Copper homeostasis in rats fed on a high-sulphide diet

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The mechanism underlying the reduced Cu status in rats fed on a high-sulphide diet was investigated. Male rats aged 6 weeks were fed *ad libitum* on purified diets containing either 0 or 500 mg S^{2-}/kg and demineralized water for a period of 2 weeks. The high-sulphide diet had no effect on feed intake, body-weight gain or weight of liver and kidney but significantly reduced Cu concentrations in plasma and kidney. Biliary Cu excretion was decreased significantly in rats fed on the high-sulphide diet. Apparent Cu absorption (Cu intake – faecal Cu) and true Cu absorption (Cu intake – (faecal Cu – biliary Cu)) were significantly lowered after sulphide feeding for 2 weeks. Rats fed on the high-sulphide diet excreted less Cu in urine than did the controls. We conclude that high sulphide intake reduces Cu status in rats through inhibition of Cu absorption which is reflected by a decrease in biliary Cu excretion as a secondary feature.

Sulphide: Copper: Biliary excretion: Rat

There is an interaction between Cu, Mo and S in ruminant nutrition which can cause Cu status to vary across the entire range between deficiency and toxicity (Suttle, 1991). The metabolic basis for the Cu–Mo–S interaction lies in the formation of unabsorbable copper thiomolybdate complexes in the rumen which remain stable during transit to the ileum and thus determine the availability of Cu for absorption (Suttle, 1974, 1991). In ruminants the sulphide (S^{2-}) that is essential to form thiomolybdates ($MoOS_3^{2-}$, MoS_4^{2-}) can be produced by rumen microbes from protein and inorganic S from the diet or saliva (Ivan *et al.* 1986). The addition of sulphide to the diet of single-stomached animals may impair their Cu status. Bremner *et al.* (1982) showed that in rats fed on a high-sulphide diet the retention by the gut-free carcass of ^{64}Cu administered as a single oral dose was reduced and that Cu concentrations in plasma and kidneys were lowered. These findings indicate that the adverse effect of high sulphide intake on Cu status in rats is mediated by inhibition of intestinal Cu absorption, but stimulation of biliary Cu excretion cannot be excluded.

The amount of Cu in the body is determined by the difference between Cu intake and excretion. Intestinal Cu absorption equals Cu input, while at the recommended dietary Cu concentration (National Research Council, 1978) Cu output is represented by excretion of biliary Cu (Cartwright & Wintrobe, 1964) which is poorly reabsorbed (Owen, 1964; Farrer & Mistilis, 1967) and by Cu elimination via the urine (Yu & Beynen, 1995). We thus hypothesized that high sulphide intake inhibits intestinal Cu absorption which leads to a depressed biliary Cu excretion in order to achieve Cu balance or, alternatively, that high sulphide intake primarily stimulates biliary Cu excretion with enhanced Cu absorption as a secondary feature. Both mechanisms would lead to the observed impaired Cu status in rats fed on diets high in sulphide.

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 Sprague–Dawley rats (SD/Hsd-Ola, Harlan CPB, Zeist, The Netherlands), aged about 3 weeks, were used. On arrival they were housed in groups of five in stainless steel cages (600 × 210 × 190 mm) with wire mesh bases. The rats were given *ad libitum* demineralized water and the control diet (Table 1), which was formulated according to the recommended nutrient requirements of rats (National Research Council, 1978). After 14 d the rats were housed individually in metabolism cages (31400 mm^2 × 120 mm) for another 7 d. Then (day 0) the rats were divided into two groups of eight animals so that the group distributions of body weight were similar. One group was randomly allocated to the high-sulphide diet containing 500 mg added S^{2-}/kg diet (Table 1), and the other group remained on the control diet. Extra sulphide was added to the test diet in the form of CaS. The control and test diets were balanced for Ca (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. The metabolism cages were placed in randomized position in a room with controlled lighting (light on: 06.00–18.00 hours), temperature (19–21°) and relative humidity (50–60%).

Collection of samples

Faeces and urine were collected separately and quantitatively during days 12–14. At the end of the experiment (day 14) 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, NJ, USA). The abdomen was opened while the rats were under anaesthesia induced by a combination of ketamine (60 mg/kg body weight) administered intramuscularly and xylazine (8 mg/kg body weight) administered subcutaneously. This combination of the two drugs was used because it has been shown not to influence bile flow in rats (Fleck & Barth, 1990). After the cannula was inserted into the common bile duct and secured with suture thread the rats were kept on a heating pad (36–38°). Bile was collected into pre-weighed vials for 1 h and the volume of bile was calculated from the weight and specific gravity of the bile. 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° until analysis.

