Influence of ascorbic acid supplementation on copper metabolism in rats

BY GERRIT J. VAN DEN BERG*

Interfaculty Reactor Institute, Delft University of Technology, Mekelweg 15, 2629 JB Delft, The Netherlands

AND ANTON C. BEYNEN

Department of Laboratory Animal Science, State University, PO Box 80.166, 3508 TD Utrecht and Department of Human Nutrition, Agricultural University, PO Box 8129, 14 6700 EV Wageningen, The Netherlands

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An attempt was made to unravel further the mechanism by which high dietary concentrations of ascorbic acid influence copper metabolism. The addition of ascorbic acid to the diet of rats caused about a twofold increase in plasma ascorbate concentrations and reduced group mean plasma and tissue concentrations of Cu. The effect of 10 g ascorbic acid/kg diet was greater than that of 1 g/kg. Ascorbic acid feeding reduced blood haemoglobin concentrations and packed cell volume values. Dietary ascorbic acid caused a significant decrease in apparent Cu absorption from the intestine. Ascorbate, intravenously administered together with ^{64}Cu, caused an increase in ^{64}Cu in the liver. Ascorbate, at concentrations occurring in plasma after ascorbic acid feeding, promoted the uptake of ^{64}Cu by isolated hepatocytes. Thus, ascorbate stimulated the efficiency of hepatic uptake of Cu. Ascorbate, intravenously administered together with ^{64}Cu, stimulated accumulation of ^{64}Cu in bile of rats with a bile duct cannula. In rats fed on ascorbic acid, intravenously administered ^{64}Cu was recovered in bile at increased rates. Dietary ascorbic acid enhanced the recovery of intraperitoneally administered ^{64}Cu in faeces. The ascorbate-induced stimulation of biliary ^{64}Cu excretion may reflect an increased hepatic uptake of ^{64}Cu and be caused by an increased specific activity of Cu in liver pools. It is suggested that dietary ascorbic acid reduces tissue Cu concentrations primarily by interfering with intestinal Cu absorption. Ascorbate increases the efficiency of hepatic uptake of Cu, but this effect may not be causatively related with the reduced tissue Cu concentrations after ascorbic acid feeding.

Tissue copper: Vitamin C: Copper metabolism: Anaemia: Rat

In laboratory animals, high intakes of ascorbic acid cause reduced plasma and liver concentrations of copper and decreased plasma activities of ceruloplasmin (*EC* 1.16.3.1) (Hunt *et al.* 1970; Milne & Omaye, 1980; Smith & Bidlack, 1980; Milne *et al.* 1981; Johnson & Murphy, 1988). In addition, rats fed on large amounts of ascorbic acid develop anaemia, which may be the result of ascorbate-induced Cu deficiency (Johnson & Murphy, 1988). In humans, ascorbic acid supplementation may induce decreased Cu concentrations and ceruloplasmin activities in serum (Finley & Cerklewski, 1983; Jacob *et al.* 1987; Milne *et al.* 1988).

In rats fed on ascorbic acid, whole-body retention of orally administered ^{64}Cu was depressed (Van Campen & Gross, 1968) and the apparent efficiency of Cu absorption reduced (Johnson & Murphy, 1988). The disappearance of ^{64}Cu from ligated intestinal segments was depressed by the addition of ascorbate to the lumen (Van Campen & Gross,

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1968). Simultaneous oral administration of ^{64}Cu and ascorbate increased the recovery of ^{64}Cu in faeces within 1 d when compared with the administration of ^{64}Cu alone (Van den Berg *et al.* 1990). Thus, it is plausible that ascorbate impairs Cu absorption.

Post-absorptively, ascorbate may also influence Cu metabolism. After intraperitoneal administration of ^{64}Cu, whole-body retention of ^{64}Cu and specific activity of ^{64}Cu in the liver were increased in rats fed on ascorbate (Van den Berg *et al.* 1990). This effect may relate to the reduced tissue Cu concentrations in rats fed on ascorbate, because in nutritionally Cu-deficient rats whole-body retention of intraperitoneally administered ^{64}Cu was also increased (Van den Berg *et al.* 1990, 1991). Further evidence supporting this concept comes from the observation that liver cells isolated from Cu-deficient rats have increased efficiency of Cu uptake (Van den Berg *et al.* 1991). Thus, dietary ascorbic acid may have indirect effects, i.e. effects caused by ascorbate-induced decreased tissue Cu concentrations.

With the use of a human erythroleukemic cell line (K562 cells) it was shown that ascorbate enhances Cu transport from ceruloplasmin into the cells (Percival & Harris, 1989). DiSilvestro & Harris (1981) have shown an enhancing effect of ascorbate, when administered together with Cu, on lysyl oxidase (*EC* 1.4.3.13) activity in chick aorta, a Cu-dependent enzyme extremely sensitive to changes in dietary Cu. These studies point to a direct effect of ascorbate. It may be caused by enhancement of Cu dissociation by reduction of Cu(II) to Cu(I), which promotes the availability of Cu for cellular uptake (Van den Berg & Van den Hamer, 1984; Ettinger *et al.* 1986). Thus, the indirect and direct effects of ascorbate on cellular Cu uptake are complementary.

