced excretion rates were lower than in the control rats. In experiment 2 (Fig. 3), copper absorption after feeding the

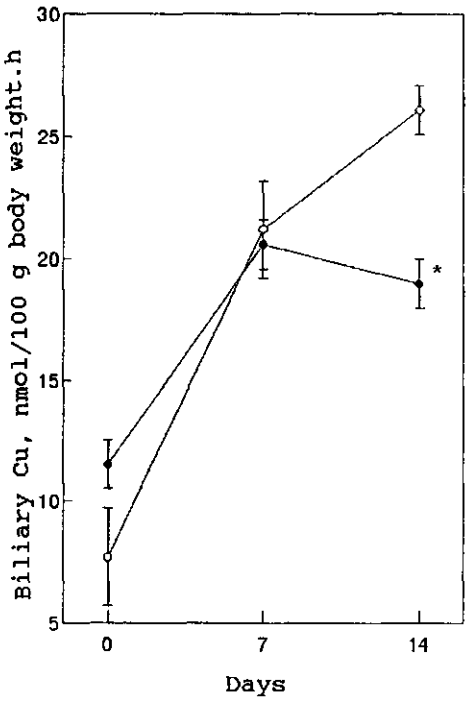

Fig. 5. Experiment 2: Biliary copper excretion in jaundiced (closed circles) and control (open circles) rats fed the high-copper diet from d 0. Each point represents the mean and SEM (vertical bar) for six jaundiced or five control rats. *: Significant strain difference ($p<0.05$).

high-copper diet was systematically higher in the jaundiced rats. Going from the normal- to the high-copper diet did not significantly change urinary copper excretion (not shown). Zinc loading tended to depress biliary copper concentrations (Table 4) and absolute copper output (Fig. 4) when the diet contained the normal amount of copper. Copper loading significantly

enhanced biliary zinc output (Table 4).

Responses to diets of copper and zinc balance

Table 5. Experiment 1: Copper and zinc balance in control (C) and jaundiced (J) rats fed the experimental diets[1]

Measure	Rats	Control	High Cu	High Zn	High Cu+Zn	SEM	ANOVA[2]
Apparent absorption[3], μmol/100 g body weight.day							
Cu	C	0.53	2.31[c]	0.53	2.11[c]		
		(0.04)	(0.33)	(0.05)	(0.09)		
	J	0.57	2.62[c]	0.60	2.56[c]		
		(0.04)	(0.14)	(0.03)	(0.31)		NP
Zn	C	1.09	1.23	5.95[z]	6.40[z]		
		(0.10)	(0.11)	(1.13)	(0.40)		
	J	1.20	1.33	7.18[z]	8.02[z]		
		(0.05)	(0.10)	(0.38)	(0.95)		NP
Urinary excretion, nmol/100 g body weight.day							
Cu	C	29.0	41.6[c]	24.2	39.6		
	J	26.1	42.6	22.7	39.2[c]	< 0.001	C
Zn	C	13.1	11.0	27.9[z]	46.7[z]		
		(1.8)	(1.1)	(4.8)	(13.2)		
	J	9.9	11.4	28.7[z]	45.8[z]		
		(0.8)	(2.6)	(3.5)	(11.1)		NP

[1]Values are means for six rats, except for the control group of jaundiced rats which consisted of five animals. Pooled SEMs (for homogeneous variances) or separate SEMs (in parentheses) are given.

[2]ANOVA significance ($p<0.05$); C = significant effect of dietary copper (high- vs. normal- copper diets); NP = ANOVA not performed because variances were not homogeneous.

[3]Apparent absorption was calculated as intake (based on chemical analysis of copper and zinc) minus fecal excretion.

[4]Group comparisons: c = significant effect of high-copper diet within strains and for diets with identical amount of zinc ($p<0.025$; high-copper vs. control diet or high-copper, high-zinc vs. high-zinc diet); z = significant effect of high-zinc diet within strains and for diets with identical amount of copper ($p<0.025$; high-zinc vs. control diet or high-zinc, high-copper vs. high-copper diet).

In experiment 1, the high-copper diets produced about a four-fold increase in copper absorption (Table 5). The high-zinc diets produced about a six-fold increase in zinc absorption. Feeding the high-copper diets resulted in higher output of copper with urine. In both strains, supplemental zinc raised urinary zinc excretion, this effect being greater when the diet was high in copper (Table 5).

DISCUSSION

Apart from the raised plasma concentrations of conjugated bilirubin, the jaundiced rats also differed from their control counterparts with regard to other variables. Body weight was lower in the jaundiced rats but absolute liver weight was not (not shown) so that relative liver weight was markedly higher in jaundiced rats. When fed the control diet, copper and zinc metabolism were altered in the jaundiced rats when compared with the control rats. Plasma ceruloplasmin activity and copper concentrations in plasma and liver were higher which was associated with higher biliary copper excretion. When compared with the control rats, the jaundiced rats had lower rates of biliary zinc output. From the present data it cannot be concluded whether the altered baseline metabolism of copper and zinc in the jaundiced rats is causally related to the defective hepatic transport of bilirubin in these rats.

On the basis of short-term experiments with anesthetized rats that were given massive doses of copper intracardially (Houwen et al., 1990), we had hypothesized that copper feeding causes more hepatic copper accumulation in jaundiced than in control rats. The data in Fig. 1 and 2 support our hypothesis: dietary copper loading produced about 50% greater liver copper pools in the jaundiced rats. Houwen et al. (1990) showed that after intracardiac administration of very high amounts of copper the rates of biliary copper excretion were lower in jaundiced than in control rats. We found this strain difference after 14

days of dietary copper challenge (Fig.5), but in experiment 1 the amounts of copper excreted in bile were similar in jaundiced and control rats (Fig. 4). Because baseline rates of biliary copper excretion were higher in the jaundiced rats, the copper-induced rise was smaller than in control rats. Thus, it is conceivable that the smaller increase in biliary copper excretion has contributed to the greater hepatic copper accumulation in the jaundiced rats after copper loading. In addition, the jaundiced rats systematically absorbed more copper when fed the high-copper diets (Table 5, Fig. 3). The strain difference was significant in experiment 2 ($p<0.05$ for pooled data for d 7 and 14) but failed to reach statistical significance in experiment 1 ($p=0.129$ for pooled data of the two high-copper diets). The more efficient intestinal copper absorption in the jaundiced rats probably also contributed to the greater copper-induced copper stores in liver.

When the jaundiced rats were fed the control diet, they had an elevated hepatic copper concentration associated with higher rates of biliary copper excretion and apparent copper absorption. Although it is known that biliary copper is poorly reabsorbed in the intestine (Klaassen, 1976; Farrer, 1967; Owen, 1964), the observed alterations in baseline copper metabolism of the jaundiced rats point to a more efficient entero-hepatic cycling of copper in these animals. This could imply that baseline rates of urinary copper excretion are greater in jaundiced than in control rats. In experiment 2 this was seen indeed (33.1 $\pm$ 3.0 vs 25.2 $\pm$ 1.0 nmol urinary copper/100 g body weight.day; mean $\pm$ SEM, $p<0.05$, $n=5$ or 6), but not so in experiment 1 (Table 5).

In individual rats, both biliary copper concentration and absolute biliary copper excretion were positively related to the hepatic copper concentration (Fig. 6). Thus, it seems that both biliary copper concentration and absolute excretion are determined by the hepatic copper concentration although the total amount of copper excreted with bile tended to plateau at higher hepatic copper concentrations. The available data do not convincingly indicate that for jaundiced rats the slopes of the

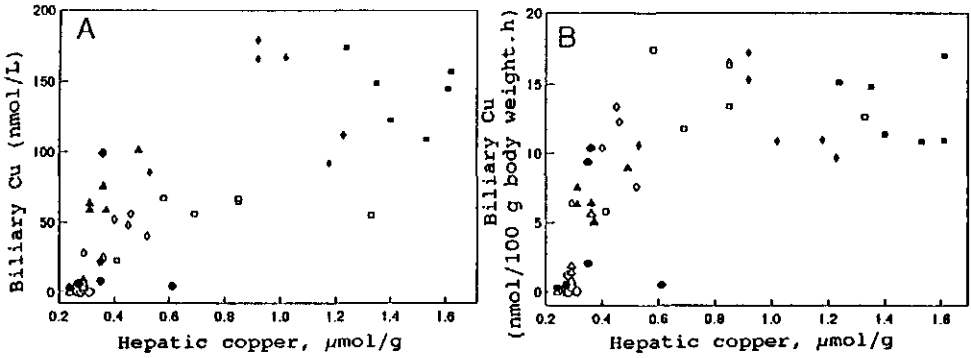

Fig. 6. Experiment 1: Biliary copper concentration (panel A) and absolute biliary copper excretion (panel B) as functions of hepatic copper concentration in individual control (open symbols) and jaundiced (closed symbols) rats fed the experimental diets for 28 days. Symbols: △, ▲ = control diet; □, ■ = high-copper diet; ○, ● = high-zinc diet; ◇, ◆ = high-copper, high-zinc diet. The regression equations are $y = 99 x - 0.8$ ($r=0.77$, $n=47$, $p<0.01$) for panel A and $y = 9 x + 2$ ($r=0.68$, $n=47$, $p<0.01$) for panel B.

relationships are smaller than for the control rats. It follows that the lesser increase in absolute biliary copper excretion in the jaundiced rats after the dietary copper challenge cannot be readily explained by a reduced efficiency of transport hepatic copper stores to bile. Possibly, the transport of copper from plasma into bile is diminished in the jaundiced rats, which would agree with the higher plasma copper concentrations in these rats. This reasoning implies that copper taken up from plasma by the liver does not mix indistinguishably with copper stores of the liver and that copper transport across the bile canalicular membrane is depressed rather than that across the sinusoidal membrane. The latter implication is supported by studies indicating that in the jaundiced rats there is impaired canalicular transport of gluthatione conjugates (Oude Elferink et al., 1989) and that gluthatione and copper transport are coupled (Alexander & Aaseth, 1980). Further support comes from the observation that zinc metabolism in the jaundiced rats was not altered so much as was copper metabolism. This can be

explained by the fact that the main route for endogenous copper excretion is the bile (Owen, 1964, 1965) whereas for zinc it is the pancreas (Cousins, 1985).

Zinc loading partly counteracted the copper-induced rise in hepatic copper concentration (Fig. 1). This effect has been reported earlier (Brewer et al., 1990; Storey & Greger, 1987; L'Abbé & Fischer, 1984; Murthy et al., 1974; Van Campen & Scaife, 1967) and is generally explained by inhibition of copper absorption. However, our data did not show inhibition of copper absorption by supplemental zinc, which could relate to the relatively mild zinc challenge imposed. The high zinc diets reduced biliary copper excretion which can be considered as an effect secondary to the lower hepatic copper concentration. Thus, it remains unknown why in the present study supplemental zinc partly counteracted the dietary-copper-induced rise in liver copper.

An interesting finding emerged from this study. The diet high in both copper and zinc produced higher rates of urinary zinc excretion than did the diet high in zinc alone (Table 5). Supplemental copper in the absence of extra zinc had no effect on urinary zinc excretion. As far as we know, the interrelated effect of zinc and copper on urinary zinc excretion has not been reported earlier. It is difficult to see how this effect is brought about but zinc balance would be maintained by the slightly raised apparent zinc absorption.

In summary, the feeding of high-copper diets caused higher liver copper concentrations in the jaundiced than in the control rats. This strain difference may be explained by a lesser increase in biliary copper excretion and slightly greater efficiency of dietary copper absorption in the jaundiced rats. Comparison of dietary effects in these strains of rats may result in new concepts in copper homeostasis and the etiology of copper toxicosis.

ACKNOWLEDGEMENTS

Shiguang Yu was supported by The Netherlands Foundation for Nutrition and Health Research. We thank Inez Lemmens for analytical assistance and Gerrit Van Tintelen for taking care of the rats.

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Chapter 5

IRON AND COPPER METABOLISM IN ANALBUMINAEMIC RATS FED A HIGH-IRON DIET

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(*submitted for publication*)

Iron and copper metabolism in analbuminaemic rats fed a high-iron diet

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Abstract: The metabolism of iron and copper in male Nagase analbuminaemic (NA) and Sprague Dawley (SD) rats was compared. The relative liver weight was higher and spleen weight significantly lower in NA than SD rats. In NA rats, red blood cell count, haemoglobin and haematocrit were lower whereas plasma transferrin, total iron-binding capacity and mean corpuscular haemoglobin were higher when compared with SD rats. Iron concentrations in plasma, liver, kidneys and heart were higher and those in spleen and tibia were lower in NA rats. The iron concentrations in liver and spleen were positively correlated with the amount of brown pigment as observed histologically. Bile flow as well as biliary iron and copper excretion were higher in NA than SD rats. Copper concentrations in liver, kidneys and plasma were higher in NA rats. Plasma levels of ceruloplasmin were about two-fold higher in NA rats. The feeding of a high-iron diet

reduced kidney copper concentrations in both strains of rats, which was associated with a decrease in the absorption and biliary excretion of copper.

INTRODUCTION

The Nagase analbuminaemic (NA) rat is a mutant Sprague Dawley (SD) rat that almost completely lacks serum albumin (Nagase et al., 1979). Apart from being a carrier of free fatty acids and hormones in the blood (Vallner, 1977; Kragh-Hansen, 1981), albumin also is a transport form of trace elements (Lau & Sarkar, 1984). Thus, it is feasible that NA rats have an aberrant metabolism of iron and copper. Indeed, Suzuki et al. (1986) showed that NA versus SD rats have lower hepatic iron and copper concentrations. In the present study selected indicators of iron and copper metabolism were compared and contrasted in NA and SD rats fed purified diets containing either a normal or high level of iron. High intakes of iron not only depress intestinal absorption but also biliary excretion of copper in rats (Yu et al., 1994), and thus challenge regulatory mechanisms of iron and copper metabolism. It was expected that this comparative nutritional study with NA and SD rats would provide further insight into the regulation of iron and copper metabolism.

MATERIALS AND METHODS

The experimental protocol was approved and its conduct supervised by the animal welfare officer of the Wageningen Agricultural University.

Animals and experimental procedures

Male Nagase analbuminaemic (NA) rats (Central Animal Facility, Utrecht University, The Netherlands) and male Sprague Dawley (SD) rats (SD/Hsd-Ola, Harlan CPB, Zeist, The Netherlands) aged about 9 weeks were used. On arrival, the rats were housed

in groups of 4 or 5 animals of the same strain in stainless steel cages (60 x 20 x 19 cm) with wire mesh bases placed in a room with controlled lighting (light on: 06.00-18.00 h), temperature (20-22 °C), and relative humidity (55-57%). The rats were fed ad libitum on a purified control diet (Table 1) and demineralized water for a period of 12 days.

Then, the rats were divided into two groups of 6 rats each per strain. Within each strain, the groups were stratified for body weight. One group within each strain remained on the control diet and the other was transferred to the high-iron diet (Table 1).

Table 1. Composition of the experimental diets

	Control	High Fe
Ingredients, g/kg		
Constant components[1]	340.6	340.6
Maize starch	329.6	328.6
Glucose	329.6	328.6
$FeSO_4.7H_2O$	0.174	2.200
Chemical analysis, mg/kg		
Iron	38.6	593.1
Copper	3.9	4.1

[1]The constant components consisted of (g): casein, 151; maize oil, 25; coconut oil, 25; molasses, 50; cellulose, 30; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; $CaCO_3$, 12.4; $NaH_2PO_4.2H_2O$, 15.1; mineral premix, 10; vitamin premix, 12. The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize starch 9853.2; The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D, L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (1200 retinol equivalents); cholecalciferol, 2; maize meal, 9826.15.

