Dietary ascorbic acid lowers the concentration of soluble copper in the small intestinal lumen of rats

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We tested the hypothesis that ascorbic acid in the diet of rats lowers the concentration of soluble Cu in the small intestine, causing a decrease in apparent Cu absorption. Male rats were fed on diets adequate in Cu (5 mg Cu/kg) without or with 10 g ascorbic acid/kg. The diet with ascorbic acid was fed for either 6 or 42 d. Ascorbic acid depressed tissue Cu concentrations after a feeding period of 42, but not after 6 d. Dietary ascorbic acid lowered apparent Cu absorption after 6, but not after 42 d. The lowering of tissue Cu concentrations after long-term ascorbic acid feeding may have increased the efficiency of Cu absorption, and thus counteracted the inhibitory effect of ascorbic acid. Dietary ascorbic acid caused a significant decrease in the Cu concentrations in the liquid phase of both the proximal and distal parts of the small intestinal lumen. This effect was due to both a decrease in the amount of Cu in the liquid digesta and an increase in the volume of the liquid phase; only the latter effect for the distal intestine was statistically significant. We conclude that ascorbic acid supplementation lowers Cu absorption by decreasing the concentration of soluble Cu in the small intestine.

Ascorbic acid: Copper: Rat

In rats fed on ascorbic acid the apparent intestinal absorption of Cu is reduced (Van Campen & Gross, 1968; Johnson & Murphy, 1988; Van den Berg *et al.* 1990; Van den Berg & Beynen, 1992). This is supported by the observation that the disappearance of ^{64}Cu from ligated intestinal segments was depressed by the addition of ascorbate to the lumen (Van Campen & Gross, 1968), but the underlying mechanism is unknown. Under *in vitro* conditions, ascorbic acid can reduce Cu^{2+} to Cu^{+} ions (Harris & Percival, 1991; McArdle, 1992), which may lower Cu solubility (Gollan *et al.* 1971). If this effect of ascorbic acid also occurs in the lumen of the intestine, the inhibition of Cu absorption caused by ascorbic acid intake can be explained because the efficiency of Cu absorption probably depends on the concentration of Cu in the liquid phase of the digesta (Van den Berg *et al.* 1993).

In the present study we tested the hypothesis that the inhibitory effect of dietary ascorbic acid on apparent Cu absorption in rats is associated with a decrease in the concentration of Cu in the liquid phase of small intestinal digesta. Any effect of dietary ascorbic acid on intestinal Cu solubility may be independent of the duration of feeding with this vitamin. In contrast, ascorbic-acid-induced inhibition of Cu absorption may become smaller with longer feeding periods due to compensatory effects of the reduced tissue Cu concentrations as produced by ascorbic acid feeding (Van den Berg & Beynen, 1992). Thus, the effect of ascorbic acid (10 g/kg diet) on apparent Cu absorption and the concentration of soluble intestinal Cu in rats was determined after short-term (6 d) and long-term (42 d) feeding periods.

MATERIALS AND METHODS

The experimental protocol was approved by the animal experiments committee of the Rotterdam Erasmus University.

Animals, housing and diets

Outbred, male Wistar rats (Hsd/Cpb:WU; Harlan, Zeist, The Netherlands), aged about 3 weeks were used. On arrival, the rats had *ad lib.* access to a commercial, pelleted diet (SRMA^{R}; Hope Farms, Woerden, The Netherlands) and tap water. After 2 d all rats entered a pre-experimental period of 10 d. They received a purified control diet containing 709·4 g glucose and 5 mg Cu/kg (Table 1), and demineralized water. The rats had free access to food and water. During the pre-experimental period the rats were housed in groups of four animals in wire-topped, polycarbonate cages (375 × 225 × 150 mm) with inlaid wire mesh floors above filter paper. The cages were placed in a room with controlled temperature (20–22°), relative humidity (40–65 %) and a 12 h light cycle (light, 06.00–18.00 hours).