To unravel further the metabolic basis for the reduced tissue Cu concentrations after ascorbic acid feeding, the influence of ascorbate on hepatic uptake and biliary excretion of Cu is of interest. Bile is the main route by which Cu leaves the body and, thus, biliary Cu excretion plays an important role in Cu homeostasis. We have carried out in vitro and in vivo experiments to study the effect of ascorbate on hepatic Cu metabolism. The previously-mentioned studies with rats (Van Campen & Gross, 1968; Johnson & Murphy, 1988; Van den Berg *et al.* 1990) have employed diets containing 10 g ascorbic acid/kg, which is equivalent to intakes of about 1 g ascorbic acid/kg body-weight. Compared with common doses of ascorbic acid supplementation in humans (Finley & Cerklewski, 1983; Jacob *et al.* 1987) such intakes are unrealistically high, which might interfere with extrapolation of the rat findings to man. Therefore, we have studied not only the effects on Cu metabolism in rats of diets containing 10 g ascorbic acid/kg, but also those of diets containing 1 g ascorbic acid/kg. The effects of 1 g ascorbic acid/kg were studied using diets containing either recommended (National Research Council, 1978) or low amounts of Cu because in animals fed on a low-Cu diet the effects of ascorbic acid feeding may be more pronounced.

MATERIALS AND METHODS

Expt 1. Cu uptake by isolated rat hepatocytes

Male Wistar rats of the HSD/Cpb:WU strain (Harlan-CPB, Zeist) were used as hepatocyte donors. The rats were aged 10 weeks and had been fed on a commercial pelleted diet (SRMA®; Hope Farms, Woerden) and tap water *ad lib.* Hepatocytes were isolated by the collagenase (*EC* 3.4.24.3) perfusion method of Berry & Friend (1969). Primary parenchymal cell cultures were obtained by selective attachment to collagenized plastic dishes (diameter, 60 mm) for 2 h at 37° in a Ham's F-10 medium (Ham, 1963) supplemented with fetal calf serum (120 ml/l). Various concentrations of ascorbate (L-ascorbate; Merck, Darmstadt, Germany) and ^{64}Cu acetate (15 μmol Cu/l) were added to the medium. After various incubation periods at 37° the radioactive medium was aspirated and the cells were

harvested and washed twice with Ham's F-10 before measurement of cellular ^{64}Cu. Net Cu uptake by hepatocytes was expressed as ng Cu/mg cellular protein, and was corrected for non-specific Cu binding or uptake, or both, by subtracting cellular radioactivity determined after incubation of hepatocytes at 4° in parallel experiments. To test whether ascorbate interacts specifically with Cu uptake, $^{65}ZnCl_2$ uptake by hepatocytes was also measured. For this purpose, $^{65}ZnCl_2$ (12 μmol Zn/l; specific activity 35 TBq ^{65}Zn/g Zn; Radiochemical Centre, Amersham, UK) was added to the medium of parallel incubations.

Expt 2. Hepatic uptake and biliary excretion of ^{64}Cu after intravenous administration of ^{64}Cu with or without ascorbate

Male Wistar rats, aged 10 weeks and weighing on average 250 g, were used. The rats had been fed on commercial pelleted diet and tap water *ad lib.* The animals were anaesthetized with pentobarbital sodium (60 mg/kg body-weight intraperitoneally; Nembutal®, Sanofi Sante Animale SA, Paris, France). The bile duct was cannulated as described elsewhere (Villalon *et al.* 1987). Body temperature was kept at 37° with the use of a thermostatically-controlled heating lamp. At 15 min after collection of the first bile, ^{64}Cu acetate (0·5 μg Cu) with or without 0·1 mg ascorbate in phosphate-buffered saline (9 g sodium chloride/l) was injected intravenously; a total volume of 0·25 ml was injected per animal. Bile was then collected for a period of 150 min after which the rats were killed by exposure to carbon dioxide. Blood was collected by aortic puncture and livers were removed. ^{64}Cu was measured in bile, liver and carcass without liver.

Expt 3. Effect of a diet containing 10 g ascorbic acid/kg on Cu metabolism

Male, specified-pathogen-free Wistar rats, aged about 3 weeks, were used. On arrival in the animal house they were kept, three animals in a cage, in wire-topped Makrolon-3 cages (UNO BV, Zevenaar) with a layer of sawdust as bedding. For 10 d they were fed *ad lib.* on a purified diet containing 5 mg Cu/kg. The diet was formulated according to the recommended nutrient requirements of rats (National Research Council, 1978); its composition is given in Table 1. After the pre-experimental period of 10 d (day 0) the rats were divided into two groups consisting of six rats each. One group remained on the pre-experimental diet and the other group was transferred to the diet containing 10 g ascorbic acid/kg (Table 1).

On day 28 the animals received a single oral dose of ^{64}Cu acetate (5 μg Cu) and on day 35 an intraperitoneal injection of ^{64}Cu acetate (5 μg Cu) in 0·25 ml sodium acetate buffer (0·05 mol/l, pH 5·4). After the administration of ^{64}Cu, urine and faeces were collected during 3 d for determination of ^{64}Cu, and ^{64}Cu whole-body measurements were extended over 96 h. True efficiency of Cu absorption was calculated according to Heth & Hoekstra (1965).

On day 42, the bile duct was cannulated while under pentobarbital anaesthesia. After another 15 min, ^{64}Cu acetate (0·5 μg Cu) was administered intravenously. Subsequently, bile was collected over a total period of 150 min. Then the rats were killed by exposure to CO_2 and livers excised. ^{64}Cu in whole liver, body (minus liver) and total bile was measured and expressed as a percentage of the administered dose. Liver metallothionein, Cu in selected tissues, plasma ascorbate, packed cell volume, blood haemoglobin and plasma ceruloplasmin (as its oxidase activity) were determined.