The groups were randomly assigned to the diets. The control diet was formulated according to the nutrient requirements of rats (National Research Council, 1978) and contained 35 mg added Fe/kg diet. The high-iron diet had a composition identical to that of the control diet but contained 443 mg added Fe/kg diet. Iron was added to the diets in the form of $FeSO_4.7H_2O$ and extra iron was added at the expense of maize starch and glucose in a 1:1 ratio. The purified diets were in powdered form and stored at 4 °C until used for feeding.

After formation of the four experimental groups, the rats were individually housed in metabolism cages (314 cm^2 x 12 cm). The rats had free access to feed and demineralized water for another four weeks. Faeces and urine of each rat were quantitatively collected during the last week of the experimental period. Feed intake and body weight were recorded regularly.

At the end of the experiment, bile was collected by common bile duct cannulation with the use of polyethylene tubing (i.d. 0.28 mm, o.d. 0.61 mm, Intramedic, Clay Adams, Parsippary, NJ, U.S.A.). The abdomen of the rats was opened while they were under anaesthesia using a combination of ketamine (6 mg/100 g body weight) administered intramuscularly and xylazine (0.8 mg/100 g body weight) administered subcutaneously. Fleck and Barth (1990) had shown that the combination of the two drugs does not affect bile flow in rats. After the tubing was inserted into the common bile duct and secured with suture thread, the rats were kept on a heating pad (36-38 °C) and bile was collected into pre-weighed vials for a period of 60 min. The volume of collected bile was calculated using its weight and specific gravity.

Following bile collection, blood samples were taken from the anaesthetized rats into heparinized tubes by abdominal aorta puncture. Then, the rats were killed by decapitation and organs were removed and weighed. A portion of the liver (about 1 g) and about half of the spleen were put into 4% (v/v) of formaldehyde for histological examination. Plasma was separated from blood samples by centrifugation at 3000 rpm at room temperature for 25

min. Bile, organ and plasma samples were stored at -20 °C until analysis.

Analytical methods

Iron and copper in organs were determined by flame atomic absorption spectrometry (Perkin-Elmer 2380, Norwalk, CT, U.S.A.) after the samples had been dried in a vacuum dryer for 48 h and wet digested in 14 M nitric acid (Suprapur, Merek, Darmstadt, Germany) at 80 °C for 2 h. Iron in the diets was measured similarly without prior drying of the samples. For the determination of iron and copper in faeces and copper in the diets, the samples were ashed at 500 °C for 17 h in a muffle furnace and subsequently dissolved in 6 M HCl; iron and copper were measured using flame atomic absorption spectrometry. Iron and copper in urine and copper in plasma were measured directly with flame atomic absorption spectrometry. The determinations of copper and iron in bile were carried out with flameless atomic absorption spectrometry (Varian SpectrAA-300, Varian Techtron Pty. Ltd., Springvale, Australia) after the samples had been properly diluted with demineralized water. Reference bovine liver sample (NBS 1577a, National Institute of Standards Technology, Gaithersburg, MD, U.S.A.) was used to assess accuracy of iron and copper analysis. Iron and copper concentrations measured in the reference sample were on average 114 and 98% of the certified values.

Haematological parameters of fresh, heparinized blood samples were measured using the Sysmex K-1000D (Sysmex-TOA, TOA Medical Electronics Co, Ltd, Kobe, Japan). Iron and total iron-binding capacity in plasma were determined with a commercially available kit and the COBAS-BIO autoanalyser (Hoffmann-La Roche BV, Mijdrecht, The Netherlands). Ceruloplasmin in plasma was assayed as p-phenylenediamine oxidase activity as described (Sunderman & Nomoto, 1970). Transferrin was measured in plasma using an electroimmunodiffusion assay (Laurell, 1972; Kaysen & Watson, 1982). Rabbit anti rat transferrin was obtained from

Cappel, Organon Teknika, Durham NC, USA. Homogenous rat transferrin was also obtained from Organon Teknika and used as a standard. Albumin in plasma was measured as described by Mancini et al. (1965). Total protein in plasma was determined colorimetrically using a commercially available kit (Bio-Rad Lab, Munich, Germany).

The formaldehyde-fixed samples of liver and spleen were processed using routine histological methods. The samples were embedded in paraplast, cut at 5 μm and stained with haematoxylin and eosin or with Perl's staining for iron. The slides were examined microscopically in random order by a person who was blinded to treatment modality.

Statistical analysis

The SPSS software package (SPSS Inc., Chicago, IL, U.S.A.) was used to evaluate the results statistically. If the variances were homogeneous (Bartlett's test), the data were subjected to two-way analysis of variance (ANOVA) with strain of rats (NA versus SD rats) and amount of iron in the diet (high versus normal) as main effects. The level of statistical significance was preset at $p<0.05$. Statistical significance of the main effects was also identified in select, direct comparisons with the use of Student's t-test or with the Mann-Whitney U test if the variances were not homogeneous (F test). The Mann-Whitney U test was also used to evaluate the scores from the histological examination of liver and spleen. The level of significance was preset at $p<0.025$ to take into account the increased probability of a type I error due to multiple comparisons (Bonferroni's adaptation).

RESULTS

Plasma albumin

As would be expected, NA rats were almost completely deficient in plasma albumin (Table 2). The total protein

concentration in plasma was significantly lower in NA than in SD rats. Dietary iron had no effect on total protein and albumin concentrations in plasma.

Table 2. Feed intake, body and organ weights, and plasma protein concentrations of SD and NA rats fed the experimental diets[1-3]

	Control diet		High-Fe diet		SEM	ANOVA
	SD rats	*NA rats*	*SD rats*	*NA rats*		
Feed intake, g/d	18.7	19.9	18.4	20.1	0.6	S
Body weight, g						
Initial	326.5	314.6	322.6	311.7	9.2	
Final	379.1	369.5	368.5	370.3	10.9	
Organ weight, g/100 g body weight						
Liver	3.27	3.98[s]	3.11	3.79[s]	0.09	S
Spleen	0.22	0.19[s]	0.21	0.19	0.01	S
Kidney	0.29	0.29	0.29	0.28[d]	0.01	
Heart	0.31	0.33	0.32	0.29	0.02	
Plasma protein, mg/ml						
Total	54.6	46.3[s]	52.5	46.0[s]	1.1	S
Albumin	28.2	0.01[s]	27.9	0.01[s]		
	(0.4)	(0.001)	(0.2)	(0.002)		

[1] Values are means for 4-6 rats per group; pooled SEMs are given for homogeneous variances and separate SEMs are given in parentheses when variances are not homogeneous.

[2] ANOVA significance ($p<0.05$): S = strain effect (NA versus SD rats); D = dietary iron effect (high versus normal dietary iron).

[3] Group comparisions ($p<0.025$): s = significant strain difference (NA versus SD rats) for rats fed the same diet; d = significant dietary iron effect (high versus normal dietary iron) for rats of the same strain.

Growth performance and organ weights

Feed intake was higher in NA than SD rats (Table 2). Initial and final body weights of the two strains were similar. Relative liver weight was greater and that of spleen lesser in NA rats. Relative kidney and heart weights did not differ between NA and SD rats. The greater relative liver weight in NA rats has been

seen previously (Suzuki et al., 1986; Joles et al., 1991; Zhang et al., 1992). Contrary to our findings, Suzuki et al. (1986) and Joles et al. (1991) reported that relative spleen and kidney weight were higher and lower, respectively, in NA rats than in SD rats. These discrepancies could relate to the use of rats with different ages. The high-iron diet did not affect feed intake and body and organ weights in either strain of rats.

Iron status

There were various significant differences in haematological parameters and iron concentrations in organs between NA and SD rats (Table 3). In NA rats, red blood cell count, haemoglobin and haematocrit were significantly lower, whereas mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were significantly higher. The concentration of iron, total iron-binding capacity and transferrin in plasma were significantly higher in NA than in SD rats. Iron concentrations in spleen and tibia were lower in NA rats, but those in liver, kidney and heart were higher. Feeding the high-iron diet produced an increase in iron concentrations in spleen, but did not elicit a strain difference in response.

Copper status

Plasma ceruloplasmin activities and copper concentrations in plasma, liver and kidneys were higher in NA rats than in their SD counterparts (Table 4). There was no strain difference in spleen, heart and tibia copper concentrations. Dietary iron loading slightly reduced copper concentrations in kidneys in both strains of rats.

Absorption and urinary excretion of iron and copper

When fed on the control diet, NA versus SD rats tended to have lower efficiencies of apparent iron and copper absorption (intake minus faecal excretion) (Table 5). Urinary excretion of iron and copper did not differ between the two strains. After

Table 3. Indicators of iron status in SD and NA rats fed the experimental diets[1-3]

	Control diet		High-Fe diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Red blood cell count, 10^{12}/L						
	8.6	7.5[S]	8.5	7.3[S]	0.1	S
Haemoglobin, mmol/L						
	11	10[S]	11	9[S]	0.1	S
Haematocrit, %						
	56	47[S]	55	46[S]	0.6	S
Mean corpuscular volume, fL						
	65	63	64	63	0.6	
Mean corpuscular haemoglobin, fmol						
	1.2	1.3[S]	1.2	1.3[S]	0.4	S
Mean corpuscular haemoglobin concentration, mmol/L						
	18	20[S]	19[d]	20[S]	0.1	S,D
Plasma						
Fe, μmol/L	28	36[S]	27	39[S]	1.5	S
Total iron-binding capacity, μmol/L						
	75	111[S]	73	113[S]	2.5	S
Transferrin, mg/ml						
	8.3	11.3[S]	8.3	11.8[S]	0.4	S
Iron in organs, mg/g dry weight						
Spleen	2.9	2.6	3.8	2.9[S]	0.23	S,D
Liver	0.53	0.66	0.55	0.69[S]	0.03	S
Kidney	0.26	0.32[S]	0.26	0.33[S]	0.01	S
Heart	0.35	0.42[S]	0.34	0.36	0.01	S
Tibia	0.10	0.07[S]	0.10	0.07[S]	<0.001	S

[1-3] See legends to Table 2.

feeding the high-iron diet, the percentages of apparent absorption of iron and copper were markedly depressed. Absolute iron intake was enhanced but that of copper diminished. In the rats fed the high-iron diet, urinary iron excretion was raised and urinary copper excretion left unchanged. Feeding the high-iron diet did not elicit qualitative strain differences in the response of absorption and urinary excretion of iron and copper.

However, in quantitative terms the group mean iron-induced depressions of iron and copper absorption were less in the NA than in SD rats.

Table 4. Indicators of copper status in SD and NA rats fed the experimental diets[1-3]

	Control diet		High-Fe diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Plasma						
Cu, μg/ml	0.81	1.29[S]	0.78	1.17[S]		
	(0.02)	(0.07)	(0.01)	(0.03)		
Ceruloplasmin[4], absorbance units						
	169	400[S]	160	330[S]		
	(5)	(21)	(5)	(23)		
Organ copper, μg/g dry weight						
Liver	13.8	16.3[S]	13.9	15.5	0.7	S
Kidney	25.5	32.8[S]	22.1	27.5[S,d]	1.0	S,D
Heart	27.9	27.5	27.5	27.8	0.3	
Spleen	5.6	6.3	5.7	5.7	0.3	
Tibia	3.4	3.2	3.3	3.3	0.2	

[1-3] See legends to Table 2.

[4] Ceruloplasmin oxidative activity is expressed as absorbance change due to formation of the coloured oxidation product of p-phenylenediamine at 530 nm (Δ absorbance/L.min).

Biliary iron and copper excretion

The bile flow was slightly greater in NA than in SD rats (Table 6). Biliary iron and copper excretion were systematically greater in NA rats. The high-iron diet had no effect on bile flow and biliary iron output but reduced biliary copper concentration and excretion significantly. The mean decrease in biliary copper excretion after feeding the high-iron diet was two-fold greater in NA rats than in SD rats (0.23 versus 0.11 μg/100 g body weight. h).

Table 5. Apparent absorption and urinary excretion of iron and copper in SD and NA rats fed the experimental diets[1-3]

	Control diet		High-Fe diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Fe absorption,						
% of intake	15.9	9.5	6.3[d]	7.9	0.7	D
mg/d	0.15	0.09	0.56[d]	0.76[d]	0.09	D
Cu absorption,						
% of intake	37.2	21.8	6.5[d]	7.0	1.8	D
µg/d	27.0	17.0	4.5[d]	5.3	2.3	D
Urinary excretion, µg/d						
Fe	3.0	4.1	8.9[d]	10.0[d]	2.4	D
Cu	5.8	5.7	6.2	5.1	2.3	

[1-3] See legends to Table 2.

Table 6. Bile flow and biliary iron and copper excretion in SD and NA rats fed the experimental diets[1-3]

	Control diet		High-Fe diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Bile flow, ml/100 g body weight.h						
	0.29	0.35[S]	0.30	0.32	0.02	S
Biliary Fe,						
µg/ml	2.3	2.6	2.4	2.8	0.2	S
µg/100 g body weight.h						
	0.67	0.89[S]	0.70	0.91[S]	0.07	S
Biliary Cu,						
µg/ml	0.90	1.54[S]	0.52	0.90	0.23	S,D
µg/100 g body weight.h						
	0.27	0.51[S]	0.16	0.28[S]	0.07	S,D

[1-3] See legends to Table 2.

Histopathology of liver and spleen

As based on the presence of Perl's positive material, the NA rats were found to have considerably more iron in the Kupffer cells than did SD rats ($p<0.025$) (Table 7). The Perl's positive material coincided with the presence of brown pigment as observed in parallel slides stained with haematoxylin and eosin. Part of the brown pigment probably represented lipofuscin. The incidence of Perl's positive hepatocytes did not differ between NA and SD

Table 7. Frequency distribution and amount of Perl's positive material in liver and spleen of NA and SD rats fed the experimental diets[1]

	Score	Control diet		High-Fe diet	
		SD rats	NA rats	SD rats	NA rats
Kupffer cells	0	4	-	3	-
	1	2	-	3	-
	2	-	3	-	3
	3	-	3	-	3
	4,5	-	-	-	-
Hepatocytes	0	4	2	5	3
	1	2	4	1	2
	2	-	-	-	1
	3-5	-	-	-	-
Spleen red pulp	0	-	-	-	-
	1	1	1	-	-
	2	2	4	-	4
	3	1	1	2	2
	4	1	-	3	-
	5	1	-	1	-
Spleen white pulp	0	1	-	4	-
	1	4	1	2	-
	2	1	5	-	6
	3-5	-	-	-	-

[1] There were 6 rats per group. The amount of Perl's positive material is expressed on a scale from 0 (none) to 5 (very high).

rats. The high-iron diet did not influence the amount of Perl's positive material in the liver of both NA and SD rats. Hepatocellular vacuolation, mainly of the centrilobular hepatocytes, was observed in NA rats only; it was not influenced by dietary iron concentration (not shown).

The amount of Perl's positive material in the red pulp of the spleen was greater in SD than in NA rats ($p<0.025$); this strain difference was somewhat more pronounced after consumption of the high-iron diet. Perl's positive material correlated with the presence of brown pigment aggregates seen in slides stained with haematoxylin and eosin. Perl's positive material in the white pulp of spleen was greater in NA rats than in SD rats fed high-iron diet ($p<0.025$). In NA rats, but not in SD rats, there was a diet-independent, decreased cellularity in the marginal zones of the spleen (not shown).