At the end of the pre-experimental period (day 0 of the experiment) the rats were divided into three groups of eight animals each, which were stratified for body weight. The groups were randomly allocated to the experimental diets: two groups remained on the control diet, and one group was transferred to the diet containing 10 g ascorbic acid/kg (Table 1). One group given the control diet received this diet for 36 d followed by the ascorbic-acid-containing diet for another 6 d. The other two groups were given their diets for 42 d. Ascorbic acid was added to the test diet at the expense of the glucose component. The control diet was formulated according to the nutrient requirements of rats (National Research Council, 1978). The composition of the diets is shown in Table 1. The purified diets, which were in powdered form, were stored at 4° until feeding. Food and demineralized water were provided *ad lib.* The experiment lasted 42 d. During the experimental period the rats were housed individually in metabolism cages (Tecniplast Gazzada, Buguggiata, Italy) which were placed in racks in randomized position. Feed consumption and body weight were recorded weekly. During the last 5 d but one of the experimental period (days 37–41), faeces of each rat were collected quantitatively.

Collection of samples

From 09.00 hours on day 42, the animals received 3 g of their food in random order at 5-min intervals. The rats had been fasted for 16 h, and each rat consumed its meal within 5 min. At 3 h after feeding, each rat was 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 in a heparinized tube. The entire small intestine, between stomach and caecum, was removed. It was divided on the basis of measured length into a proximal and a distal half. Total intestinal contents of both halves of the intestine were collected separately in pre-weighed tubes by gently squeezing the intestine between finger and thumb. The intestinal contents were immediately centrifuged for 10 min at 10000 ***g*** and supernatant and pellet were separated. The weights of the pellet and the supernatant fraction were determined. Plasma was collected immediately from the heparinized blood samples by low-speed centrifugation. Liver, heart, kidneys, spleen and muscle (*flexor digitorum longus*) were excised and weighed. All samples were frozen at −20° until analysis.

Chemical analyses

Food and faeces samples and pellets from digesta were freeze-dried, homogenized and weighed, and subsequently ashed at 500° for 18 h and dissolved in 6 M-HCl. The

Table 1. *Composition of the purified diets*

Dietary supplement...	None	Ascorbic acid
Components (g/kg)		
Glucose	709·4	699·4
Ascorbic acid	—	10·0
Constant components*	290·6	290·6
Chemical analysis		
Cu† (mg/kg)	5·1	5·1
Ascorbate† (g/kg)	0	10·5

* The constant components consisted of (g): casein 151, maize oil 25, coconut fat 25, cellulose 30, $CaCO_3$ 12·4, $MgCO_3$ 1·4, KCl 1·0, $KHCO_3$ 7·7, NaH_2PO_4 15·1, mineral premix 10, vitamin premix 12. The mineral premix consisted of the following (mg): $CuSO_4.5H_2O$ 15·7, MnO_2 79, $FeSO_4.7H_2O$ 174, $ZnSO_4.H_2O$ 33, $NiSO_4.6H_2O$ 13, NaF 2, $CrCl_3.6H_2O$ 1·5, $SnCl_2.2H_2O$ 1·9, NH_4VO_3 0·2, KI 0·2, $Na_2SeO_3.5H_2O$ 0·3, maize meal 9679·2. The vitamin premix consisted of the following (mg): thiamin 4, riboflavin 3, nicotinic acid 20, DL-calcium panthothenate 17·8, pyridoxine 6, cyanocobalamin 50, choline chloride 2000, pteroylmonoglutamic acid 1, biotin 2, menadione 0·05, DL-α-tocopheryl acetate 60, retinyl acetate and retinyl palmitate 8 (1200 retinol equivalents), cholecalciferol 0·025, maize meal 9828·125.

† Average values of four measurements.