Expt 4. Effects of diets containing 1 g ascorbic acid/kg on Cu metabolism

Male Wistar rats aged about 3 weeks were used. All animals were fed on the purified diet containing 5 mg Cu/kg. After 10 d, on day 0 of the experimental period, the rats were divided into four groups of equal size so that group mean body-weights were similar. Each

Table 1. *Expts 3 and 4. Composition of the purified diets used*

	Expt 3		Expt 4			
Copper* (mg/kg)	5·0	5·0	5·0	5·0	1·0	1·0
Ascorbic acid* (g/kg)	—	10·0	—	1·0	—	1·0
Components (/kg diet)						
Glucose (g)	702·6	692·6	702·6	701·6	702·6	701·6
Ascorbic acid (g)	—	10·0	—	1·0	—	1·0
$CuSO_4.5H_2O$ (mg)	15·7	15·7	15·7	15·7	—	—
Constant components† (g)	297·4	297·4	297·4	297·4	297·4	297·4
Chemical analysis						
Cu‡ (mg/kg)	5·2	5·2	5·3	5·1	0·8	0·8
Ascorbate‡ (g/kg)	—	9·1	—	1·0	—	0·9

* Calculated values.

† The constant components consisted of (g): ovalbumin 151, maize oil 25, coconut fat 25, cellulose 30, magnesium carbonate 1·4, potassium chloride 1·0, potassium bicarbonate 7·7, sodium dihydrogenphosphate 15·1, sodium carbonate 6·8, calcium carbonate 12·4, mineral premix 10, vitamin premix 12. The mineral premix consisted of the following (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 meal 9694·9. The vitamin premix consisted of the following (mg): thiamin 4, riboflavin 3, nicotinamide 20, DL-calcium panthothenate 17·8, choline chloride 2000, pyridoxine 6, cyanocobalamin 50, folic acid 1, biotin 2, menadione 0·05, DL-α-tocopheryl acetate 60, retinyl acetate and retinyl palmitate 8 (4000 IU), cholecalciferol 2 (1000 IU), maize meal 9826·15.

‡ Average values of six measurements.

group was randomly assigned to one of four experimental diets. The diets contained 5 mg Cu/kg with or without 1 g ascorbic acid/kg or 1 mg Cu/kg with or without 1 g ascorbic acid/kg. The former diet was identical to the pre-experimental diet.

The composition of the diets is given in Table 1. The experiment was carried out with three cohorts of twelve rats each, that is three rats per dietary group. The interval between the experiment with the first and second cohort was 1 week, and that between the second and third cohort was 16 weeks. During the experimental period (days 0–28) the rats were housed individually in metabolism cages (Techniplast Gazzada, Buguggiate, Italy). One batch of diet was used for the three cohorts. The diets, which were in powdered form, were stored at −20° until feeding. The concentration of ascorbic acid was checked before the study of each cohort.

From days 11 to 13 and days 24 to 26 urine and faeces of each rat were collected quantitatively. The tubes for collecting faeces and urine had been cleaned with 0·1 mol hydrochloric acid/l. Urinary and faecal Cu were analysed.

On day 24 each non-starved rat was injected intraperitoneally with ^{64}Cu acetate (25 μg Cu/kg body-weight) and ^{64}Cu whole-body retention was determined. Urine and faeces were collected during 3 d for determination of ^{64}Cu.

On day 28 the animals were killed by exposure to CO_2. Blood samples were taken by aortic puncture. Tissues were collected, weighed, and frozen at −20° until analysis. Plasma ascorbate, packed cell volume, blood haemoglobin and plasma ceruloplasmin were determined.

^{64}Cu and radiochemical analyses

^{64}Cu was obtained by irradiating a Cu wire (purity 99·999%; Ventron, Karlsruhe, Germany) in a thermal neutron flux of $10^{17}/m^2$ per s for 36 h in the reactor of the Interfaculty Reactor Institute of the Delft University of Technology. Following irradiation, the wire was dissolved in 25 μl nitric acid (undiluted) and diluted with sodium acetate

Table 2. *Expt 1. Effect of ascorbate on copper uptake by isolated hepatocytes from rats**

(Values are means for triplicate determinations; the pooled SE was 2·3. Results for one cell preparation are shown: similar results were found with other preparations (n 3))

Ascorbate in incubation medium (μmol/l)...	Cu uptake (ng Cu/mg cellular protein)			
Incubation period (min)	0	10	100	1000
30	16	28	33	50
60	29	38	48	76
120	59	66	76	136

* For details of procedures, see pp. 702–703.

There were significant effects of ascorbate, incubation period and interaction (two-way analysis of variance; $P < 0{\cdot}001$).

buffer (0·05 mol/l, pH 5·4) resulting in a final Cu concentration of 1 mg/ml. The specific activity of the ^{64}Cu solution at the start of the experiments was 320 GBq ^{64}Cu/g Cu (8·5 Ci/g).

^{64}Cu in urine, faeces, bile and tissues was determined by gamma counting (Philips Model PW4800 with a 3 × 3 inch sodium iodide crystal detector; overall efficiency of 6%).

^{64}Cu whole-body retention was determined with a whole-body counter specially designed for rats (Van Barneveld & Van den Hamer, 1985). The overall efficiency of this counter for ^{64}Cu was 14%. Whole-body counting of the animals was performed within 2 h post-injection and at regular intervals for another 96 h.