DISCUSSION

The analbuminaemic rats differed from SD rats with regard to various haematological indicators of iron metabolism. NA rats had decreased values of red blood cell count, haemoglobin and haematocrit and increased values of plasma iron concentration, mean corpuscular haemoglobin concentration and total iron-binding capacity. This is in agreement with findings of Sugiyama et al. (1984). The altered haematological indices in NA rats were associated with an altered distribution of iron in organs. Lower iron concentrations were observed in tibia and spleen, but liver, kidney and heart had higher concentrations. These data are at variance with those of Suzuki et al. (1986) who found that hepatic iron concentrations in NA rats were lower than in SD rats, whereas other organs did not show a strain difference. The rats studied by Suzuki et al. (1986) were fed on a commercial rat diet, which probably contained an iron concentration much higher than that of our control diet. However, this does not explain the discrepancy because feeding the high-iron diet did not influence

the strain difference in distribution of iron between organs. The higher hepatic iron concentration in NA versus SD rats corroborates the greater amount of Perl's positive material in the Kupffer cells. Likewise, in NA rats a lower iron concentration in spleen was associated with lower amounts of Perl's positive material in the red pulp of the spleen.

As to the iron stores in organs, in the NA rats there was a decrease in tibia iron concentration. It is tempting to speculate that this is responsible for the lower red blood cell counts, haemoglobin level and haematocrit values in the NA rats. The lower tibia iron concentration in NA rats may not be related to a lower efficiency of iron absorption because feeding the high-iron diet did not elevate tibia iron in NA rats. As reported earlier (Emori et al., 1983), plasma transferrin was increased in NA versus SD rats, resulting in a higher plasma total iron-binding capacity. In NA rats there is enhanced synthesis of transferrin in the liver (Sugiyama et al., 1987). This effect is amplified by the higher relative liver weight in NA rats. It is not clear what the stimulus is for the increased level of plasma transferrin in NA rats. The degree of saturation of transferrin with iron was similar in NA and SD rats, the values being on average 33 and 38%.

Copper concentrations in plasma, liver and kidney were higher in NA rats than in SD rats. This is surprising in the light that plasma albumin plays an important role as copper carrier (Sarkar & Kruck, 1966; Lau & Sarkar, 1971). However, there were markedly higher plasma ceruloplasmin activities in the NA rats as has been shown earlier by Sugiyama et al. (1982), which may be explained by enhanced synthesis and secretion of ceruloplasmin by hepatocytes of NA rats. The higher ceruloplasmin level and higher plasma and liver copper concentrations in NA rats could be associated with a higher whole-body turnover of copper because biliary copper excretion was higher in the NA rats. However, NA and SD rats had similar rates of absorption and urinary excretion of copper. Thus, it is possible that in the NA

rats there is an enhanced flux of copper through the enterohepatic cycle.

In both strains of rats, feeding the high-iron diet did not have a marked impact on the indicators of iron status. This is difficult to understand. Although the efficiency of iron absorption was depressed by iron loading, the absolute amount of iron absorbed was drastically increased. The increase in urinary iron excretion was negligible when compared with the increase in iron absorption. As reported earlier for another strain of rats (Yu & Beynen, 1992), dietary iron loading did not affect biliary iron excretion. Thus, there must have been accumulation of iron in organs other than those measured. In any event, the question remains what is the fate of the extra iron that was absorbed after iron loading.

In keeping with a previous study (Yu et al., 1994), the dietary iron challenge produced a decrease in apparent copper absorption. This effect was accompanied by a fall of biliary copper excretion. This can be interpreted as a compensatory response in an attempt to maintain copper homeostasis. Thus, apart from the slight reduction in kidney copper, the indicators of copper status were not influenced by feeding the high-iron diet. In this regard there was no difference between the two strains. This is somewhat surprising because the group mean iron-induced reduction of copper absorption was smaller (11.7 versus 22.5 μg/d) and that of biliary copper excretion was greater (0.23 versus 0.11 μg/100 g body weight.h) in NA than in SD rats. Since there was no clear strain difference in copper distribution between organs, the origin of the extra copper excreted in the bile of NA rats is unknown.

In conclusion, the NA and SD rats were found to differ concerning various indicators of iron and copper status. The differences cannot be readily explained by the higher plasma levels of transferrin and ceruloplasmin in the analbuminaemic rats. Likewise, the observed intestinal absorption, and urinary and biliary excretion of iron and/or copper did not provide

clues. Possibly, strain differences in iron and copper fluxes within the body are involved.

ACKNOWLEDGEMENTS

Shiguang Yu was supported by The Netherlands Foundation for Nutrition and Health Research. This work was supported in part by a grant from the National Institutes of Health RO1 DK 42297 and in part by the research service of the United States Department of Veterans Affairs. We thank Inez Lemmens for assistance with chemical analyses and Gerrit Van Tintelen for taking care of the rats.

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Chapter 6

COPPER METABOLISM IN ANALBUMINAEMIC RATS FED A HIGH-COPPER DIET

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(submitted for publication)

Copper metabolism in analbuminaemic rats fed a high-copper diet

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Abstract: Copper metabolism in male Nagase analbuminaemic (NA) rats was compared with that in male Sprague Dawley (SD) rats fed purified diets containing either 5 or 100 mg Cu/kg diet. Dietary copper loading increased hepatic and kidney copper concentrations in both strains to the same extent, but baseline values were higher in the NA rats. There was no strain difference in true and apparent copper absorption nor in faecal endogenous and urinary copper excretion. The NA rats had higher levels of radioactivity in kidneys at two hours after intraperitoneal administration of ^{64}Cu. As based on the distribution of added ^{64}Cu about 70% of plasma copper appeared to be in the non-protein compartment in the NA rats whereas in SD rats it was only about 1%. It is conluded that the NA rats are able to maintain a relatively normal metabolism of copper even after dietary copper challenge. In the NA rats, zinc concentrations in kidneys, liver and urinary zinc excretion were elevated when compared with SD rats. The high-copper diet did not affect tissue zinc concentrations and apparent zinc absorption in both strains of rats.

INTRODUCTION

Nagase et al. (1979) have reported on an analbuminaemic,

mutant strain of Sprague Dawley (SD) rats. In this so-called Nagase analbuminaemic (NA) rat, there is a deletion of seven base pairs from base 5 to 11 from the 5' end of intron HI of the albumin gene (Esumi et al., 1983). Despite the absence of plasma albumin, NA rats do not show overt clinical abnormalities and they share similarities in this respect with human patients with analbuminaemia (Nagase, 1987). The NA rat is an interesting animal model to study the functions of albumin in plasma.

Plasma albumin plays an important role in the transport of absorbed copper from the intestine to the liver (Peters & Hawn, 1967; Gordon et al., 1987). Surprisingly, NA rats were found to display higher rates of biliary copper excretion than their SD counterparts (Yu et al., 1994), pointing to a higher whole-body turnover of copper in NA rats. In the present study, we further examined various aspects of copper metabolism in NA versus SD rats. In an attempt to modulate copper metabolism, the rats of both strains were fed purified diets with either a normal (5 mg/kg) or a high (100 mg/kg) copper concentration. Since plasma albumin also participates in zinc transport (Lau & Sarkar, 1984), the monitoring of selected aspects of zinc metabolism was added to this experiment.

MATERIALS AND METHODS

The experimental protocol was approved by the animal experiments committee of the Rotterdam Erasmus University, serving for the Delft University of Technology.

Animals, housing and diets

Male Nagase analbuminaemic (NA) rats (Central Animal Facility, Utrecht University, The Netherlands) and male Sprague Dawley (SD) rats (SD/Hsd-Ola, Harlan CPB, Zeist, The Netherlands) aged about 3 weeks were used. On arrival, the rats were housed in groups of 3 or 4 animals of the same strain in wire-topped, polycarbonate cages (37.5 x 22.5 x 15.0 cm) with inlaid wire

bottoms above a layer of sawdust. The cages were placed in a room with controlled lighting (light on: 06.00-18.00 h), temperature (20-22 °C), and relative humidity (55-57%). The rats had free access to demineralized water and the purified control diet (Table 1) for the pre-experimental period of 11 days.

Table 1. Composition of the experimental diets

	Normal Cu	High Cu
Ingredients		
Constant components[1], g	290.6	290.6
Glucose, g	709.4	709.1
$CuSO_4.5H_2O$, mg	15.7	314.0
Chemical analysis, mg/kg		
Copper	4.8	102.6
Zinc	17.2	17.2

[1]The constant components consisted of (g): casein, 151; maize oil, 25; coconut oil, 25; cellulose, 30; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; $CaCO_3$, 12.4; $NaH_2PO_4.2H_2O$, 15.1; mineral premix, 10; vitamin premix, 12. The mineral premix consisted of (mg): $FeSO_4.7H_2O$, 174; MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize starch 9694.9. The vitamin premix consisted of (mg) thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (1200 retinol equivalents); cholecalciferol, 2; maize starch, 9826.15.

Then, the rats were divided into two groups of 6 rats per strain so that within-strain body weight distributions of the groups were similar. One group of each strain was transferred to the high-copper diet (Table 1) while the other remained to be fed on the control diet. The control diet was formulated according to the nutrient requirements of rats (National Research Council, 1987) and contained 4 mg added Cu/kg diet. The high-copper diet

contained 80 mg added Cu/kg. Copper was added to the diets in the form of $CuSO_4.5H_2O$. The experimental diets, which were in powdered form, were stored at 4 °C until used for feeding.

After allocation to the dietary groups (day 0 of the experiment), the rats were kept individually in metabolism cages (314 cm^2 x 12 cm) and had free access to the diets and demineralized water. Feed intake, corrected for spillage, and body weights were recorded at regular intervals.

Measurement of true copper absorption

A cross-over design and a whole-body counting method were employed to measure true copper absorption. On day 14, three animals per dietary group of each strain received [^{64}Cu]Cu-acetate in an extrinsically labelled meal. The remaining three animals of each group were injected with the radiotracer intraperitoneally. To equalize handling and treatment of the rats, those receiving the radiotracer orally were injected intraperitoneally with stable copper in saline and those injected with ^{64}Cu were given a meal to which stable copper was added. On day 21, the administration route of the radiotracer for each animal was alternated. On the days of radiotracer administration, treatment order was randomized.

The radioactive meals were prepared by adding 5 μg of ^{64}Cu (1.5 MBq) in 0.20 ml of sodium acetate buffer (0.05 mol/L, pH 5.4) to 2 g of experimental diet. For intraperitoneal administration, 0.20 ml of the radiotracer solution was injected. The meals with the radiotracer or with an identical amount of stable copper were presented to the rats after a 16-h fast. The meals were consumed within 5 min. Subsequently, the intraperitoneal injection was given.

Radioactivity in individual rats was counted within 5 min after administration of ^{64}Cu. Thereafter, all rats were given free access to their experimental diets. For another 4 days (days 14-17 and 21-24), the animals were counted periodically. Faeces and urine of individual rats were collected quantitatively every

day for 4 days and radioactivity as well as the amount of copper were measured. All animals were also measured on day 20, i.e. one day before the second administration of the radiotracer; whole-body activity was found not to differ from background measurements.

The retention of ^{64}Cu in the rats was measured in a specially designed whole-animal liquid scintillation counter (Van Barneveld & Van den Hamer, 1984). The efficiency of the whole-body counter for detection of ^{64}Cu was 14%, and its stability was monitored by counting a ^{65}Zn source. ^{64}Cu was obtained by irradiating a copper wire (purity 99.999%, Ventron, Karlsruhe, Germany) in a thermal neutron flux of 1×10^{17} $m^{-2}.s^{-1}$ for 36 h in the research reactor of the Interfaculty Reactor Institute, Delft University of Technology. Following irradiation, the wire was dissolved in 25 µl of HNO_3 (35%) and diluted with sodium acetate buffer (0.05 mol/L, pH 5.4), resulting in a final copper concentration of 1 g/L.

Distribution of radioactive copper in organs

On day 29 half of the rats of each group were injected intraperitoneally with [^{64}Cu]Cu-acetate (1 µg Cu, 0.3 MBq) in 0.25 ml of sodium acetate buffer (0.05 mol/L, pH 5.4). Two hours later the rats were anaesthetized by intraperitoneal administration of 15 mg pentobarbital (Nembutal®, Sanofi Sante Animale SA, Paris, France), and blood was obtained from the abdominal aorta and collected into heparinized tubes. Plasma was isolated immediately by low-speed centrifugation. Liver, heart, kidneys, spleen and muscle (left *flexor digitorum longus*) were excised, weighed, counted for radioactivity, and subsequently stored at -20 °C until chemical analysis. On day 30 the procedures were repeated with the remaining rats. ^{64}Cu in tissues, faeces and urine were determined by gamma counting (Philips model PW4800 with a 3 x 3 inch NaI(Tl) crystal detector, overall efficiency of 6%).

In-vitro distribution of radioactive copper between the protein and non-protein compartments of plasma

The distribution of ^{64}Cu was determined in pooled plasma samples from male growing SD rats fed on a purified diet (5 mg Cu/kg diet) and male adult Wistar rats (Hsd/Cpb:WU) and NA rats fed on a commercial pelleted diet (RMH-B^R, Hope Farms, Woerden, The Netherlands). ^{64}Cu-acetate (0.01 μg Cu) was mixed with histidine at a molar ratio of 1:10 in a final volume of 0.1 ml and then added to 1 ml of thawed plasma. The plasma of Wistar and SD rats had been frozen at -20 °C for 1 week and that of the NA rats at -70 °C for 4 weeks. To equilibrate the ^{64}Cu with endogenous copper, the mixture was incubated at 37 °C for 1 h. Then, the samples (0.25 ml) were introduced into a MPS-system (Amicon, Danvers, MA; Technical data publication No. 460C) using YMT membranes (cut off, approximately 30 KD) and centrifuged at 2000 rpm for 10 min at an angle of 45°. Radioactivity was counted in the ultrafiltrate and in the incubation mixture prior to ultrafiltration. The ultrafiltrated fraction was calculated as the radioactivity in 1 ml of ultrafiltrate divided by that in 1 ml of the mixture and multiplied by 100. The ultrafiltration assays were done in triplicate.

Chemical analyses

Organ samples were dried in a vacuum dryer at 60 °C for 48 h and subsequently digested in 14 M HNO_3 at 80 °C for 2 h. Copper and zinc were measured using flame atomic absorption spectrometry (Perkin-Elmer 2380, Norwalk, CT, U.S.A.). Faeces and feed were ashed in a muffle furnace at 500 °C for 17 h and dissolved in 6 M HCl prior to measurement of copper and zinc, but urine and plasma were introduced directly into the flame atomic absorption spectrometry. Histidine in pooled plasma samples was analysed as described by Turnell and Cooper (1982).

Calculation of apparent and true copper absorption

Apparent copper absorption was calculated as intake minus

faecal excretion and expressed as percentage of intake or μg/d. The apparent absorptions measured during days 14-17 and 21-24 did not differ significantly (Student's t-test, $p \geq 0.08$) within dietary groups of the same strain and thus were pooled.

True copper absorption was calculated according to Heth and Hoekstra (1965). Counting measurements were corrected for background and radioisotope decay, and then expressed as percentage of administered dose. Plots of the logarithm of percentage radioactivity retention after intraperitoneal and oral administration of the radiotracer versus time were constructed. The zero-time intercepts were determined by extrapolation of the linear parts of the curves. Within the dietary groups of the same strain, the slopes of the retention curves for the same administration route on day 14 versus 21 were not significantly different (Student's t-test, $p \geq 0.23$), and thus the data were pooled. The percentage true absorption was calculated by dividing the intercept of the retention curve for the orally administered radiotracer by that for the intraperitoneally injected radiotracer and multiplying 100. This calculation was executed for each animal. The absolute true absorption was calculated by multiplying intake by the percentage of true copper absorption. Faecal excretion of endogenous losses was calculated as absolute true absorption minus absolute apparent absorption.