Table 2. *Effect of feeding ascorbic acid for either 6 or 42 d on growth performance and plasma ascorbate concentrations of rats**

(Mean values for eight rats per dietary group)

Dietary supplement...	None (control)	Ascorbic acid				
Feeding period...	Days 0–42	Days 37–42		Days 0–42		
	Mean	Mean	Statistical significance of difference from control: $P =$	Mean	Statistical significance of difference from control: $P =$	Pooled SE (df 23)
Body wt (g)						
Day 0	111	111	0·92	109	0·61	2·2
Day 42	319	312	0·37	319	1·00	7·1
Feed intake (g/d)						
Days 37–41	18·9	18·4	0·26	18·7	0·81	0·46
Faecal output (g wet wt/d)						
Days 37–41	8·1	8·1	1·00	8·5	0·34	0·35
Faecal dry wt (g/kg)						
Days 37–41	582	576	0·72	565	0·25	11.3
Plasma ascorbate (μmol/l)	53	86	0·00	81	0·00	4·9

* For details of diets and procedures, see Table 1 and pp. 702–703.

supernatant fractions of intestinal contents were used without further pre-treatment. Plasma and tissues were freeze-dried and then digested in 14 M-HNO_3 (Suprapur; Merck, Darmstadt, Germany). Cu in the samples was determined by atomic absorption spectrophotometry using either an acetylene–air flame or graphite furnace (Varian AA-475 and Varian Spectra AA-3300; Varian Techtron, Springvale, Australia). Accuracy of Cu analysis was checked with a reference sample (Standard Reference Material 1577 Bovine Liver; US National Institute of Standards and Technology, Gaithersburg, MD, USA); the

Table 3. *Effect of feeding ascorbic acid for either 6 or 42 d on indicators of Cu status and apparent Cu absorption in rats**

(Mean values for eight rats per dietary group)

Dietary supplement...	None (control)	Ascorbic acid				
Feeding period...	Days 0–42	Days 37–42		Days 0–42		
	Mean	Mean	Statistical significance of difference from control: $P =$	Mean	Statistical significance of difference from control: $P =$	Pooled SE (df 23)
Cu concentrations						
Plasma (μmol/l)	15·99	14·99	0·21	14·26	0·02	0·496
Liver (nmol/g dry wt)	234	232	0·99	209	0·04	7·0
Kidney (nmol/g dry wt)	396	347	0·05	316	0·00	16·1
Heart (nmol/g dry wt)	310	316	0·61	271	0·03	10·6
Spleen (nmol/g dry wt)	79	78	0·77	66	0·04	4·0
Muscle (nmol/g dry wt)	52	55	0·44	43	0·05	2·5
Faecal Cu output (μg/d) Days 37–41	65	72	0·06	67	0·57	2·7
Apparent Cu absorption (% intake) Days 37–41	32·5	23·4	0·00	29·3	0·31	2·07

For details of diets and procedures, see Table 1 and pp. 702–704.

bias was always less than 5%. Precinorm (Boehringer, Mannheim, Germany) was analysed along with the plasma and soluble digesta, and analysed Cu concentration differed less than 10% from the target. The combined between- and within-run variation of analysed Cu concentrations in the reference samples was less than 5% (coefficient of variation).

Ascorbic acid in diet samples was quantified after extraction with 0·68 M-metaphosphoric acid by HPLC with electrochemical detection (Yoshiura & Iriyama, 1986). The recovery of added ascorbic acid was 95% (SE 1·10, n 6) and the combined within- and between-run variation coefficient for a reference sample (8 g ascorbic acid/kg feed) was 8%. For the analysis of plasma ascorbate, plasma was mixed with 0·54 M-metaphosphoric acid (1:4, v/v) in order to precipitate proteins and to prevent oxidation of ascorbate (Parviainen *et al.* 1986). Clear supernatant (100 μl) was injected into a reversed-phase column (125 mm × 4 mm; Merck Lichrospher 100-5RP18 cat. no. 50943; Merck, Darmstadt, Germany) and eluted with 0·08 M-metaphosphoric acid at a flow rate of 1 ml/min. Ascorbic acid was detected electrochemically at +0·7 V and the amount determined by peak-height measurement. The detection limit was 5 μmol/l and linearity was observed up to 400 μmol/l. The recovery of ascorbic acid added to plasma samples was on average 98% (SE 0·61, n 9) and the day-to-day reproducibility for a reference sample containing 25 μmol ascorbate/l was < 5% (coefficient of variation, n 6).