Chemical analyses

Urine and faeces were pretreated for Cu analysis. Urine was acidified to pH 1 with 6 mol HCl/l, and centrifuged for 10 min. The supernatant fraction was used for Cu analysis. Faeces were freeze-dried, ashed at 500° for 18 h and dissolved in 6 mol HCl/l. Tissues were freeze-dried and then digested with HNO_3 (Suprapur; Merck, Darmstadt, Germany) and hydrogen peroxide (Aristar; BDH Chemicals, Poole, UK). The mixture consisted of 1 g tissue/l HNO_3–H_2O_2 (13:6, v/v). Feed samples were pretreated for Cu analysis as described for faeces. All Cu analyses were performed by flame atomic absorption spectrometry with the use of a Varian AA-475 (Varian Technotron, Springvale, Australia). The accuracy was evaluated by concurrent analysis of Standard Reference Material 1577 Bovine Liver (US National Institute of Standards and Technology, Gaithersburg, Maryland, USA). We found 156 (SE 3) μg Cu/g (n 6 runs), while the certified value was 158 μg Cu/g.

Ascorbic acid in diet samples was quantified after extraction with 0·68 mol metaphosphoric acid/l by high-performance liquid chromatography (HPLC) with electrochemical detection (Yoshiura & Iriyama, 1986). For the analysis of plasma ascorbate, plasma was mixed with 0·54 mol metaphosphoric acid/l (1:4, v/v) in order to precipitate proteins and to stabilize ascorbate (Parviainen *et al.* 1986). Ascorbate was then determined by a HPLC method applying pre-column derivatization and spectrofluorometry (Speek *et al.* 1984).

Ceruloplasmin in plasma was measured by its enzymic oxidase activity, using p-phenylenediamine as substrate. The p-phenylenediamine oxidase activity of rat ceruloplasmin was converted to a concentration of ceruloplasmin (g/l) as described by

Table 3. *Expt 2. ^{64}Cu distribution (% dose) in tissues and bile 150 min after intravenous administration of ^{64}Cu (0·5 μg Cu/rat) in the absence or presence of ascorbate (0·1 mg/rat)*†

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

Intravenous supplement...	None		Ascorbate	
Site of ^{64}Cu	Mean	SE	Mean	SE
Liver	42	1·7	53*	4·6
Bile	11	1·2	16*	1·7
Carcass	43	2·9	25*	2·2

Mean values were significantly different from those of animals not given ascorbate (two-tailed Student's *t* test): * $P < 0·05$.

† For details of procedures, see p. 703.

Sunderman & Nomoto (1970). Blood haemoglobin was measured spectrophotometrically as metcyanohaemoglobin at 540 nm using Lyse S (Coulter Electronics, Krefeld, Germany). Liver metallothionein was determined by the 'Cd-hem' method reported by Onosaka & Cherian (1981).

Statistical analysis

The Kolmorgorov–Smirnov one-sample test was used to check normality of the data. For data distributed normally either Student's *t* test, one-way or two-way analysis of variance was applied to disclose statistically significant effects of treatments as indicated in the tables. Data not distributed normally were transformed logarithmically and then checked for homogeneity of variances (Cochran's C test); subsequently, statistically significant differences were evaluated as indicated previously for normally distributed data.

RESULTS

Expt 1. Cu uptake by isolated hepatocytes

Hepatocytes accumulated ^{64}Cu (Table 2) with time and this was a temperature-dependent process because at 4° Cu uptake rates were less than 5% of those measured at 37° (values not shown). When ascorbate was added to the incubation medium a marked increase in ^{64}Cu uptake occurred which depended on the ascorbate concentration (Table 2). On the other hand, the addition of ascorbate to the incubation medium (1000 μmol/l) stimulated uptake of ^{65}Zn by hepatocytes on average by 12%, but this effect did not reach statistical significance.

Expt 2. Hepatic uptake and biliary excretion of ^{64}Cu after intravenous administration of ^{64}Cu without or with ascorbate

Table 3 shows that rats injected with ascorbate accumulated more ^{64}Cu in liver and excreted more in bile, whereas their remaining carcass contained less ^{64}Cu compared with controls.

Expt 3. Effect of a diet containing 10 g ascorbic acid/kg on Cu metabolism

The addition of ascorbic acid to the diet at a concentration of 10 g/kg caused a more than twofold increase in plasma ascorbate levels (Table 4). Ascorbate did not influence body-weight gain. Packed cell volume and blood haemoglobin concentrations were significantly reduced by ascorbate intake. Rats fed on ascorbic acid showed significantly decreased

Table 4. *Expt 3. Effect of feeding a diet containing 10 g ascorbic acid/kg for 6 weeks on body-weight, haematological variables and tissue copper concentrations of rats*†

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

Dietary ascorbic acid (g/kg)...	None		10	
	Mean	SE	Mean	SE
Body-wt (g)				
Initial	80	0·8	79	1·2
Final	249	7·8	248	5·7
Plasma				
Ascorbate (μmol/l)	100	13·9	254***	22·0
Ceruloplasmin‡ (*EC* 1.16.3.1) (g/l)	0·67	0·05	0·54*	0·03
Packed cell volume	0·483	0·012	0·430**	0·004
Haemoglobin (mmol/l)	9·1	0·2	6·5**	0·5
Cu concentrations§ (μg/g)				
Plasma (μg/ml)	1·16	0·06	0·85**	0·05
Liver	12·33	0·71	9·39*	0·74
Kidney	19·27	0·78	17·20*	0·49
Heart	24·67	0·33	22·30*	0·93
Spleen	5·30	0·36	4·11*	0·29
Muscle	3·97	0·19	3·32*	0·12
Bone	2·87	0·06	2·11***	0·07
Skin	5·00	0·36	4·10	0·21
Metallothionein (μg/g liver)	35	4·1	32	3·7

Mean values were significantly different from those of animals not given ascorbic acid (two-tailed Student's *t* test): * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

† For details of procedures, see p. 703.