Statistical analyses

The data were subjected to two-way analysis of variance (ANOVA) with strain (NA versus SD rats) and copper intake (high versus normal dietary copper) as main effects. The level of statistical significance was preset at $p<0.05$. Homogeneity of the variances was verified with the use of Bartlett's test. Statistical significance of the main effects was also identified in selected, direct comparisons with the use of Student's t-test or Mann-Whitney test if variances were not homogeneous (F test). The level of significance was preset at $p<0.025$ to take into account the increased probability of a type I error due to

multiple comparisons (Bonferroni's adaptation). All statistical analyses were performed using SPSS/PC+ software (SPSS Inc., 1988).

RESULTS

Feed intake, body and organ weights

Dietary copper concentration did not affect feed intake, body and organ weights in both strains of rats (Table 2).

Table 2. Feed intake, body and organ weights of SD and NA rats fed the experimental diets[1-3]

	Normal-Cu diet		High-Cu diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Feed intake, g/d						
d 1-7	14.4	15.0	14.6	14.9	0.3	
d 21-28	17.2	17.5	16.6	16.9	0.6	
Body weight, g						
d 0	103.7	101.0	100.8	101.8	5.0	
d 29-30	217.3	225.3	215.8	219.0	7.7	
Organ weight, g/100 g body weight						
Liver	3.51	4.28[s]	3.78	4.44[s]	0.18	S
Kidney	0.65	0.66	0.63	0.67[s]	0.01	S
Spleen	0.28	0.28	0.27	0.26[d]	0.01	
Heart	0.35	0.36	0.36	0.37	0.01	

[1] Values are means for 6 rats per group.

[2] ANOVA significance ($p<0.05$): S = strain effect (NA versus SD rats); D = copper effect (high versus normal dietary copper).

[3] Group comparisons ($p<0.025$): s = significant strain difference (NA versus SD rats) for rats fed the same diet; d = significant dietary copper effect (high versus normal dietary copper) for rats of the same strain.

There were no differences in feed intake and body weight between NA and SD rats. NA rats had markedly higher liver and slightly higher kidney weights than did SD rats. Relative spleen and heart weights were similar for the two strains of rats.

Copper in organs

Copper concentrations in liver and kidneys were raised by high copper intake in both NA and SD rats (Table 3).

Table 3. Copper concentrations in organs of SD and NA rats fed the experimental diets[1-3]

	Normal-Cu diet		High-Cu diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Plasma, μg/ml	0.79	1.01[s]	0.79	1.09[s]	0.04	S
Liver						
μg/g dry matter	14.8	16.5	26.2[d]	25.3[d]	1.90	D
μg/liver	35.8	50.8[s]	67.7[d]	78.0[d]	5.26	S,D
μg/liver.100 g body weight	16.5	22.6[s]	31.4[d]	35.7[d]	2.20	S,D
Kidney[4]						
μg/g dry matter	19.2	29.0[s]	22.4[d]	33.7[s]	1.44	S,D
μg/kidney[4]	6.8	10.8[s]	7.7	12.5[s]	0.52	S,D
μg/kidney.100 g body weight[4]	3.2	4.8[s]	3.6	5.7[s]	0.24	S,D
Heart, μg/g dry matter	24.2	25.0	25.0	25.1	0.57	
Spleen, μg/g dry matter	4.6	4.8	5.0	5.2	0.21	
Muscle, μg/g dry matter	3.9	5.2	4.6	4.4	0.40	

[1-3] See legends to Table 2.

[4] Mean of left and right kidney.

The NA rats had higher contents of copper in liver, plasma and kidneys than did the SD rats. There was no strain difference in copper concentrations in spleen, heart and muscle. The concentration of histidine in plasma pooled per strain tended to be lower in NA (8.5 mg/L) than in SD (10.1 mg/L) rats.

Copper absorption and excretion

Table 4 shows that there was no strain difference as to

apparent and true copper absorption, as well as faecal endogenous and urinary copper excretion. The percentage of apparent copper absorption was significantly reduced after dietary copper loading, but the absolute amount of absorbed copper was drastically raised in both strains of rats. Feeding the high-copper diet also increased faecal endogenous and urinary copper excretion.

Table 4. Absorption and excretion of copper in SD and NA rats fed the experimental diets[1-3]

	Normal-Cu diet		High-Cu diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Apparent absorption,						
% of intake	33	38	22	19[d]	3.1	D
μg/d	27	32	369[d]	331[d]	23.7	D
True absorption,						
% of intake	42	47	39	36	3.9	
μg/d	35	39	661[d]	653[d]	21.6	D
Faecal endogenous excretion,						
% of intake	9.0	9.3	16.2	18.5	5.9	
μg/d	8.1	7.4	278.3[d]	337.8[d]	31.8	D
Urinary excretion, μg/d						
	2.8	3.0	6.0	6.8	0.3	D

[1-3] See legends to Table 2.

The integrated recovery of ^{64}Cu in the live rats and in their cumulated faeces and urine at 4 days after administration of the radiotracer was on average more than 85% of the dose given. The excretion of ^{64}Cu in faeces and urine is presented in Fig. 1. Irrespective of the route of administration, most of the ^{64}Cu was excreted with faeces and only a small portion with the urine. This agrees with earlier observations (Owen, 1964; Linder & Roboz, 1986; Van den Berg & Beynen, 1992). More ^{64}Cu was recovered in faeces after oral than after intraperitoneal administration. Dietary copper loading accelerated the excretion

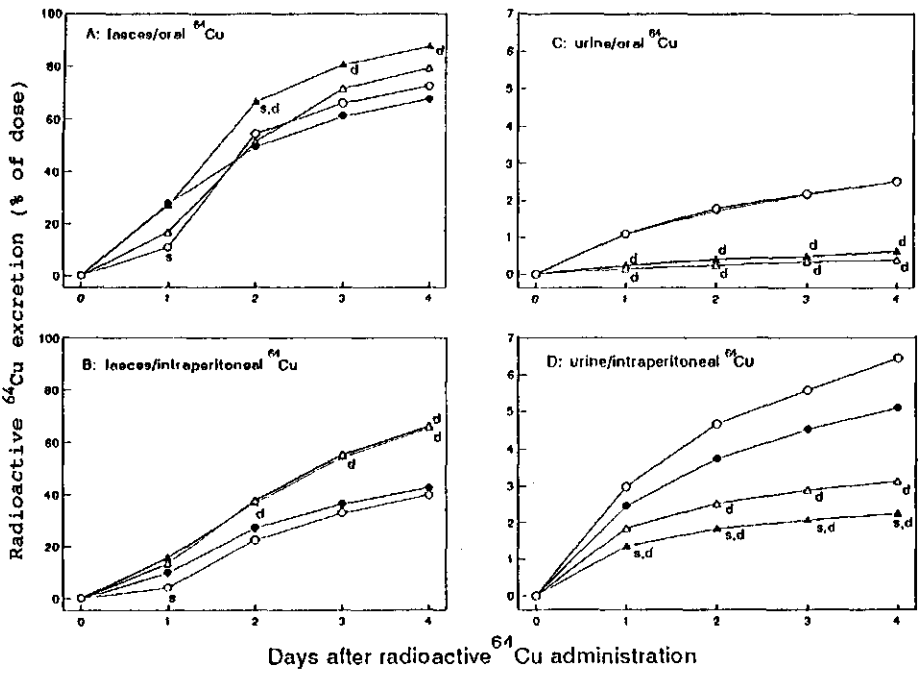

Fig. 1. Cumulative excretion of radioactivity with faeces (panels A and B) and urine (panels C and D) after either oral (panels A and C) or intraperitoneal (panels B and D) administration of ^{64}Cu as a function of post-administration days. Circles: normal-copper diet; triangles: high-copper diet; open symbols: SD rats; closed symbols: NA rats. Within each panel significant ($p<0.05$) strain differences (s, NA versus SD rats) and diet effects (d, high versus normal copper diet) are indicated.

of orally administered ^{64}Cu with faeces in the NA but not in the SD rats. With intraperitoneal administration of ^{64}Cu, feeding the high-copper diet raised faecal excretion of radioactivity in the two strains of rats.

Urinary excretion of ^{64}Cu was greater when it was injected intraperitoneally instead of administered orally. After feeding the high-copper diet the excretion of ^{64}Cu with urine was depressed irrespective of the route of administration. There was no strain difference in urinary excretion of radioactivity after oral administration of ^{64}Cu, but after intraperitoneal

administration the NA rats excreted significantly less radioactivity than did the SD rats when the diet was high in copper.

After oral administration of ^{64}Cu, whole-body retention as a function of time was similar in NA and SD rats (Fig. 2).

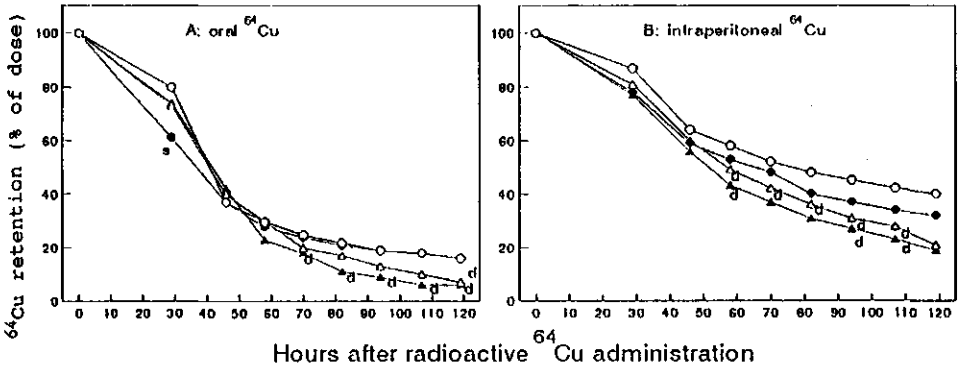

Fig. 2. Retention of ^{64}Cu administered orally (panel A) or intraperitoneally (panel B) by the SD and NA rats fed either the normal- or high-copper diet. Circles: normal-copper diet; triangles: high-copper diet; open symbols: SD rats; closed symbols: NA rats. Within each panel significant ($p<0.05$) strain differences (s, NA versus SD rats) and diet effects (d, high versus normal copper diet) are indicated.

However, after intraperitoneal administration, the NA rats tended to retain less radioactivity than the SD rats. Feeding the high-copper instead of the normal-copper diet depressed the retention of ^{64}Cu irrespective of the route of administration. The average biological half life as based on whole body retention of ^{64}Cu administered intraperitoneally, was 81 and 71 h in SD rats fed the normal- and high-copper diets, respectively. For the NA rats these values were 60 and 51 h. In the two-way ANOVA the pooled SEM was 4.6 h, and a significant effect of diet, but not of strain, was disclosed. Thus, dietary copper loading shortened the biological half life of copper. This is consistent with the observations of other investigators (Linder & Roboz, 1986; Johnson & Lee, 1988).

The biological half lives of intraperitoneally administered ^{64}Cu in the SD and NA rats fed the diet with normal amount of copper were 81 and 60 h. These values are slightly longer than those found by Johnson and Lee (1988) in male weanling Long Evans rats but shorter than the values of Van den Berg and Beynen (1992) and Marceau and Aspin (1972) for male Wistar rats and the values of Linder and Roboz (1986) for female Fischer and Sprague Dawley rats.

Distribution of radioactive copper between organs

Table 5. Distribution of ^{64}Cu between selected organs in SD and NA rats fed the experimental diets[1-3]

	Normal-Cu diet		High-Cu diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
% of the intraperitoneally administered dose at 2 h post-administration						
Liver	15.7	18.6	21.2	24.2	1.85	D
Kidneys	5.0	7.9[s]	4.9	7.3[s]	0.50	S
Spleen	0.31	0.25	0.20	0.21	0.03	D
Heart	0.21	0.18	0.16[d]	0.15	0.01	D
Muscle, per g	0.11	0.10	0.10[d]	0.07[d]	0.01	S,D
Plasma, per ml	0.75	0.67	0.52[d]	0.47[d]	0.03	D

[1-3] See legends to Table 2.

Table 5 presents the results of ^{64}Cu distribution between organs as measured at 2 h after intraperitoneal injection. Feeding the high-copper diet raised the percentage of ^{64}Cu in liver but lowered that in spleen, heart, muscle and plasma in both rat strains. The percentage of ^{64}Cu was higher in kidneys but lower in muscle of the NA rats when compared with the SD rats.

Zinc metabolism

Dietary copper concentration had no significant effect on

zinc concentrations in organs but reduced urinary zinc excretion in both NA and SD rats (Table 6).

Table 6. Zinc concentration in organs and apparent zinc absorption in SD and NA rats fed the experimental diets[1-3]

	Normal-Cu diet		High-Cu diet		SEM	ANOVA
	SD rats	NA rats	SD rats	NA rats		
Plasma, μg/ml	1.5	1.6	1.4	1.5	0.1	
Liver						
μg/g dry matter	119	115	113	116	4.7	
μg/liver	289	349[S]	295	354[S]	14.5	S
μg/liver.100 g body weight	133	156[S]	137	162[S]	5.8	S
Kidney[4]						
μg/g dry matter	117	131	116	121	4.0	S
μg/kidney[4]	42	49	40	45	2.2	S
μg/kidney.100 g body weight	19	22	19	21	0.8	S
Spleen, μg/g dry matter	120	119	118	114	4.2	
Heart, μg/g dry matter	106	105	105	102	2.4	
Muscle, μg/g dry matter	56	61	51	54	3.9	
Apparent absorption, % of intake	32	34	34	33	2.5	
Urinary excretion, μg/d	0.88	1.23[S]	0.65	1.09[S]	0.08	S,D

[1-3] See legends to Table 2.

[4] Mean left and right kidney.

Zinc contents of whole liver and kidney as well as urinary zinc excretion were higher in the NA than SD rats. Plasma zinc concentration was not altered in the NA rats. There was no strain difference in zinc concentration of spleen, heart and muscle and in the percentage of apparent zinc absorption.

DISCUSSION

In accordance with our previous study (Yu et al., 1994), the NA versus SD rats were found to have higher copper contents in liver, kidney and plasma. As would be expected, the feeding of extra copper raised hepatic and nephric copper concentrations, but there was no strain difference in response. The efficiency of intestinal copper absorption was similar in the NA and SD rats. Thus, despite the absence of plasma albumin in the NA rats, their tissue copper levels did not point to disturbed copper transport. This is supported by a similar distribution of radioactivity at 2 h after intraperitoneal administration of ^{64}Cu between the organs in NA and SD rats.

The question forces itself how copper is transported in the plasma of NA rats. We showed earlier that NA rats have about two-fold higher levels of ceruloplasmin than do SD rats (Yu et al., 1994). This could explain why the NA rats did not display signs of abnormal copper transport. Alternatively, copper may also be transported in a form not bound to plasma protein. To test this idea, the distribution of copper between protein and non-protein constituents of plasma was determined. The ultrafiltrated fractions of ^{64}Cu in plasma were 1.0, 0.8 and 71% in the Wistar, SD and NA rats, respectively. Thus, low molecular weight substances in the plasma of NA rats might play an essential role in copper transport. The concentration of histidine in plasma, which may be related to copper transport (Sarkar & Kruck, 1966; Neumann & Sass-Kortsak, 1967), was somewhat lower in the NA than SD rats. Thus, the form by which copper is transported in the plasma of NA rats remains unclear.