Calculations

Solubility of Cu was estimated as Cu present in the liquid phase of the intestinal contents. The pellet obtained after centrifugation comprises the solid phase contaminated with liquid phase. The weight of the solid phase was obtained after freeze-drying the pellet. The weight of the liquid phase was calculated as the sum of the weight of liquid phase in the pellet (total pellet weight minus solid phase) and that of the supernatant. The concentration of Cu in the supernatant was assumed to be identical to that of the liquid phase. The amount of Cu in the liquid phase was obtained after multiplying Cu concentration (μmol/l) in the supernatant with the weight of the liquid phase. The amount of Cu in the solid phase was calculated as that in the total pellet minus that in the liquid phase of the pellet.

Apparent absorption of Cu was calculated as Cu intake minus faecal excretion and expressed as a percentage of intake.

Statistical analyses

The data are summarized as group means and pooled SE. One-way analysis of variance was used to calculate pooled SE; homogeneity of variances was verified with the use of Bartlett's test. Observations within groups were found to be consistent with having a normal distribution (Kolmogorov–Smirnov test). The statistical significance of differences between the two test groups and the control group were evaluated with two-sided Student's t test, and significance probabilities are given. The level of statistical significance used to indicate an effect was pre-set at $P < 0{\cdot}025$ instead of 0·05 (Bonferroni's adaptation). Calculations were performed using the SPSS/PC$^+$ statistical package (SPSS Inc., 1988).

RESULTS

Growth performance and plasma ascorbate

Final weight and food consumption did not differ between the dietary groups (Table 2). Faecal output and percentage dry matter were similar for the three groups. The addition of ascorbic acid to the diet caused a significant increase in plasma ascorbate levels, which was similar for the two feeding periods.

Indicators of copper status and copper absorption

Cu concentrations in plasma and kidney were significantly lowered by ascorbic acid feeding for 42 d, but not by feeding for 6 d (Table 3). In rats given ascorbic acid for 42 d, Cu concentrations in liver, heart, spleen and muscle tended to be reduced. Faecal Cu excretion tended to be enhanced after short-term but not after long-term feeding of ascorbic acid. Thus, addition of ascorbic acid to the diet significantly decreased apparent Cu absorption after 6 d, but not after 42 d.

Intestinal soluble copper concentration

The amounts of Cu in liquid and solid phases in the proximal part of the small intestine did not differ significantly between the dietary groups (Table 4). However, dietary ascorbic acid slightly reduced the group mean amount of Cu in the liquid phase and slightly raised the group mean liquid phase weight. Thus, ascorbic acid in the diet significantly decreased the concentration of Cu in the liquid phase of the proximal part of the small intestine.

In the distal part of the small intestine the amount of Cu in the liquid phase was not significantly affected by dietary ascorbic acid, but the group mean was reduced by about 15%. In addition, in rats given ascorbic acid the volume of the liquid phase was significantly raised so that Cu concentration in the liquid phase of the distal part of the small intestine was significantly decreased after ascorbic acid feeding. The pH in the liquid phase of the distal part of the small intestine was significantly lower in rats fed on ascorbic

Table 4. *Effect of feeding ascorbic acid on the distribution of Cu between the liquid and solid phases of digesta in the small intestine of rats**

(Mean values for eight rats per dietary group)