‡ As measured by its oxidase activity.

§ As μg Cu/g dry weight.

activities of plasma ceruloplasmin. Control values of ceruloplasmin-protein concentration correspond well with those reported by other workers (DiSilvestro *et al.* 1988). Ascorbic acid feeding lowered Cu concentrations in plasma, liver, kidney, heart, spleen, muscle and bone. Group mean concentrations of Cu in skin were also lowered by ascorbic acid intake, but this effect just failed to reach statistical significance.

Whole-body retention of ^{64}Cu after oral and intraperitoneal administration of ^{64}Cu is shown in Fig. 1. Orally administered ^{64}Cu was not retained as efficiently as intraperitoneally injected ^{64}Cu. Biological half-lives of orally and intraperitoneally administered ^{64}Cu were 4·7 (SE 0·3) d and 5·2 (SE 0·4) d (n 5) respectively for rats fed on the diet without ascorbic acid. The differences induced by ascorbic acid were not statistically significant. However, Cu retention, as indicated by whole-body ^{64}Cu levels, was systematically higher in rats fed on ascorbic acid (Fig. 1). Cu absorption, as calculated from the y intercept values after extrapolation of the linear part of the retention curves (42–96 h) for orally administered *v.* injected ^{64}Cu, was 62 (SE 2)% for control rats and 59 (SE 2)% (n 5) for rats fed on ascorbic acid.

Ascorbic acid in the diet did not influence faecal and urinary excretion of orally administered ^{64}Cu (Table 5). The excretion of ^{64}Cu in the faeces after intraperitoneal administration of ^{64}Cu was significantly decreased in rats fed on ascorbic acid. After intravenous administration of ^{64}Cu the amount of ^{64}Cu in the liver was similar for rats fed

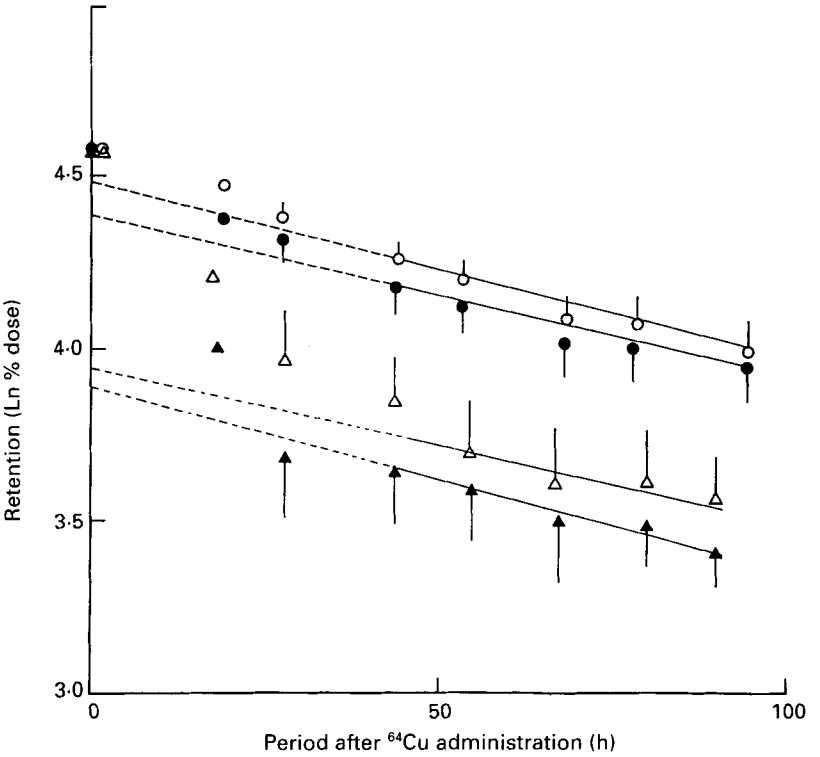

Fig. 1. Expt 3. Whole-body retention of orally (△, ▲) and intraperitoneally (○, ●) administered ^{64}Cu in rats fed on diets with recommended Cu concentrations without (▲, ●) or with (△, ○) 10 g ascorbic acid/kg. Results are means and standard deviations represented by vertical bars for five animals per dietary group. Linear fits were calculated over the time-period 42–96 h after ^{64}Cu administration. For details of procedures, see p. 703.

Table 5. *Expt 3. Recovery of administered ^{64}Cu (% dose) in urine, faeces, liver and bile of rats fed on a diet containing 10 g ascorbic acid/kg*†

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

Dietary ascorbic acid (g/kg)...		None		10	
Route of ^{64}Cu administration‡	Site of recovery	Mean	SE	Mean	SE
Oral	Urine§	3	0·5	3	0·6
	Faeces§	60	1·2	59	3·7
Intraperitoneal	Urine§	7	0·7	5	0·6
	Faeces§	34	1·9	27*	2·0
Intravenous	Liver¶	43	3·1	45	3·0
	Bile¶	10	0·9	13*	0·9
	Carcass¶	40	2·7	35	1·8

Mean values were significantly different from those of animals given the diet without ascorbic acid (two-tailed Student's *t* test): * $P < 0{\cdot}05$.

† For details of procedures, see p. 703.