A major portion of endogenous copper in rats is excreted with the bile fluid (Klaassen, 1976; Johnson & Lee, 1988) while biliary copper is poorly reabsorbed (Owen, 1964; Farrer & Mistilis, 1967). We observed that NA rats excrete more copper with bile than do SD rats (Yu et al., 1994). Thus, it would be expected that NA rats excrete larger amounts of endogenous copper

with faeces. However, this was not seen. Perhaps reabsorption of biliary copper is more efficient in NA than SD rats, but the lower rates of urinary excretion of intraperitoneally administered ^{64}Cu in the NA rats speak against this. Alternatively, in the NA rats there is an enhanced flux of copper through the enterohepatic cycle.

Whole-body retention of intraperitoneally administered ^{64}Cu was less in the NA than in the SD rats. This points to a faster turnover of copper in NA rats as was indeed calculated. It is difficult to reconcile a faster turnover of copper with the observation that NA rats had lower rates of urinary excretion of radioactivity after intraperitoneal administration of ^{64}Cu, while faecal loss of radioactivity in the NA rats was similar to that in SD rats. The lower efficiency of urinary excretion of intraperitoneally administered ^{64}Cu in the NA rats was neither associated with lower rates of urinary excretion of orally administered ^{64}Cu nor with lower rates of urinary excretion of copper mass. Nevertheless, kidney copper metabolism appeared to be aberrant in the NA rats. They had greater copper stores in their kidneys, irrespective of the copper concentration of the diet, while their kidneys had a greater affinity for intraperitoneally administered ^{64}Cu.

In summary, NA rats appear to be able to maintain a relatively normal metabolism of copper even after feeding a high-copper diet. The results indicate that copper can be transported efficiently in the NA rats, but the form of copper transported in plasma is not clear.

ACKNOWLEDGEMENTS

Shiguang Yu was supported by The Netherlands Foundation for Nutrition and Health Research and by Dr. L.H.J.C. Danse.

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Chapter 7

INTERACTIONS OF DIETARY COPPER AND SELENIUM IN RELATION TO SELENIUM STATUS IN RATS

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(*submitted for publication*)

Interactions of dietary copper and selenium in relation to selenium status in rats

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Abstract: The question addressed was whether the influence of dietary copper concentration on selenium status depends on the amount of selenium in the diet. Weanling, male rats were fed purified diets containing either 1, 4 (normal) or 42 mg Cu/kg diet and either 0.03, 0.05 (normal) or 1.0 mg Se/kg diet in a 3^2 factorial design. Extra copper was added to the diets in the form of $CuSO_4.5H_2O$ and selenium as $Na_2SeO_3.5H_2O$. In rats fed either the low or normal amounts of selenium, higher intakes of copper decreased apparent intestinal selenium absorption and increased urinary selenium excretion. Increasing dietary copper concentrations elevated selenium concentrations in liver and kidneys but slightly lowered those in spleen of rats fed the diets with the normal level of selenium. The effects of copper on selenium metabolism were not seen in rats fed the high-selenium diets. Glutathione peroxidase activity in erythrocytes was raised by feeding the diets either normal or high instead of low in copper. It is concluded that the amount of selenium in the diet determines whether or not dietary copper concentration affects selenium status.

INTRODUCTION

Studies concerning the influence of dietary copper concentration on selenium metabolism in various animal species have yielded conflicting results. The retention of orally administered ^{75}Se was depressed in liver, lungs, heart and whole blood in rats fed a high-copper diet (Rahim et al., 1986), but hepatic retention of intraperitoneally administered ^{75}Se was depressed also in rats with copper deficiency (Jenkinson et al., 1982). The toxic effect of high intakes of selenium in chicks as evidenced by growth retardation and increased mortality could be partially alleviated by raising the copper content of the diet (Hill, 1974), but a dietary copper challenge by itself resulted in a considerable accumulation of selenium in the liver of chicks (Jensen, 1975). A significant increase in liver selenium concentration has been found in copper-loaded, adult sheep (Gooneratne & Howell, 1982), but there was no effect of supplemental copper on selenium status of ewes and lambs (White et al., 1989). Studies using growing pigs (Dove & Ewan, 1990) and sheep with adequate intakes of selenium (White et al., 1982) have failed to detect an influence of copper intake on selenium status.

The differences between literature data on the impact of copper intake on selenium metabolism and status may be due to differences in duration of dietary treatment, the forms of dietary copper, selenium source and animal species used. The above-mentioned studies pointing to impaired selenium status in both copper excess and deficiency might be explained also by an interaction of dietary copper and selenium that depends on both the ratio and amounts of the two trace elements in the diet. To verify this explanation, we fed rats on purified diets containing three different concentrations of copper and selenium in a 3^2 factorial design. After 28 days, plasma and organ selenium concentrations and glutathione peroxidase activity in erythrocytes were determined as indicators of selenium status.

To see whether differences in selenium status can be explained by differences in selenium balance, we also measured faecal and urinary excretion of selenium.

MATERIALS AND METHODS

The experimental protocol was approved and its conduct supervised by the animal welfare officer of the Wageningen Agricultural University.

Animals and housing

We used male Wistar rats (HSD/Cpb:WU), aged 3 weeks. On arrival, the rats were housed in groups of 4 or 5 rats in stainless steel cages (60 x 21 x 19 cm) with wire floors for a period of 10 days. Then, for the experimental period of 4 weeks the rats were housed individually in metabolism cages (314 cm^2 x 12 cm). The cages were placed in a room with controlled temperature (19-21 °C), light-dark cycle (light: 06:00-18:00 h) and relative humidity (50-60 %).

Experimental diets

Table 1 shows the composition of the purified diets used. The reference diet (added Se:normal; added Cu:normal), which was given to all rats during the run-in period of 10 days, was formulated according to the nutrient requirements of rats (National Research Council, 1978) and contained added amounts of 4 mg Cu and 0.1 mg Se/kg feed. Copper was added in the form of $CuSO_4.5H_2O$ and selenium as $Na_2SeO_3.5H_2O$. In the experimental diets, copper was either omitted or added to levels of 4 or 60 mg/kg feed and selenium was also either omitted or added to levels of 0.1 or 1.5 mg /kg. Thus, there were nine experimental diets including the reference diet. The experimental diets, which were in powdered form, were stored at 4 °C until used for feeding. The animals had free access to feed and demineralized water throughout.

Table 1. Composition of the experimental diets

Added Se:	None			Normal			High		
Added Cu:	None	Normal	High	None	Normal	High	None	Normal	High
Ingredients									
Glucose (g)	704.3	704.3	704.1	704.3	704.3	704.1	704.3	704.3	704.1
$CuSO_4.5H_2O$ (mg)	-	15.7	235.5	-	15.7	235.5	-	15.7	235.5
$Na_2SeO_3.5H_2O$ (mg)	-	-	-	0.30	0.30	0.30	4.50	4.50	4.50
Constant components (g)[1]	295.7	295.7	295.7	295.7	295.7	295.7	295.7	295.7	295.7

[1]Constant components consisted of (g): casein, 151; maize oil, 25; coconut oil, 25; cellulose, 30; $CaCO_3$, 12.5; $NaH_2PO_4.2H_2O$, 20.1; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; mineral premix, 10; and vitamin premix, 12. The mineral premix consisted of (mg): $FeSO_4.7H_2O$, 174; MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2 and maize starch 9695.2. The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (1200 retinol equivalents); cholecalciferol, 0.025; maize starch, 9828.125.

The analysed copper and selenium concentrations of the diets without added $CuSO_4.5H_2O$ or $Na_2SeO_3.5H_2O$ were 1.2 and 0.032 mg/kg, respectively. The normal- and high-copper diets were found to contain 4.2 and 41.9 mg/kg. Analysed selenium concentrations in the normal- and high-selenium diets were 0.054 and 0.995 mg Se/kg. Thus, the analysed copper and selenium concentrations were lower than expected.

Experimental design

After the pre-experimental period (d 0), the rats were divided into 9 groups of 6 rats each. The groups, which were stratified for body weight, were randomly assigned to the experimental diets in a 3^2 factorial design. During the experimental period, the rats were housed individually in metabolic cages placed in racks in randomized position.

The experiment lasted 4 weeks. Feed intake and body weights were recorded regularly. Selenium balances were determined from d 2 to 4 and d 25 to 27; feed intake was monitored, and faeces and urine were collected quantitatively. On d 28, the rats were anaesthetized by exposure to diethyl ether. Blood was taken by orbital puncture into heparinized polyethylene tubes and the rats were killed by decapitation while they were still under anaesthesia. The liver, spleen, heart and kidneys were excised and weighed. Plasma samples were prepared from fresh blood by centrifuging (2000 rpm, 15 min). The collected organs and plasma samples were stored at -20 °C. The erythrocytes were washed three times with saline and stored at -80°C until analysis.

Analytical methods

Selenium in diets, urine and plasma was analysed by the method of Koh and Benson (1983) with a Perkin-Elmer fluorescence spectrophotometer (Model 1000, Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, U.K.). Selenium in organs and faeces was measured by the same method after the samples had been dried at 75 °C for 48 h in a vacuum dryer. A reference sample (NBS bovine

liver 1577a, National Institute of Standards Technology, Gaithersburg, MD, U.S.A.) was used to assess accuracy of the selenium determinations. Analysed values for selenium in the reference sample were on average 111 % (SE = 0.017, n = 15) of the certified value.

Glutathione peroxidase (EC · 1.11.1.9) activity in erythrocytes was measured within one week of storage according to the coupled assay of Paglia and Valentine (1967) with the following modifications. Hydrogen peroxide was used as substrate and the CoBAS-BIO auto-analyser (Hoffmann-La Roche BV, Mijdrecht, The Netherlands) was employed to measure the decrease in NADPH at 10-sec intervals for a period of 60 sec. The linear portion of the NADPH disappearance curves was used for calculation of glutathione peroxidase activity which was expressed as units (μmoles of NADPH oxidized per min) per g of haemoglobin.

For copper analysis, the organ samples were dried at 75 °C for 48 h under vacuum prior to wet ashing in 14 M nitric acid at 80°C for 2 h. Feed samples were dry ashed in a muffle furnace at 500°C for 17 h, dissolved in 6 M HCl and properly diluted with demineralized water. Copper in organs and feed samples was measured using flame atomic absorption spectrometry (Perkin-Elmer 2380, Norwalk, CT, U.S.A.). Determinations of the reference bovine liver sample produced values which were on average 103% of the certified value for copper (SE = 3.7, n = 7). Plasma copper was determined using a flameless atomic absorption spectrometry with auto-sampler (Varian SpectrAA-300, Varian Techtron Pty. Ltd., Springvale, Australia) after the plasma was properly diluted with demineralized water. Ceruloplasmin in plasma was assayed as p-phenylenediamine oxidase activity as described by Sunderman and Nomoto (1970).

Statistical analysis

Two-way analysis of variance (ANOVA) was applied to identify statistically significant effects of dietary copper and selenium concentrations and of their interaction. Original or

logarithmically transformed data were used. The variances were verified to be homogenous (Bartlett's test). One-way ANOVA was used to evaluate the effect of variable copper concentrations in diets with same level of selenium. If statistical significance was detected, the differences between group means were further examined for statistical significance using Tukey's test. $P < 0.05$ was preset as level of significance. Statistical analyses were performed by computer using the SPSS/PC+ statistical package (SPSS Inc., 1988).

RESULTS

Growth performance and organ weights

Feed intake and final body weight were not affected by the dietary treatments (Table 2). Spleen weight was raised at normal and high copper intakes versus the feeding of the diets without added copper. High selenium intake slightly, but significantly, increased liver weight. Dietary copper and selenium concentrations did not affect heart and kidney weight.

Copper status

To check copper status of the rats, copper concentrations in plasma and organs and the level of plasma ceruloplasmin were determined. Copper intake significantly influenced the indicators of copper status. In rats fed the low-, normal- and high-copper diets, respectively, the values (n = 18) were as follows: plasma Cu, 0.01 (SE 0.003), 0.87 (SE 0.04) and 0.89 (SE 0.04) µg/ml; plasma ceruloplasmin, 0.01 (SE 0.001), 0.48 (SE 0.01) and 0.48 (SE 0.01) g/L; liver Cu, 6.1 (SE 0.3), 13.1 (SE 0.3) and 30.0 (SE 1.8) µg/g dry wt; kidney Cu, 13.0 (SE 0.2), 22.4 (SE 0.6) and 26.8 (SE 0.7) µg/g dry wt and heart Cu, 13.1 (SE 0.3), 24.0 (SE 0.2) and 24.3 (SE 0.3) µg/g dry wt. Thus, copper status was modulated by dietary copper concentration as would be anticipated. None of the indicators of copper status was significantly influenced by selenium intake ($p \geq 0.10$, three-way

Table 2. Feed intake, and body and organ weights of rats fed the experimental diets[1]

Added Se:	None			Normal			High			Pooled	
Added Cu:	None	Normal	High	None	Normal	High	None	Normal	High	SEM	ANOVA[2]
Feed intake, g/d											
d 0-7	17.0	17.9	17.4	17.9	16.9	17.1	17.2	16.7	16.7	0.7	
d 21-28	20.1	21.4	20.7	20.3	21.2	20.0	20.2	19.5	20.3	0.7	
Body weight, g											
d 0	119	122	119	123	118	123	119	119	119	4.7	
d 28	285	311	297	311	298	293	283	282	286	11.3	
Organ weight, g/100 g body wt											
Liver	3.99	4.07	4.03	3.90	4.19	3.93	4.26	4.18	4.33	0.08	Se
Spleen	0.18	0.18	0.21	0.16[a,3]	0.20[b]	0.19[b]	0.17	0.18	0.19	0.02	Cu
Heart	0.35	0.33	0.35	0.36	0.35	0.33	0.35	0.34	0.35	0.01	
Kidney[4]	0.32	0.31	0.31	0.31	0.30	0.30	0.30	0.31	0.30	0.01	

[1]Values are means for 6 rats.

[2]Significance based on two-way ANOVA ($p<0.05$), Se = selenium effect, Cu = copper effect.

[3]Values with different superscripts for groups with the same dietary selenium concentration differ significantly ($p<0.05$, Tukey's test).

[4]Means of left and right kidneys.

ANOVA).

Selenium status

Selenium concentrations in plasma and selected organs were systematically elevated with increasing amounts of dietary selenium (Table 3). This also held true for glutathione peroxidase activities in erythrocytes. The contents of selenium in liver and kidney were most sensitive to selenium intake.

Glutathione peroxidase activities in erythrocytes were increased when the rats were fed either the normal- or high-copper diets when compared with the low-copper diets. A similar pattern was seen for hepatic and nephric selenium concentrations but these effects were not apparent in rats fed the high-selenium diets.

In rats fed the low-selenium diets, plasma selenium was lowered and hepatic selenium concentration was raised by increasing copper intake. The effect of dietary copper concentration was most striking in the rats fed the normal-selenium diets. Increasing copper intakes produced elevated liver and kidney selenium concentrations but lowered spleen copper concentrations.

Selenium balance

Not only absolute apparent intestinal absorption, but also the percentages of apparent selenium absorption increased with increasing selenium concentrations of the diets (Table 4). Urinary excretion and retention of selenium also rose with higher intakes of selenium.