Dietary supplement...	None (control)	Ascorbic acid				
Feeding period...	Days 0–42	Days 37–42		Days 0–42		
	Mean	Mean	Statistical significance of difference from control: $P =$	Mean	Statistical significance of difference from control: $P =$	Pooled SE (df 20–23)
Proximal intestine						
Liquid phase wt (mg)	191	208	0·79	208	0·77	38·6
Solid phase wt (mg)	40	40	0·99	36	0·73	8·5
Cu						
Amount in liquid phase (nmol)	2·99	2·26	0·40	2·18	0·38	0·528
Amount in solid phase (nmol)	2·16	2·62	0·33	2·81	0·31	0·370
Concentration in liquid phase (μmol/l)	16·34	11·17	0·02	10·43	0·02	1·301
Distal intestine						
Liquid phase wt (mg)	433	614	0·04	628	0·01	49·3
Solid phase wt (mg)	98	93	0·72	98	1·00	11·2
pH	7·03	6·57	0·02	6·69	0·03	0·104
Cu						
Amount in liquid phase (nmol)	7·12	5·90	0·38	5·99	0·39	0·949
Amount in solid phase (nmol)	2·20	2·97	0·00	2·94	0·07	0·206
Concentration in liquid phase (μmol/l)	17·96	9·52	0·02	9·35	0·03	2·093

* For details of diets and procedures, see Table 1 and pp. 702–704.

acid. Irrespective of the feeding period, ascorbic acid in the diet produced an increase in the amount of Cu in the solid phase of the distal small intestine.

DISCUSSION

The present results confirm that a high concentration of ascorbic acid in the diet of rats lowers tissue Cu concentrations and intestinal absorption of Cu (Johnson & Murphy, 1988; Van den Berg *et al.* 1990; Van den Berg & Beynen, 1992). Decreased tissue Cu concentrations, as seen in rats fed on a diet deficient in Cu, are associated with increased efficiencies of Cu absorption. Thus, we have put forward the idea that after longer periods of ascorbic acid feeding the reduced tissue Cu concentrations elicit compensatory mechanisms so that Cu absorption is enhanced, and thereby masks the inhibitory effect of ascorbic acid (Van den Berg & Beynen, 1992). Indeed, ascorbic acid consumption by the rats depressed apparent Cu absorption within 6 d, but this effect was not seen after 42 d. On the other hand, tissue Cu concentrations were reduced after 42 d, but not after 6 d. Thus, when studying effects of ascorbic acid on Cu metabolism, rebound effects of impaired Cu status should be considered.

Although there is very limited information on the mechanism and principal site(s) of Cu absorption (Owen, 1964; Van Campen & Mitchell, 1965), it could be suggested that the concentration of soluble Cu in the intestinal lumen and the activity of an undefined Cu carrier in the mucosa are important determinants of the efficiency of Cu absorption. With regard to Cu solubility, alimentary secretions may form soluble Cu complexes which enhance Cu absorption (Gollan, 1975). Fructose feeding lowered both intestinal concentrations of soluble Cu and apparent Cu absorption in rats (Van den Berg *et al.* 1993). As far as we know, the present study demonstrates for the first time that ascorbic acid feeding lowered Cu concentrations in the liquid phase of small intestinal contents. It is likely that the short-term feeding of ascorbic acid reduced apparent Cu absorption by decreasing the concentration of soluble Cu in the intestinal lumen.

It is not known why ascorbic acid in the diet reduced the concentration of soluble intestinal Cu. Ascorbic acid may reduce Cu^{2+} to Cu^{+} (Harris & Percival, 1991; McArdle, 1992) which could impair Cu binding to physiological ligands so that it becomes less soluble (Gollan *et al.* 1971). The observed, systematic tendency for the amount of Cu in the liquid phase of intestinal contents to be reduced and that in the solid phase to be raised after feeding with ascorbic acid might have resulted from such an effect. The decrease in soluble Cu concentration in the distal part of the small intestine can be explained in part by the enlargement of the liquid phase. The basis for the greater liquid phase after ascorbic acid feeding is not known.

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