‡ ^{64}Cu was administered after feeding the diets for the following periods (weeks): oral administration 4, intraperitoneal administration 5, intravenous administration 6.

§ ^{64}Cu accumulated for 3 d after administration.

¶ ^{64}Cu accumulated for 150 min after administration.

Table 6. *Expt 4. Effect of feeding a diet containing 1 g ascorbic acid/kg for 4 weeks on body-weight, haematological variables and tissue copper concentrations in rats**

(Mean values for nine rats per dietary group)

						Statistical significance of effect of‡:	
Cu† (mg/kg)...	5·0	5·0	1·0	1·0	Pooled	Ascorbic	
Ascorbic acid† (g/kg)...	—	1·0	—	1·0	SE	acid	Cu
Body-wt (g)							
Initial	79	79	80	81	0·1		$P < 0·001$
Final	201	197	194	183	1.1		
Feed intake (g/d)	14·0	14·1	14·2	14·0	0·1	—	—
Plasma							
Ascorbate (μmol/l)	92	163	96	153	11·1	$P < 0·01$	—
Ceruloplasmin (*EC* 1.16.3.1) (g/l)	0·60	0·59	0·04	0·06	1·62	—	$P < 0·001$
Packed cell volume	0·452	0·427	0·386	0·354	0·010	$P < 0·01$	$P < 0·001$
Haemoglobin (mmol/l)	8·6	8·1	6·9	6·1	0·9	$P < 0·001$	$P < 0·001$
Cu concentrations§ (μg/g)							
Plasma (μg/ml)	1·09	0·98	< 0·1	< 0·1	1·10	—	$P < 0·001$
Liver	10·33	9·22	6·49	6·06	1·90	$P < 0·05$	$P < 0·001$
Heart	20·44	19·19	11·76	11·64	1·67	—	$P < 0·001$
Kidney	17·01	15·75	8·84	8·16	3·46	—	$P < 0·001$
Spleen	5·29	4·47	1·50	1·48	3·88	—	$P < 0·001$
Muscle	4·52	4·34	1·48	1·23	1·44	—	$P < 0·001$
Bone	2·74	2·48	2·05	1·63	3·73	—	$P < 0·001$
Skin	3·65	3·07	0·90	0·71	4·94	—	$P < 0·001$

* For details of procedures, see pp. 703–704.

† Calculated values.

‡ Two-way analysis of variance; plasma and spleen Cu concentrations were subjected to ANOVA after log transformation of the data.

§ As μg Cu/g dry weight.

on diets without or with ascorbic acid (Table 5). Biliary excretion of ^{64}Cu was significantly increased by ascorbic acid feeding.

Expt 4. Effects of diets containing 1 g ascorbic acid/kg on Cu metabolism

The addition of ascorbic acid to the diet at a concentration of 1 g/kg caused a significant increase in plasma ascorbate levels (Table 6). Ascorbic acid tended to lower body-weight when fed in combination with the diet low in copper. Feed intakes were not influenced significantly by ascorbic acid or copper concentration of the diet (Table 6). Ascorbic acid reduced packed cell volume values and blood haemoglobin concentrations both in rats given diets with recommended and those with low Cu concentrations, the effect being somewhat more pronounced in the latter. Plasma ceruloplasmin (activity) was not influenced by ascorbate, but almost completely suppressed by low Cu intake.

In rats fed on the diets with the recommended Cu concentration, high ascorbic acid intake induced decreased group mean Cu concentrations in plasma and liver. Likewise, group mean concentrations of Cu in other tissues were lowered by dietary ascorbic acid. Feeding the diets with the low Cu concentration resulted in markedly lowered Cu concentrations in all tissues.

Table 7. *Expt 4. Apparent absorption of copper by rats fed on diets containing 1 g ascorbic acid/kg**

(Mean values for nine rats per dietary group)

						Statistical significance of effect of‡:	
Cu† (mg/kg)...	5·0	5·0	1·0	1·0	Pooled	Ascorbic	
Ascorbic acid† (g/kg)...	—	1·0	—	1·0	SE	acid	Cu
Cu intake (μg/d)	74	71	12	11	0·3	—	—
Faecal Cu (μg/d)	43	55	4	5	1·9	$P < 0·01$	$P < 0·001$
Apparent absorption§							
μg/d	31	16	8	7	0·7	$P < 0·01$	$P < 0·001$
% of intake	42	23	70	59	2·1	$P < 0·01$	$P < 0·001$

* Values for days 11–13 and days 24–26 of the experiment. For details of procedures, see pp. 703–704.
† Calculated values.
‡ All data were subjected to ANOVA after log transformation.
§ Apparent absorption is expressed in absolute (intake − faecal output) and relative terms (100 × (intake − faecal output)/intake).

Table 8. *Expt 4. Excretion of intraperitoneally administered ^{64}Cu in urine and faeces of rats fed on diets containing 1 g ascorbic acid/kg**

(Mean values for nine rats per dietary group)

	^{64}Cu recovery (% dose)					Statistical significance of effect of‡:	
Cu† (mg/kg)...	5·0	5·0	1·0	1·0	Pooled	Ascorbic	
Ascorbic acid† (g/kg)...	—	1·0	—	1·0	SE	acid	Cu
Urine	7	8	2	2	0·4	—	$P < 0·001$
Faeces	21	30	6	7	1·1	$P < 0·01$	$P < 0·001$

* Values for days 24–26 of the experiment. For details of procedures, see pp. 703–704.
† Calculated values.
‡ All data were subjected to ANOVA after log transformation.