In rats fed the low-selenium diets, apparent selenium absorption was significantly increased ($p < 0.05$, Student's t-test) after feeding the diets for 3 weeks instead of a few days. Urinary selenium excretion was significantly ($p < 0.05$, Student's t-test) elevated after 25-27 days versus 2-4 days in rats fed either the normal- or high-selenium diets. Such a time trend was not seen in rats fed the low-selenium diets. When compared with the first balance period, selenium retention during the second

Table 3. Selenium status of rats fed the experimental diets[1]

Added Se:	None			Normal			High			Pooled SEM	ANOVA[2]
Added Cu:	None	Normal	High	None	Normal	High	None	Normal	High		
Plasma											
Se, μg/ml	0.27[a,3]	0.26[ab]	0.23[b]	0.45	0.47	0.45	0.54	0.52	0.52	0.13	Se
GSH, U/g Hb	168	184	172	270	306	296	288	368	361	18.6	Se, Cu
Selenium in organs, μg/g dry weight											
Liver	0.52[a]	0.62[b]	0.62[ab]	1.80[a]	2.27[ab]	2.49[b]	3.21	3.18	3.56	0.17	Se, Cu
Spleen	1.30	1.30	1.25	1.71[a]	1.66[ab]	1.62[b]	2.33	2.23	2.16	0.09	Se
Heart	1.03	1.06	1.03	1.52	1.61	1.61	1.78	1.82	1.73	0.05	Se
Kidney	1.46	1.79	1.39	2.37[a]	3.23[ab]	4.14[b]	6.41	5.95	6.14	0.27	Se, CuxSe

[1] Values are means for 6 rats.

[2]Significance based on two-way ANOVA ($p<0.05$), Se = selenium effect, Cu = copper effect, CuxSe = interaction.

[3]Values with different superscripts for groups with the same dietary selenium concentration differ significantly ($p<0.05$, Tukey's test).

Table 4. Selenium balance in rats fed the experimental diets[1]

Added Se:	None			Normal			High			Pooled SEM	ANOVA[2]
Added Cu:	None	Normal	High	None	Normal	High	None	Normal	High		
Apparent absorption, % of intake											
d 2-4	71.0[a,3]	69.8[a]	58.8[b]	84.5[a]	75.6[b]	71.5[b]	84.3[a]	89.8[a]	79.0[b]	1.950	Se,Cu,CuxSe
d 25-27	79.2[a]	76.1[a]	69.0[b]	81.3[a]	73.8[b]	66.3[c]	82.6	84.4	80.8	2.109	Se,Cu,CuxSe
Absorbed amount, μg/d											
d 2-4	0.39[ab]	0.40[a]	0.33[b]	1.85[a]	1.59[b]	1.53[b]	19.99	21.67	18.39	0.525	Se,Cu
d 25-27	0.51	0.51	0.45	1.99[a]	1.84[ab]	1.60[b]	22.55	22.68	22.42	0.618	Se,Cu
Urine excretion, μg/d											
d 2-4	0.11[a]	0.13[ab]	0.17[b]	0.38[a]	0.96[b]	1.19[b]	2.66	2.01	2.23	0.119	Se,Cu,CuxSe
d 25-27	0.12	0.14	0.13	0.73[a]	1.47[b]	1.58[b]	3.65	2.93	2.93	0.184	Se,Cu,CuxSe
Retention, μg/d											
d 2-4	0.28[a]	0.26[a]	0.16[b]	1.47[a]	0.63[b]	0.34[b]	17.33[ab]	19.65[a]	16.16[b]	0.489	Se,Cu,CuxSe
d 25-27	0.40	0.37	0.33	1.26[a]	0.37[b]	0.02[b]	18.90	19.74	19.50	0.534	Se,Cu,CuxSe

[1]Values are means for 6 rats.

[2]Significance based on two-way ANOVA ($p<0.05$), Se = selenium effect, Cu = copper effect, CuxSe = interaction.

[3]Values with different superscripts for groups with the same dietary selenium concentration differ significantly ($p<0.05$, Tukey's test).

balance period was increased in the low-selenium groups, decreased in the normal-selenium groups, and essentially unchanged in the high-selenium groups.

Dietary copper concentration significantly influenced selenium balance. In rats fed the low- and normal-selenium diets, increasing intakes of copper produced a decrease in apparent selenium absorption and an increase in urinary selenium excretion so that selenium retention showed a decreasing trend. The effects of dietary copper on selenium balance were most pronounced in rats fed the normal-selenium diets. In rats fed the high-selenium diets, the amount of copper in the diet had no systematic effect on intestinal absorption, urinary excretion and whole-body retention of selenium.

DISCUSSION

The levels of dietary copper and selenium used markedly affected copper and selenium status, but were within the range of tolerance by the rats, at least for the duration of the experiment. Feed intake and body-weight gain were not affected by the dietary treatments and the rats showed no clinical signs of copper and/or selenium deficiency or toxicity. In contrast, in many previous trials the interactions of dietary copper and selenium in relation to selenium status were studied in animals of deficient or poisonous copper and/or selenium status (Hill, 1974; Jensen, 1975; Jenkinson et al., 1982; Gooneratne & Howell, 1982).

This study shows that the interaction between dietary copper and selenium with respect to indicators of selenium status is quite complex. The type of indicator and dietary selenium concentration determine the interaction. In rats fed the low-selenium diets, increasing copper intakes reduced plasma selenium but raised liver selenium. In rats fed the normal-selenium diets, increasing copper intakes slightly reduced spleen selenium but markedly elevated liver and kidney selenium without affecting

plasma selenium concentrations. These observations are generally in line with previous studies (Jensen, 1975; Gooneratne & Howell, 1982; Jenkinson et al., 1982). In rats fed the high-selenium diets the amount of copper in the diet had no influence on selenium status. Thus, the ratio of copper:selenium in the diet determines the effect of copper intake on selenium status.

The complexity of the impact of dietary copper concentration on selenium status is further illustrated by the results on whole-body selenium retention. In rats fed either the low- or normal-selenium diets, increasing copper intakes depressed selenium retention. However, in rats fed the low-selenium diets this was not reflected by selenium concentrations in the organs analysed. More surprisingly, in rats fed the normal-selenium diets the effects of dietary copper on selenium retention and selenium concentrations in liver and kidney appear to be at variance. In copper-deficient rats, ^{75}Se retention was increased in brain and lungs but decreased in liver (Jenkinson et al., 1982). High intakes of copper did not affect whole-body retention of selenium but lowered selenium retention in liver, lungs, heart and whole blood (Rahim et al., 1986). Thus, increasing copper intakes in rats can cause differences in the distribution of selenium between tissues.

The percentage of apparent selenium absorption was increased with increasing selenium intakes. This has also been reported in humans (Van der Torre et al., 1991). Brown et al. (1972) observed that 95 to 100 % of administered ^{75}Se was absorbed by rats fed diets containing either 0, 0.5 or 4.0 mg Se/kg diet. The percentage absorption of selenium was not altered in chicks by supplementing the diet with either 0.4 or 4.0 ug Se/kg (Humaloja & Mykkänen, 1986). The discrepancies between the various studies cannot be readily explained.

The effects of dietary copper on intestinal absorption and urinary excretion of selenium in rats fed the normal-selenium diets are difficult to reconcile. Increasing copper intakes reduced apparent selenium absorption and enhanced urinary

excretion of selenium. This would imply that increasing copper intakes do not allow for a new steady-state of selenium to be attained, at least not at the level of the whole body. It also suggests that copper affects selenium metabolism both at the pre- and post-absorptive level.

In conclusion, the effect of dietary copper on selenium status and selenium metabolism depends on the dietary selenium concentration. When the diets contained the normal amount of selenium, increasing copper intakes reduced selenium absorption, raised urinary selenium excretion and altered selenium concentrations in certain organs, but with the high selenium intake copper became ineffective.

ACKNOWLEDGEMENT

Shiguang Yu was supported by The Netherlands Foundation for Nutrition and Health Research.

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Chapter 8

THE COMBINED EFFECT OF HIGH IRON AND ZINC INTAKE ON COPPER STATUS IN RATS

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(*submitted for publication*)

The combined effect of high iron and zinc intake on copper status in rats

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Abstract: The interactions between copper, zinc and iron intakes in rats were investigated with regard to copper status. Weanling male rats were fed purified diets containing two levels of each of the three elements in a 2^3 factorial design. The added amounts of copper, zinc and iron in the diets were 5, 12 and 35 mg/kg feed or were ten times as high. After feeding on the experimental diets for four weeks, the rats were killed and copper concentrations in plasma and organs measured. Plasma copper concentration was lowered by high zinc and iron intakes but this was seen only in the rats fed the normal-copper instead of the high-copper diets. In essence, the effects of zinc and iron were additive. Neither in rats fed the normal-copper diets nor in those fed the high-copper diets did extra iron or zinc intake alter copper concentrations in liver, spleen, kidney and tibia.

INTRODUCTION

The interactions between dietary concentrations of copper and zinc on the one hand and copper and iron on the other, have been well documented (Johnson, 1989; Hambidge et al., 1986). Supplementation of the diet with high amounts of iron decreases copper status in rats. Excess dietary zinc also lowers copper status in rats. However, little is known about the combined effect of zinc and iron on copper status. It could be suggested

that the effects of supplemental zinc and iron are additive or synergistic. Thus, we fed rats on diets with either the recommended concentrations of copper, iron and zinc (National Research Council, 1978) or with concentrations that were about 10 times higher. In contrast to earlier studies (Kinnamon, 1966; Hall et al., 1979; Bremner & Young, 1981; Storey & Greger, 1987) we used only moderately elevated dietary concentrations of copper, zinc and iron so as to avoid unrealistic dietary conditions.

MATERIALS AND METHODS

Animals and housing

Male Wistar rats (Cpb:WU), aged about 3 wk were used. They were kept in a room with controlled temperature (19-21 °C), lighting (light on: 06:00-18:00 h) and relative humidity (50-60 %). During the pre-experimental period of 10 d, the rats were housed in groups of 4 or 5 animals in stainless steel cages (60 x 21 x 19 cm) with wire bottoms. During the experimental period, the rats were housed individually in metabolism cages (314 cm^2 x 12 cm).

Experimental design

During the pre-experimental period, the rats had free access to demineralized water and a purified diet containing 6 mg Cu, 18 mg Zn and 43 mg Fe/kg diet (Table 1). This diet was formulated according to the nutrient requirements of rats (National Research Council, 1978). At the end of the pre-experimental period (d 0), the rats were divided into 8 groups of 6 rats each so that body weight distributions in the groups were similar. Each group was then randomly assigned to one of the eight purified experimental diets, including the pre-experimental diet (Table 1). The diets either had the added, recommended concentrations of copper, zinc and iron, or had added levels of these elements that were 10 times higher. Thus, the experimental diets were used in a 2^3

Table 1. Composition of the experimental diets

Cu:	Normal				High			
Supplement:	None	Zn	Fe	Zn+Fe	None	Zn	Fe	Zn+Fe
Ingredients								
Glucose (g)	704.1	703.8	702.5	702.2	703.9	703.6	702.4	702.1
$CuSO_4.5H_2O$ (mg)	15.7	15.7	15.7	15.7	157	157	157	157
$ZnSO_4.H_2O$ (mg)	33	330	33	330	33	330	33	330
$FeSO_4.7H_2O$ (mg)	174	174	1740	1740	174	174	1740	1740
Constant components (g)[1]	295.7	295.7	295.7	295.7	295.7	295.7	295.7	295.7
Chemical analysis (mg/kg)								
Copper	6	7	5	6	43	47	36	43
Zinc	18	140	18	139	18	140	17	143
Iron	43	45	376	368	49	38	352	346

[1]Constant components consisted of (g): casein, 151; corn oil, 25; coconut oil, 25; cellulose, 30; $CaCO_3$, 12.5; $NaH_2PO_4.2H_2O$, 20.1; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; mineral premix, 10; and vitamin premix, 12. The mineral premix consisted of (mg): MnO_2, 79; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2 and corn starch 9901.9. The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; nicotinamide, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; retinylacetate and retinylpalmitate, 8 (4000 IU); cholecalciferol, 2 (1000 IU); corn starch, 9826.15.

factorial design. Copper, zinc and iron were added to the diets in the form of sulphate salts. Chemical analysis showed that copper, zinc and iron concentrations in the experimental diets were within the expected range (Table 1).

Demineralized water and food were supplied ad libitum. The diets, which were in powdered form, were stored at 4 °C until used for feeding. Feed consumption, which was corrected for spillage, and body weights were recorded regularly. On d 28, the rats were anesthetized by exposure to diethyl ether. Blood was taken by abdominal aorta puncture and collected in heparinized polyethylene tubes and the rats were killed by decapitation while they were still under anesthesia. The liver, heart, spleen, kidneys (left and right) and tibias (left and right) were removed, weighed and stored at -20 °C until analysis.

Analytical methods

Copper in all organs collected, zinc in the two tibias pooled per animal, and iron in liver were measured by flame atomic absorption spectrometry (Perkin-Elmer 2380, Norwalk, CT, U.S.A.) after the samples had been dried in a vacuum dryer at 75 °C for 48 h, wet digested in 14 M nitric acid (Suprapur, Merck, Darmstadt, FRG) at 80 °C for 2 h and properly diluted with demineralized water. Feed samples for iron measurement were not dried in the vacuum dryer but were directly digested. Copper and zinc in feed samples were determined by atomic absorption spectrometry after the samples had been ashed at 500 °C for 17 h in a muffle furnace and then dissolved in 6 M HCl. Plasma samples were prepared by centrifuging the fresh heparinized blood samples at room temperature (2000 rpm, 15 min) and copper in plasma was measured directly by atomic absorption spectrometry. Ceruloplasmin in plasma was assayed as p-phenylenediamine oxidase activity as described by Sunderman and Nomoto (1970). An external control in the form of a bovine liver sample (NBS 1577a, National Institute of Standards Technology, Gaithersburg, MD, U.S.A) was used to assess bias of copper, zinc and iron analysis. Analyzed

copper, zinc and iron concentrations were 106% (SE=2.2, n=7), 111% (SE=4.4, n=7) and 102% (SE=12.5, n=7) of the certified values, respectively.

Statistical analysis

The data from feed intake and body and organ weights were subjected to three-way analysis of variance (ANOVA) with dietary copper, zinc and iron concentrations as main effects. The data were logarithmically transformed prior to analysis of variance if the variances were heterogeneous according to Bartlett's test. The data from indicators of copper status at constant copper intake were analyzed by two-way ANOVA with dietary iron and zinc concentrations as main effects. Differences between groups were also evaluated using one-way ANOVA which, if statistical significance was detected, was followed by Tukey's test. The probability of a type I error < 0.05 was taken as the criterion of statistical significance. All tests were performed by computer using the SPSS/PC$^+$ statistical package (SPSS Inc., 1988).

RESULTS

Growth performance and organ weights

Final body weight and feed intake were not affected by copper and iron intake, but supplemental zinc had a slight, lowering effect (Table 2). Dietary zinc loading with levels > 2000 mg Zn/kg feed is known to impair feed intake of rats (Kinnamon, 1966; Storey & Greger, 1987), but apparently this effect is also seen with moderately increased amounts of dietary zinc. The decrease in final body weight in the high-zinc groups may be due to the decreased feed intake. Extra zinc in the diet also decreased relative liver weight. The dietary treatments did not significantly influence relative spleen, kidney and heart weights.