Analytical methods

The concentrations of Cu in organs, faeces, urine and feed samples were determined by flame atomic absorption spectrometry (Perkin-Elmer 2380, Perkin-Elmer Corporation, Norwalk, CT, USA). For the determination of Cu in organs, samples were dried in a vacuum dryer for 48 h and digested in 14 M-HNO_3 at 80° for 2 h. 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° for 17 h in a muffle furnace and then dissolved in 6 M-HCl. The determination of Cu in bile and plasma was carried out using flameless atomic absorption spectrometry (Varian AA-300, Varian Techtron Pty. Ltd., Springvale, Australia) after appropriate 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,

Table 1. *Composition of the experimental diets*

Ingredients (g/kg)	Control	High S^{2-}
Constant components*	276·57	276·57
Glucose	711·03	711·505
$CaCO_3$	12·4	10·8
CaS	—	1·125

* Constant components consisted of (g/kg diet): casein 151, maize oil 25, coconut oil 25, cellulose 30, $NaH_2PO_4.2H_2O$ 15·1, $MgCO_3$ 1·4, KCl 7·0, Na_2CO_3 0·07, mineral premix 10, and vitamin premix 12. The mineral premix consisted of (mg/kg diet): $FeSO_4.7H_2O$ 174, MnO_2 79, $ZnSO_4.H_2O$ 33, $CuSO_4.5H_2O$ 15·7, $NiSO_4.6H_2O$ 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 maize starch 9679·2. The vitamin premix consisted of (mg/kg diet): thiamin 4, riboflavin 3, nicotinamide 20, D,L-calcium pantothenate 17·8, pyridoxine 6, cyanocobalamin 50 (0·1% purity), choline chloride 2000, pteroylglutamic acid 1, biotin 2, menadione 0·05, D,L-α tocopheryl acetate 60, retinyl acetate and retinyl palmitate 8 (1200 retinol equivalents), cholecalciferol 0·025 and maize starch 9828·125.

Gaithersburg, MD, USA) was used to assess bias of Cu analysis. Analysed Cu concentration was 100·1 (SE 0·89)% (*n* 6) of the NBS certified value.

Statistical analyses

All data were found to be normally distributed (kurtosis and skewness test) except for those on biliary Cu excretion. The normally distributed data of the control and test groups were subjected to Student's *t* test to identify statistically significant differences. The variances of biliary Cu excretion were not homogeneous (*F* test) while logarithmic transformation of the data was ineffective. To evaluate the group difference on biliary Cu excretion the non-parametric Mann–Whitney U test was used. The level of significance was pre-set at $P < 0·05$. All data were processed using a computer program (Statistical Package for the Social Sciences, 1988).

RESULTS

Feed consumption, body and organ weights

The high-sulphide diet had no effect on feed consumption and body weight of the rats (Table 2). Likewise, there was no sulphide effect on the weights of liver and kidney at day 14 of the experiment.

Indicators of copper status

Table 3 shows the Cu concentrations in liver, kidney and plasma. The high-sulphide diet produced significantly reduced Cu concentrations in plasma and kidney but had no effect on hepatic Cu concentration.

Apparent absorption and urinary excretion of copper

Analysed Cu concentrations in the control and high-sulphide diets were found to be 4·0 and 3·3 mg/kg respectively. Thus, the rats fed on the high-sulphide diet consumed less Cu than did the control rats (Table 3). After 2 weeks the high-sulphide diet had significantly reduced apparent Cu absorption (Cu intake minus faecal Cu excretion) and urinary Cu excretion (Table 3).

Biliary copper excretion

The high-sulphide diet had no effect on bile flow, the values being 3·3 (SE 0·3) and 3·8 (SE 0·2) ml/kg body weight per h for the control and high-sulphide groups respectively. Sulphide feeding drastically reduced the amount of Cu excreted in bile (Table 3).

Table 2. *Feed intake, body and organ weights of rats fed on either a purified control diet or a high-sulphide diet**

(Mean values with their standard errors for eight rats per dietary group)

Diets...	Control		High S^{2-}	
	Mean	SE	Mean	SE
Feed intake (g/d)				
Days −7–0	20·4	0·8	20·3	0·6
Days 0–7	18·9	0·7	19·0	0·4
Days 7–14	19·0	0·8	18·9	0·5
Body wt (g)				
Day 0	146·0	3·3	147·5	3·8
Day 7	182·8	4·2	184·9	4·1
Day 14	220·3	5·7	220·5	5·3
Organ wt (g/kg body wt)				
Liver	38·3	0·8	38·3	1·0
Kidney	3·0	0·1	3·0	0·1

* For details of diets see Table 1.