Dietary ascorbic acid increased faecal loss of Cu both in rats fed on diets with recommended and those with low Cu concentrations (Table 7). Cu in urine was not detectable. The absolute and percentage apparent absorption values of Cu were significantly decreased by dietary ascorbic acid. Low intake of Cu increased the apparent efficiency of Cu absorption.

Ascorbic acid in the diet significantly increased faecal excretion of intraperitoneally administered ^{64}Cu in rats given the diet with the recommended Cu concentration, but did not influence urinary excretion of ^{64}Cu (Table 8). The excretion of ^{64}Cu in faeces and urine was significantly decreased in rats fed on the low-Cu diet; dietary ascorbic acid did not affect ^{64}Cu excretion in these rats.

Whole-body retention of intraperitoneally administered ^{64}Cu is shown in Fig. 2. A significantly higher retention was found in rats fed on the low-Cu diets compared with rats fed on diets with the recommended Cu concentration. Biological half-lives of the

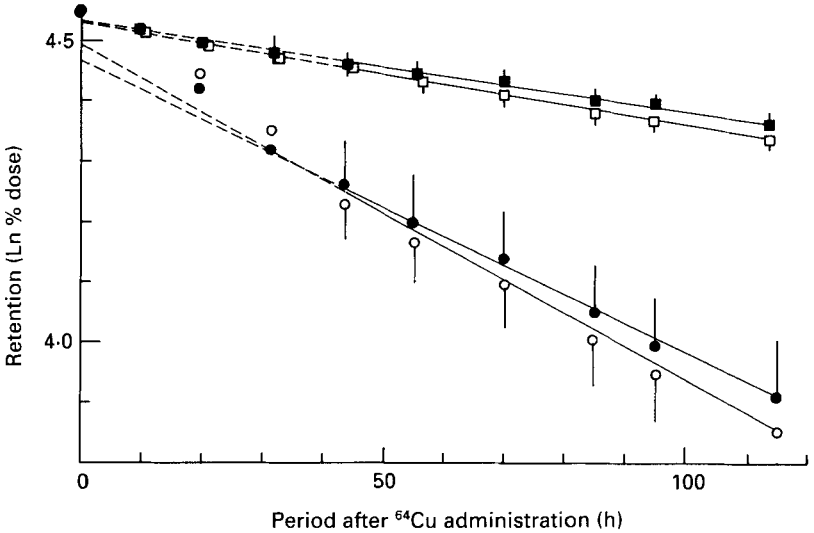

Fig. 2. Expt 4. Whole-body retention of intraperitoneally administered ^{64}Cu in rats fed on diets containing either recommended (○, ●) or low (□, ■) amounts of Cu without (●, ■) or with (○, □) 1 g ascorbic acid/kg. Results are means and standard deviations represented by vertical bars for nine animals per dietary group. Linear fits were calculated over the time-period 42–96 h after ^{64}Cu administration. For details of procedures, see pp. 703–704.

administered ^{64}Cu were 6·0 (SE 0·3) and 19·0 (SE 0·7) d (*n* 9) respectively for rats fed on the recommended and low-Cu diets without added ascorbic acid. For the rats fed on the diets containing ascorbic acid, these values were 5·0 (SE 0·3) and 20·0 (SE 0·7) d respectively. Thus, dietary ascorbic acid tended to diminish Cu retention in rats given the diet with recommended Cu concentration, but this effect was very small.

DISCUSSION

The rat as model

We have used the rat as a model to study the effects of ascorbic acid supplementation on Cu metabolism. It could be argued that the rat is not a suitable model because this animal species, unlike humans, can synthesize adequate amounts of this vitamin. The feeding of rats with diets enriched with ascorbic acid resulted in a significant increase in plasma ascorbate concentrations. These results indicate that in the rat ascorbate status can be modulated by ascorbic acid intake, which may imply that the rat is a suitable model to study the effects of ascorbic acid supplementation on Cu metabolism. This is further supported by the observation that ascorbic acid feeding reduces plasma Cu concentrations in both humans (Finley & Cerklewski 1983; Jacob *et al.* 1987; Milne *et al.* 1988) and rats (Johnson & Murphy, 1988; present study).

Ascorbic acid and Cu status

Addition of ascorbic acid to diets with recommended Cu concentrations resulted in depressed plasma ceruloplasmin activity and decreased Cu concentrations in plasma and various tissues, especially liver. This agrees with findings of other studies using diets containing ascorbic acid concentrations in the range of 10–50 g/kg (Van Campen & Gross, 1968; Smith & Bidlack, 1980; Johnson & Murphy, 1988; Van den Berg *et al.* 1990). The present study shows that the feeding of a diet with 5 mg Cu/kg containing only 1 g ascorbic acid/kg for 28 d produced essentially the same effects on tissue Cu concentrations as did

supplying a diet with ten times as much ascorbic acid for 42 d. Feeding the high ascorbic acid diet for the longer period only produced slightly greater decreases of Cu concentrations in plasma, liver and other tissues. Thus, tissue Cu concentrations are clearly affected by ascorbic acid intake at levels as low as 0·1 g/kg body-weight.