Table 2. Feed intake, body and organ weights of rats fed the experimental diets[1]

Cu:	Normal				High				Pooled SEM	ANOVA[2]
Supplement:	None	Zn	Fe	Zn+Fe	None	Zn	Fe	Zn+Fe		
Body weight, g										
d 0	94	95	95	94	94	93	94	95	1.8	
d 28	263	262	282	244	261	256	267	252	9.5	Zn
Feed intake, g/d										
d 0-7	14.8	15.3	15.7	14.9	15.2	15.0	15.9	14.3	0.7	
d 21-28	20.3	20.2	22.3	19.0	21.3	20.7	20.9	19.8	0.9	Zn
Organ weight, g/100 g body wt										
Liver	4.38	4.25	4.65	4.20	4.48	4.15	4.25	4.40	0.13	Zn, CuxZnxFe
Spleen	0.20	0.19	0.21	0.20	0.20	0.19	0.22	0.21	0.01	
Kidney[3]	0.34	0.32	0.35	0.34	0.33	0.33	0.34	0.33	<0.01	
Heart	0.36	0.36	0.37	0.37	0.39	0.35	0.39	0.37	0.01	

[1] Values are means for 6 rats per dietary group.

[2]Significance based on three-way ANOVA ($p<0.05$): Zn = dietary zinc effect; CuxZnxFe = three-way interaction.

[3]Means for left and right kidneys.

Zinc and iron status

To check zinc and iron status, concentrations of zinc in tibias and iron in liver were determined. The high-zinc versus normal-zinc diets significantly ($p<0.05$, Student's test) raised tibia zinc concentrations, the values being 192 ± 2.4 and 177 ± 1.5 μg/g dry wt (mean ± SE, n=24). Feeding the high-iron instead of normal-iron diets significantly ($p<0.05$, Student's t-test) elevated liver iron concentrations from 249 ± 4.8 to 336 ± 9.8 μg/g dry wt (mean ± SE, n=24). Thus, it may be concluded that the differential dietary concentrations of zinc and iron indeed modulated zinc and iron status. Copper intake did not significantly ($p\geq0.34$, three-way ANOVA) influence tibia zinc and liver iron concentrations.

Copper status

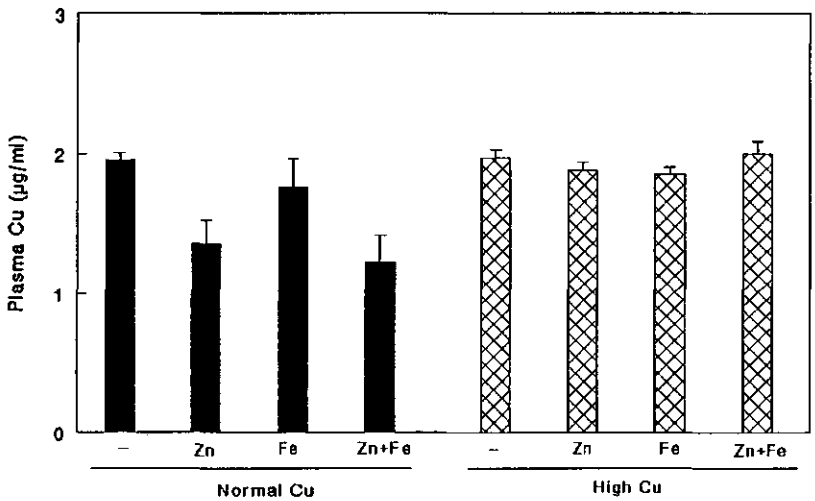

Fig. 1. Plasma copper concentrations in rats fed the experimental diets. The results are presented as means and SEMs (vertical bars) for 6 rats per dietary group; the pooled SEM is 0.151 μg/ml. The high-copper diets significantly ($p<0.05$, three-way ANOVA) raised plasma copper concentrations. In rats fed the normal-copper diets, the high zinc intake significantly ($p<0.05$, two-way ANOVA) reduced plasma copper; there was no zinc effect in the rats fed the higher-copper diets.

Table 3. Copper status of rats fed the experimental diets[1]

Cu:	Normal						High					
Supplement:	None	Zn	Fe	Zn+Fe	SEM	ANOVA[2]	None	Zn	Fe	Zn+Fe	SEM	ANOVA[2]
Plasma ceruloplasmin, g/L												
	0.64	0.41	0.54	0.36	0.07	Zn	0.68	0.58	0.57	0.59	0.14	
Copper in organs, μg/g dry wt												
Liver	15	15	15	14	0.7		31	32	32	33	5.8	
Spleen	11	10	10	10	0.4		12	11	12	11	0.3	
Kidney[3]	24	24	23	23	1.4		30	29	29	29	2.1	
Heart[4]	26	24	24	23	1.3		26^{ab}	27^{a}	25^{b}	26^{ab}	0.4	Zn
Tibia	5.3	5.1	5.0	5.0	0.1		5.3	5.3	5.1	5.2	0.1	

[1]Values are means and pooled SEMs for 6 rats per dietary group.

[2]Significance based on two-way ANOVA ($p<0.05$): Zn = dietray zinc effect.

[3]Values for right kidney.

[4]Heart copper concentrations with different superscripts differ significantly ($p<0.05$, Tukey's test).

Supplemental copper significantly elevated plasma copper concentrations (Fig. 1) and copper concentrations in all five organs and also raised plasma ceruloplasmin levels (Table 3).

Plasma copper concentrations in rats fed the normal-copper diets were significantly ($p<0.05$, two-way ANOVA) lowered by extra zinc in the diet (Fig. 1). This zinc effect was not seen after feeding the high-copper diets. The level of ceruloplasmin in plasma responded to supplemental zinc in a similar way as did plasma copper (Table 3). Extra iron in the diet did not significantly lower plasma copper and ceruloplasmin levels in rats fed the normal-copper diets ($p\geq0.36$, two-way ANOVA). The effects of supplemental zinc and iron in the normal-copper diets were essentially additive.

Feeding either the high-zinc or the high-iron diets had no significant effect on copper concentrations in liver, heart, kidney, spleen and tibias in rats fed the normal-copper diets. This was also seen in rats fed the high-copper diets, except that dietary zinc concentration had a very small effect on heart copper concentration.

DISCUSSION

In keeping with earlier studies using zinc concentrations ranging from 120 to 2441 mg Zn/kg diet (STorey & Greger, 1987; Fisher et al., 1981), supplemental zinc in this study lowered plasma copper concentration in rats fed the normal-copper diets. The adverse effect of extra zinc on plasma copper concentration was abolished by supplemental dietary copper. Since the effect of high zinc intake on feed intake did not depend on copper intake, it can be excluded that the zinc-induced lowering of plasma copper concentration in rats fed the normal-copper diets was caused by the reduced feed intake. Thus, there is an interaction of dietary copper and zinc at concentrations ranging between the requirement and a 10-fold higher level. It has been shown that zinc loading inhibits copper absorption in rats (Hall

et al., 1979).

The slight reduction of plasma copper concentration as produced by the high dietary iron level was only apparent when dietary copper concentration was adequate and not when it was raised. This indicates that the adverse effect of high iron intake on plasma copper can be counteracted by high copper intake. The earlier observation that increasing iron intakes impair copper absorption in rats (Storey & Greger, 1987) agrees with the present results.

The present study shows that in rats fed diets with the recommended concentration of copper moderately raised dietary zinc and iron concentrations reduce plasma copper concentration. The effect of supplemental zinc was somewhat greater than that of iron, but the two effects were additive. The effects of zinc and iron were not seen in rats fed the high-copper diets. The ratios of zinc:copper and iron:copper in the diet appear to determine plasma copper concentration rather than the absolute amounts of zinc and iron.

ACKNOWLEDGEMENT

Shiguang Yu was supported by The Netherlands Foundation for Nutrition and Health Research.

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Chapter 9

GENERAL DISCUSSION

General discussion

This thesis shows that copper metabolism in rats can be influenced by various trace elements in the diet, including copper itself. Below, an attempt is made to integrate selected portions of the results obtained and to draw conclusions.

High intakes of iron or tin reduce copper absorption in rats

High intakes of iron (Johnson, 1989; Bremner & Young, 1981) or tin (Pekelharing et al., 1993; Greger et al., 1981) impair copper status of rats as indicated by lowered copper concentrations in tissues and plasma. It was not clear how dietary iron or tin alters copper status. Bremner and Young (1981) suggested that the intake of excessive amounts of iron stimulates the excretion of stored copper because apparent copper absorption was found to be unchanged (Johnson & Hove, 1986; Johnson & Murphy, 1988). In the present experiments, high intakes of iron or tin inhibited apparent copper absorption, which probably led to the observed lowered copper concentrations in tissues and plasma as well as to the decrease in biliary copper excretion (Chapters 2 & 3). How iron or tin inhibits copper absorption in the gastrointestinal tract is not known and this question needs further investigation.

Copper metabolism in jaundiced rats with hereditary conjugated hyperbilirubinemia is altered

The jaundiced rats used were derived from a mutant Wistar rat and displayed hereditary conjugated hyperbilirubinemia (Jansen et al., 1985; Kuipers et al., 1988). The autosomal recessive defect in this jaundiced rat is impaired canicular transport of organic anions (Oude Elferink et al., 1989; Kuipers et al., 1988; Jansen et al., 1985). After feeding on a high-copper diet, the jaundiced rats had elevated copper concentrations in liver when compared with their control counterparts (Chapter 4). Because the jaundiced rats have enlarged livers, the increase in absolute copper content of the liver was even more impressive. The accumulation of hepatic

copper in jaundiced rats after administration of a high-copper diet most likely is the result of a diminished increase in biliary copper excretion and a greater efficiency of copper absorption. Since bile fluid inhibits copper absorption (Gollan, 1975; Farrer & Mistilis, 1967), the reduced bile flow in the jaundiced rats could have contributed to the greater copper absorption. The canicular transport of glutathione is impaired in the jaundiced rats (Oude Elferink et al., 1989). Since the transport of glutathione and copper across the canicular membrane may be coupled (Alexander & Aaseth, 1980), the lesser increase in biliary copper excretion seen after dietary copper loading in the jaundiced rats could relate to defective canicular transport.

Copper metabolism in analbumianemic rats is not markedly altered

The Nagase analbuminaemic (NA) rat is derived from a mutant Sprague Dawley (SD) rat (Nagase et al., 1979). The NA rat lacks albumin in plasma which is due to the deletion of seven base pairs from base 5 to 11 from the 5' end of intron HI of the albumin gene (Esumi et al., 1983). Albumin is thought to play an important role in copper transport to liver after absorption by the gut (Gordon et al., 1987; Mason, 1979). However, copper contents in liver, kidney and plasma increased in NA rats to a similar extent as in control rats after feeding a high-copper diet. The NA rats had higher levels of radioactivity in kidneys and liver at two hours after intraperitoneal administration of ^{64}Cu (Chapter 6). The present results suggest that apart from albumin other substances, possibly amino acids (Zeumann & Sass-Kortsak, 1967; Sarkar & Kruck, 1966), take part in the transport of copper, at least in the absence of albumin.

Iron metabolism was found to be altered in NA rats (Chapter 5). NA rats had higher values of red blood cell count, haemoglobin, haematocrit, mean corpuscular haemoglobin concentration and total iron binding capacity, but lower values of mean corpuscular volume. Iron concentrations in plasma, liver,

kidney and heart were higher but those in spleen and tibia were lower in NA rats. Whether these changes of iron metabolism in NA are related to the absence of albumin is not clear.

Interactions of copper with selenium, iron and zinc

The effect of dietary copper on selenium status and metabolism of rats was found to depend on the dietary selenium concentration (Chapter 7). When the diets contained a low or normal amount of selenium, increasing copper intakes reduced selenium absorption, raised urinary selenium excretion and altered selenium concentrations in certain organs, but with higher selenium intakes dietary copper became ineffective. Copper might affect selenium metabolism both at the pre- and post-absorptive level.

The interactions of copper metabolism with dietary zinc or iron have been well documented (Johnson, 1989). Supplementation of the diet with high amounts of iron or zinc can impair the copper status of animals. However, the combined effect of iron and zinc on copper status was not well known. Moderately elevated concentrations of iron and zinc in the diet reduced the copper concentration in the plasma of rats. In essence, the effects of iron and zinc were additive (Chapter 8).

Relationship of hepatic and biliary copper

The liver is a central organ in copper metabolism (Linder & Goode, 1991; Evans, 1973). Copper is mainly stored in the liver and is incorporated into ceruloplasmin in this organ (Evans et al., 1970; Owen, 1964). From the liver, copper is secreted into bile, which is the main route for copper disposal from the body (Owen, 1965). Biliary copper concentration was found to be directly related to hepatic copper concentration in rats (Chapters 2-4). This also holds true for pigs (Skalicky et al., 1978). Thus, it seems that biliary copper concentrations are determined by hepatic copper concentration. Nevertheless, the total amount of copper excreted with bile tended to plateau at

high hepatic copper concentrations (Chapter 4).

Conclusions

1. High intakes of iron or tin impair copper status of rats through inhibition of copper absorption.

2. Excessive copper accumulation in the liver of jaundiced rats after feeding a high-copper diet is caused by a lesser rise in biliary copper excretion and greater copper absorption.

3. Copper metabolism is altered in analbuminaemic rats, but despite the lack of albumin in plasma, the analbuminaemic rats were able to transport copper from the gut to liver and to maintain copper homeostasis after either iron or copper loading.

4. The ratio of copper:selenium in the diet determines whether or not increasing intakes of copper affect selenium metabolism.

5. The lowering effects of moderately increased intakes of iron and zinc on plasma copper concentration are additive.

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Summary

Copper is an essential trace element for animals and man. It is a component of many enzymes and plays an important role in numerous physiological processes. Insufficient or redundant intake of copper may result in impaired health. Endogenous and exogenous factors can influence copper metabolism. The exogenous factors generally are ingested with the food. Thus, it is relevant to understand the interactions of copper metabolism with exogenous factors.

Various experiments have been conducted to investigate the metabolism of copper and its interactions with dietary iron, zinc, tin and selenium in rats, including hereditary jaundiced and analbuminemic rats. The rats were fed purified diets and housed under controlled conditions.

High intakes of iron or tin reduced copper concentrations in plasma, liver and kidneys as well as biliary copper excretion. Evidence is presented that these effects are brought about by inhibition of copper absorption in the gastrointestinal tract. How dietary iron or tin affects copper absorption is not known and this question needs further investigation.

The jaundiced rats used are characterized by conjugated hyperbilirubinemia and impaired canicular transport of organic anions. Copper metabolism was found to be altered in the jaundiced rats. After feeding a diet with a normal level of copper, the jaundiced rats had higher (baseline) values for copper concentrations in plasma and liver and for the rates of copper excretion with bile when compared with control rats. The jaundiced rats had a lower bile flow but significantly higher biliary copper concentrations. When fed on a high-copper diet, the jaundiced rats displayed a greater rise in copper concentrations in liver which was associated with a lesser increase in biliary copper excretion and greater copper absorption from the gastrointestinal tract.

The analbuminemic rats used are derived from a mutant Sprague Dawley rat and completely lack albumin in plasma. Copper concentrations in liver, kidney and plasma were increased in

analbuminemic rats fed either adequate- or high-copper diets. The analbuminemic rats also had higher levels of radioactivity in kidneys and liver at two hours after intraperitoneal administration of ^{64}Cu. The results obtained suggest that analbuminemic rats can maintain a relatively normal transport of copper.

The effects of dietary copper on selenium status and metabolism depended on the ratio of copper:selenium in the diet. When the diets contained either a low or normal amount of selenium, increasing copper intakes reduced selenium absorption, raised urinary selenium excretion and altered selenium concentrations in certain organs. However, with higher selenium intakes dietary copper became ineffective. It is suggested that dietary copper can affect selenium metabolism both at the pre- and post-absorptive level.

Supplementation of the diet with moderate amounts of iron or zinc reduced plasma copper concentrations of rats. The combined effects of iron and zinc were additive.