Table 3. *Copper concentrations in plasma, liver and kidney, biliary excretion of copper, apparent and calculated true copper absorption in rats fed on either a purified control diet or a high-sulphide diet for 14 d*†

(Mean values with their standard errors for eight rats per dietary group)

Diets...	Control		High S^{2-}	
	Mean	SE	Mean	SE
Indicators of Cu status, day 14				
Plasma Cu (μmol/l)	11·00	0·83	2·05**	0·36
Liver Cu (μmol/g dry wt)	0·24	0·009	0·23	0·006
Kidney Cu (μmol/g dry wt)	0·38	0·010	0·34**	0·000
Cu balance (days 12–14)				
Cu intake (μmol/d)	1·50	0·06	1·49	0·04
Faecal Cu excretion (μmol/d)	0·88	0·07	1·11*	0·06
Apparent Cu absorption (% of intake)	41·0	3·92	25·0*	3·90
Urinary Cu excretion (nmol/d)	73·7	3·87	56·4*	6·02
Biliary Cu excretion (day 14), (nmol/kg body wt per h)	16·4	8·3	0·3*	0·1
True Cu absorption (μmol/kg body wt per d)	3·3	0·3	1·7**	0·1

Mean values were significantly different from those for the control group: * $P < 0·05$, ** $P < 0·01$.
† For details of diets and procedures, see Table 1 and pp. 910–911.

DISCUSSION

In the rats fed on the high-sulphide diet for 2 weeks, feed intake, body-weight gain and organ weights were not affected, but sulphide feeding lowered plasma and kidney Cu concentrations without affecting the hepatic Cu stores. These results are in agreement with those reported by Bremner *et al.* (1982).

By the end of the experiment the control rats apparently absorbed about 40% of the Cu ingested. The efficiency of Cu absorption corroborates that seen in earlier work (Van den Berg & Beynen, 1992). Feeding the high-sulphide diet significantly reduced apparent Cu absorption. At the same time, biliary Cu excretion was markedly depressed. Since biliary

Cu is only poorly reabsorbed (Owen, 1964; Farrer & Mistilis, 1967), the excretion of endogenous Cu with the faeces must have been reduced in the rats fed on the high-sulphide diet. As a result, the effect of sulphide feeding on Cu absorption, as assessed by calculation of apparent Cu absorption, was underestimated. The diurnal rate of biliary Cu excretion is not constant (Dijkstra *et al.* 1991), but when assuming that it is and that Cu excreted with bile is not reabsorbed, then true Cu absorption can be calculated as Cu intake − (faecal Cu − biliary Cu) which equals apparently absorbed Cu + biliary Cu. Table 3 shows the values for true Cu absorption under conditions of zero biliary Cu reabsorption. The values are almost identical to those that would be obtained if reabsorption of biliary Cu was either 25 or 50%. As would be anticipated, sulphide feeding reduced true Cu absorption. The calculation of true Cu absorption would suggest that biliary Cu is the only source of endogenous Cu in faeces. It should be noted that gastrointestinal excretions of Cu other than biliary excretion do occur (Mason, 1979). Although biliary Cu excretion had virtually ceased on the sulphide regimen, apparent Cu absorption should not equal true absorption.

It may be suggested that sulphide loading impairs Cu status in rats through inhibition of Cu absorption. In an attempt to maintain whole-body Cu balance, biliary and urinary excretion of Cu are diminished. Bremner *et al.* (1982) gave control and sulphide-fed rats a single oral dose of ^{64}Cu and determined radioactivity in the gut-free carcass 4 h later. Sulphide feeding was found to depress the recovery of radioactivity. The present results indicate that the observation by Bremner *et al.* (1982) is explained by inhibition of intestinal Cu absorption rather than by enhanced biliary Cu excretion. It is not known how sulphide feeding inhibits Cu absorption in rats, but it is tempting to speculate that, analogously to the situation in the rumen (Suttle, 1991), there is formation of insoluble CuS complexes in the rat intestine which cause a decrease in the amount of soluble Cu. Lower concentrations of soluble Cu in the small-intestinal lumen of rats are associated with lower efficiencies of Cu absorption (Van den Berg *et al.* 1993, 1994).

As mentioned earlier, the sulphide-induced decrease in biliary Cu excretion may be a compensatory response to the reduced Cu status. On the basis of Fe and Sn loading studies in rats we have suggested that biliary Cu excretion is determined by the Cu concentration in the liver (Yu *et al.* 1994; Yu & Beynen, 1995). However, in the present study the decrease in biliary Cu excretion seen after the dietary sulphide challenge was not associated with a decrease in hepatic Cu. It appears that the magnitude of Cu transport across the bile canalicular membrane and the size of the hepatic Cu pool are not causally related.

In conclusion, the feeding of the high-sulphide diet to rats inhibited intestinal Cu absorption which led to reduced Cu concentrations in plasma and kidney and depressed rates of biliary and urinary Cu excretion.

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