The biliary copper concentration was directly related to the hepatic copper concentration. However, the total amount of biliary copper excretion tended to plateau at high hepatic copper concentrations.

The following conclusions can be drawn. 1. High intakes of iron or tin impair copper status of rats through inhibition of copper absorption. 2. Excessive copper accumulation in the liver of jaundiced rats fed a high-copper diet was caused by a lesser rise in biliary copper excretion and greater copper absorption. 3. Copper metabolism in analbuminemic rats was not markedly altered, suggesting that albumin does not play a crucial role in copper transport from the gastrointestinal tract to the liver. 4. The ratio of copper:selenium in the diet determines the effect of dietary copper on selenium metabolism. 5. The effects of elevated dietary iron and zinc on plasma copper concentration are additive.

Samenvatting

Koper is een essentieel spoorelement voor mens en dier. Het is een component van vele enzymen en speelt een belangrijke rol in talrijke fysiologische processen. Onvoldoende of overtollige opneming van koper is nadelig voor de gezondheid. Endogene en exogene factoren kunnen de koperhuishouding beïnvloeden. De exogene factoren worden normaliter met het voedsel opgenomen. Het is derhalve relevant om de interacties tussen het kopermetabolisme en de exogene factoren te kennen.

Diverse experimenten zijn uitgevoerd teneinde de koperhuishouding en haar interacties met ijzer, zink, tin en selenium in de voeding te bestuderen in ratten, inclusief ratten met erfelijke geelzucht of afwezigheid van albumine in het bloedplasma. De ratten kregen semisynthetische voeders en werden onder gecontroleerde condities gehuisvest.

Een hoge opneming van ijzer of tin reduceerde de koperconcentraties in plasma, lever en nier evenals de excretie van koper met de gal. Er wordt bewijs geleverd dat deze effecten veroorzaakt worden door een verminderde koperabsorptie in de darm. Op welke wijze ijzer en tin met de koperabsorptie interfereren is niet bekend en dient nader onderzocht te worden.

De gebruikte geelzuchtige ratten worden gekarakteriseerd door geconjugeerde hyperbilirubinemie en een defect caniculair transport van organische anionen. De koperstofwisseling bleek gewijzigd in de geelzuchtige ratten. Tijdens verstrekking van een voeder met normaal kopergehalte, hadden de geelzuchtige dieren hogere niveaus van koper in het plasma en de lever en hogere excretiesnelheden van koper met de gal vergeleken met controleratten. De geelzuchtige ratten hadden een geringere produktie van galvloeistof maar de koperconcentratie in de gal was significant verhoogd. Na verstrekking van een koperrijk voeder toonden de geelzuchtige ratten een grotere toename van het leverkopergehalte hetgeen samenging met een geringere toename van de excretie van galkoper en een grotere absorptie van koper in de darm.

De gebruikte analbuminemische ratten zijn ontstaan uit een

Sprague Dawley mutant en hebben geen albumine in het bloedplasma. De koperconcentraties in lever, nier en plasma van de analbuminemische ratten waren verhoogd vergeleken met die van controleratten; dit stamverschil werd niet beïnvloed door het koperniveau van het voer. Twee uur na intraperitoneale toediening van ^{64}Cu hadden de analbuminemische ratten een verhoogde hoeveelheid radioaktiviteit in de nier en lever. De verkregen resultaten duiden erop dat analbuminemische ratten een relatief normaal kopertransport kunnen handhaven.

De effecten van koperopneming op de seleniumstatus en -huishouding waren afhankelijk van de koper:selenium-verhouding in het voer. Wanneer het voer een lage of normale seleniumconcentratie bevatte, leidde een verhoogde koperopneming tot een verminderde seleniumabsorptie, verhoogde seleniumexcretie met de urine en gewijzigde seleniumconcentraties in diverse organen. Echter bij een hogere seleniumconcentratie van het voer was koper niet meer effectief. Gesuggereerd wordt dat opgenomen koper de seleniumhuishouding op zowel pre- als post-absorptie-niveau kan beïnvloeden.

Suppletie van het voer met matige hoeveelheden zink of ijzer reduceerde de plasmakoperconcentratie bij ratten. De effecten van zink en ijzer waren additief.

De koperconcentratie van de galvloeistof was direct gecorreleerd met de koperconcentratie van de lever. Echter, de absolute koperexcretie met de gal neigde een constant niveau te bereiken bij hogere koperconcentraties van de lever.

De volgende conclusies kunnen worden getrokken. 1. Een hoge opneming van ijzer of tin reduceert de koperstatus door remming van de koperabsorptie. 2. De overmatige koperstapeling in de lever van geelzuchtige ratten na verstrekking van een koperrijk voeder werd veroorzaakt door een geringe toename van de koperexcretie met de gal en een verhoogde koperabsorptie. 3. De koperhuishouding bij analbuminemische ratten was niet drastisch gewijzigd hetgeen duidt dat albumine geen cruciale rol in het kopertransport speelt. 4. De verhouding koper:selenium in het

voeder bepaalt de invloed van het niveau van koperopneming op de seleniumhuishouding. 5. De effekten van ijzer en zink in de voeding op de plasmakoperconcentratie zijn additief.

小　结

铜是动物及人类所必需的微量元素。它是许多酶的组成成分并在诸多生理过程中发挥重要作用。铜摄入不足或过量均可损害健康。许多内外因子可影响铜代谢。外源性因子常随同食物一起被摄入体内。因此，很有必要了解铜与影响其代谢的外源性因子的相互作用。

在可控条件动物室中，通过对大鼠进行合成饲料喂养实验，对铜代谢及铜与膳食铁，锌，锡和硒的相互作用进行了研究。所用大鼠包括遗传性黄胆鼠和遗传性无白蛋白鼠。

结果表明，增加铁和锡摄入可降低血浆，肝和肾铜浓度及减少胆汁铜排出量。其原因是铁或锡拮抗胃肠道铜吸收。膳食铁或锡拮抗铜吸收的机理尚不清楚，有待进一步研究。

遗传性黄胆鼠的特征是高直接胆红素血症和有机阴离子向胆汁转运异常。遗传性黄胆鼠的铜代谢也有改变。当饲以铜含量正常的饲料，黄胆鼠基础血浆和肝铜浓度以及胆汁铜排出速率较对照高。虽然黄胆鼠胆汁流量明显下降，但是胆汁铜浓度显著增高。当饲以高铜饲料，黄胆鼠表现出肝铜浓度增加并伴有胃肠道铜吸收增高和胆汁铜排出相对减少。

无白蛋白鼠 (analbuminemic rats) 源于 Sprague Dawley 突变鼠。无白蛋白鼠缺乏血浆白蛋白. 无论饲以充足或高铜饲料，无白蛋白鼠的肝，肾和血浆铜浓度均较对照高。腹腔注射放射性同位素铜二小时后，无白蛋白鼠肝和肾的放射性也较对照高。这些结果提示无白蛋白鼠仍能维持较正常的体内铜的转运。

膳食铜对硒代谢的作用依赖于膳食中铜与硒的比例。当膳食硒含量正常或降低时，增加铜含量可减少硒的吸收，增加尿硒排出及改变某些脏器硒的浓度。然而，当同时增加硒的摄入时，高铜膳食对硒的作用消失。结果表明，膳食铜对硒代谢的作用可发生在硒被吸收前及吸收后。

增加膳食铁或锌可降低大鼠血浆铜浓度。铁和锌对血浆铜浓度的作用为相加作用。

胆汁铜浓度和肝铜浓度呈直线正相关。然而，当肝铜浓度较高时，胆汁铜排出量趋于饱和。

根据实验结果可得出以下结论：1. 增加铁或锡摄入量可抑制铜的吸收并进而影响大鼠铜代谢。2. 当饲以高铜饲料，黄胆鼠的过量肝铜潴留是由于其胆汁铜排出增加较少和胃肠道铜吸收增加。3. 无白蛋白鼠铜代谢没有显著改变。这表明白蛋白在铜从胃肠道向肝脏的转运中的作用有限。4. 膳食铜对硒代谢的影响取决于膳食中铜和硒的比例。5. 增加膳食铁和锌对血浆铜浓度的作用为相加作用。

Acknowledgements

This thesis is the result of three-years, intensive work with the help of many people and financial support from different sources. The thesis could never have been completed without the help of people from the Department of Human Nutrition and the Small Laboratory Animal Centre (C.K.P.), Wageningen Agricultural University, and the Department of Laboratory Animal Science and the Department of Large Animal Medicine and Nutrition, Faculty of Veterinary Medicine, Utrecht University. I therefore would like to express my general thanks and gratitude to those who helped me in one way or another.

The work described in this thesis started in 1990 when I was granted a fellowship of The State Education Commission of The People's Republic of China. At that moment, I did not know much about nutrition research in Holland and had no idea which university or institute I should contact. Then, I started to search the literature because I felt that the department with a high production of good papers should be the best one for me to work in. I thus selected the Department of Human Nutrition, Wageningen Agricultural University and wrote a letter to Professor Dr. J.G.A.J. Hautvast who then gave me the permission to do research in his department.

Professor Hautvast, I thank you very much for your hospitality and also for appointing two excellent supervisors, Professors Anton C. Beynen and Clive E. West. In addition, I have benefited a great deal from you through discussing current literature with you and participating in the 'AIO lunch'. It is amazing that you are not only knowledgeable in the field of nutrition but also well-informed about the related disciplines so that you reliably can predict the future trends in nutrition research and policy.

The person to whom I am indebted most is my promotor, Professor Dr. ir. Anton C. Beynen. During the past three years he gave me guidance and advice from the initial stage of drawing hypotheses from the literature, to making the proper experimental design, interpreting and processing data, writing papers and

submitting them for publication. Not only did you help me with respect to my research itself, but you also repeatedly arranged for financial support. "No problem" always was your answer whenever I asked you for help. No words in the world could express my gratitude for what you have done for me. What I have learned from you in relation to the approaches of doing research and the ways of interacting, communicating and stimulating people, will certainly influence my future scientific career as well as my personal life. I am also greatly indebted to your wife Ingrid and your daughter and son. Many evenings, nights and weekends, which should belong to them, you have used for my research work.

My thanks of course are due to those who work or worked in the Department of Human Nutrition. Professor Clive E. West, you are the first person who I met when I arrived at the department. I am very glad that you were my supervisor during the beginning of my research. Your English tongue helped me to improve my English. I also learned a lot from you through participating in your 'Micronutrient Club' meetings. I find it remarkable that you combine hard working and a good sense of humour. I thank you very much for everything you did for me. My special thanks I wish to tender to Annet Roodenburg. Annet, with you I did the first experiment in the C.K.P.. You made me familiar with the way of doing laboratory animal research here in Wageningen. You are also my most important teacher of Dutch, cultural history as well as of the Dutch language for which I am extremely grateful. I also thank your son Tim for the happy times that we spent together.

I do appreciate the help I received from all the people working in the laboratory of the Department of Human Nutrition, especially Jan Harryvan, Peter van de Bovenkamp, Robert Hovenier and Frans Schouten. Jan, thank you for everything you did for me. Most of my chemical analyses could never have been finished without your help. Peter, thank you for showing me how to operate the instruments in the laboratory and arranging for me to be able to use instruments in other departments. Robert and Frans, I am

very grateful for your kindness and for always being ready whenever I asked for help. I would also like to give my thanks to Arnold Timmer, Leslie Boogerd, Joke Barendse, Paul Hulshof and Truus Kosmeijer.

I thank Ben Scholte and Hannie van Oosten for their help in using the computer and thank Marie Jansen, Lidwien van der Heijden and Sioe-Kie Kroes for their help which greatly facilitated my research work. The staff members, AIOs, OIOs and Ph.D. fellows in the Department of Human Nutrition are highly acknowledged for their friendship, hospitality and willingness to help me.

The members of the Small Laboratory Animal Centre contributed substantially to this thesis. Jo Haas, you always arranged animal rooms, ordered or bred animals for me as soon as possible and always fulfilled requirements of my research. You are an excellent manager of the centre. Gerrit van Tintelen, with whom I conducted most of my experiments, I thank for his patience and reliability, and also for taking care of the animals and organizing the autopsies of each experiment that we did. Gerrit, I enjoyed it very much to work with you. Frank van den Broek, in your capacity as animal welfare officer, I am deeply grateful for your advice which ensured that my research work went smoothly. Frank, I also appreciated your friendship and tolerance. The other members of the C.K.P. I also thank for their help: Maria Peters, René Bakker, Bert Weyers, Fokke Berghuis, Inge Ruisch, Jos ter Hedde, Anneke Fleurke and Joke Hoogland.

The help and assistance from the various staff members of the Department of Laboratory Animal Science are invaluable. My special thanks are due to Professor Dr. Bert van Zutphen for letting me attend his course on laboratory animal science so that I was able to obtain the legal status of animal experimenter. I also thank Professor van Zutphen for his tolerance, kindness and consideration. Many of the chemical analyses would not have been possible without the assistance and advice from Inez Lemmens, whose attention to all details ensured the reliability of the

results. Inez, dank U. Francis Pastoor, thank you very much for many lifts between Wageningen and Utrecht so that my travelling was much easier while saving money and time too. The various aspects of the Dutch culture that I learned from you while travelling has been very useful in my everyday life in Holland. Francis, please allow me to take this opportunity to congratulate you on your newly received status of Ph.D. - Gefeliciteerd, Dr. Pastoor. Those who I also wish to acknowledge gratefully are Ineke Zaalmink, Xizhong Zhang, Koen Wienk, Hein van Lith, Marianne Albers, Anita Verdonk, Ria den Bieman and Freek Schlingmann.

Many people of other departments or universities made contributions to my research. My sincere thanks are given to professor Dr. A. Th. van 't Klooster for his cordial welcome to the Department of Large Animal Medicine and Nutrition, Utrecht University and also for his continuous interest in the progress of my research. I thank Jaap Joles, Academic Hospital, Utrecht University for his collaboration in the studies with analbuminemic rats and I thank Anth. M. de Bruijn of the Department of Epidemiology and Biostatistics, Erasmus University Medical School, Rotterdam, for his advice concerning some chemical analyses. Many of my thanks go to Gerrit Jan van den Berg at the Interfaculty Reactor Institute, Delft Technology University. I enjoyed it very much conducting the various experiments together. I do appreciate your efforts in arranging my participation in the course on isotope technology in your institute. Gertjan, I am especially grateful for the useful advice and prompt help you always gave to me.

During the first ten months in The Netherlands I was supported by the State Education Commission of The People's Republic of China for which I will always be very grateful. I also thank the Xi'an Medical University for giving me the opportunity to study in The Netherlands.

Finally, I would like to take this opportunity to express my hearty thanks to my wife Xeuping Qian, my parents, brothers

and sisters for their support and encouragement.

Wageningen, 20 December 1993. Shiguang Yu

List of publications

(*: Incorporated into this thesis)

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Curriculum vitae

Shiguang Yu was born on September 27, 1957 in Linshi, Shanxi province, The People's Republic of China. He attended primary and high school in Shuiyu. He graduated from high school in 1974. From 1974 to 1978, he worked as an electrician in Duizhen. He started his higher education at Shanxi Medical College in 1978 and graduated with a bachelor degree in medicine in August, 1983. Then, he went to Harbin Medical University, studied for three years there, and received his master degree in medicine in 1986. From 1986 to 1990, he worked as a teacher of nutrition and food hygiene in the Department of Nutrition and Food Hygiene, Xi'an Medical University. In October 1990, he came to The Netherlands and started his research work described in this thesis.