Dietary ascorbic acid lowered blood haemoglobin concentrations and packed cell volume values (Tables 4 and 6). It could be suggested that these effects are caused by ascorbate-induced lowering of tissue Cu concentrations. Diets containing 1 mg Cu/kg not only lowered Cu concentrations in tissues but also caused depressed haemoglobin and packed cell volume (Table 6). Anaemia in rats as induced by Cu deficiency has been reported (Johnson & Murphy, 1988). There was no statistically significant interaction of ascorbic acid and Cu intake with regard to haemoglobin concentrations and packed cell volume, but the effect of high ascorbic acid intake tended to be somewhat more pronounced against a dietary background low in Cu. Such a tendency was also seen concerning growth performance. Final body-weight was reduced only with the combination of high ascorbic acid and low Cu concentrations in the diet (Table 6).

Cu absorption

It has been suggested (Van Campen & Gross, 1968; Johnson & Murphy, 1988; Van den Berg *et al.* 1990) that dietary ascorbic acid lowers tissue Cu concentrations through interference with the absorption of Cu from intestine. Indeed, in the present study we also found that the apparent absorption of Cu was significantly decreased after feeding a diet supplemented with 1 g ascorbic acid/kg (Table 7). This may be related to an interaction of ascorbate and Cu at the level of the intestinal lumen. Ascorbate depressed the intestinal absorption of ^{64}Cu when the two materials were administered by stomach tube (Van den Berg *et al.* 1990). Furthermore, ascorbate reduced the disappearance of ^{64}Cu from ligated duodenal segments (Van Campen & Gross, 1968). If ascorbate depresses intestinal Cu absorption, then retention of orally administered ^{64}Cu should be diminished in rats fed on ascorbic acid. This has indeed been shown earlier (Van Campen & Gross, 1968). However, we found higher whole-body levels of ^{64}Cu at each time-point after oral administration of ^{64}Cu in rats fed on ascorbic acid (Fig. 1), while ascorbic acid did not influence calculated true Cu absorption. This might be explained by ascorbate-induced decreased tissue Cu concentrations. Low Cu intake caused an increased apparent absorption of Cu and an enhanced ^{64}Cu retention after intraperitoneal injection of ^{64}Cu (Fig. 2). Thus, the reduced tissue Cu concentrations as induced by ascorbic acid feeding may have masked effects of ascorbate at the level of intestinal absorption. This would also explain the lack of effect of ascorbate feeding on the recovery of orally administered ^{64}Cu in faeces (Table 5).

Hepatic uptake of Cu

Ascorbate, intravenously administered together with ^{64}Cu, caused an increase of ^{64}Cu recovery in liver (Table 3). This effect of ascorbate was observed at a dose of 0·1 mg/rat. This caused a maximum increase of plasma ascorbate by about 115 μmol/l, assuming that the rats had about 5 ml plasma. Within this concentration range ascorbate clearly increased Cu uptake by isolated hepatocytes (Table 2).

Ascorbic acid feeding may also stimulate hepatic Cu uptake because it caused an increase of plasma ascorbate concentrations by about 100 μmol/l (Tables 4 and 6). However, no effect on accumulation of ^{64}Cu in liver was observed in rats fed on ascorbic acid and given ^{64}Cu intravenously (Table 5). This could be related to the reduced tissue Cu concentrations of rats fed on ascorbic acid. Low Cu intake induces increased efficiency of Cu uptake by various tissues (Van den Berg *et al.* 1990) and increased whole-body retention of ^{64}Cu in such rats (Fig. 2). Thus, circulating ascorbate and decreased cellular Cu concentrations

both trigger Cu uptake by cells. Any specific effect of ascorbate on the distribution of ^{64}Cu between liver and other tissues after intravenous administration of ^{64}Cu may be masked by reduced concentrations of Cu in extrahepatic tissues. Moreover, the effect of ascorbate on the cellular uptake of Cu may not be specific for liver. Ascorbate has also been shown to stimulate Cu transport from ceruloplasmin into a human erythroleukemic cell line (Percival & Harris, 1989).

Biliary excretion of Cu

Intravenously administered ascorbate stimulated ^{64}Cu accumulation in bile of rats with a bile duct cannula (Table 3). In rats fed on a diet containing 10 g ascorbic acid/kg, more intravenously administered ^{64}Cu was recovered in bile than in rats fed on no ascorbic acid (Table 5). In keeping with ascorbate-induced stimulation of biliary Cu excretion, 1 g ascorbic acid/kg in a diet with recommended Cu concentration enhanced the recovery of intraperitoneally administered ^{64}Cu in faeces (Table 8). However, an opposite effect was seen in rats fed on a diet containing 10 g ascorbic acid/kg (Table 5). This may be explained by the Cu-retaining effect of reduced tissue Cu concentrations induced by ascorbate, an effect being more pronounced in rats fed on 10 g instead of 1 g ascorbic acid/kg diet.

The ascorbate-induced stimulation of biliary ^{64}Cu excretion seen in Expt 3, and indirectly in Expt 4, probably reflects the increased hepatic uptake of ^{64}Cu and, thus, may be the result of an increased specific activity of Cu in liver pools. This is supported by the observation that in rats fed on ascorbic acid the specific activity of liver Cu was increased by about 30% (Tables 4 and 5), while the increase in biliary ^{64}Cu excretion was of the same order of magnitude. Furthermore, it is unlikely that ascorbate promotes biliary excretion of Cu mass because the combination of impaired intestinal absorption of Cu and increased biliary excretion of Cu would not allow for a new steady-state of body Cu to be reached. As a consequence the animals would soon be fully depleted. We speculate that biliary excretion of Cu mass is depressed in rats fed on ascorbic acid. In any event, the ascorbate-induced lowering of tissue Cu concentrations will by itself reduce biliary Cu excretion. This is supported by the observation that low Cu intakes diminish biliary Cu excretion (Owen & Hazelrig, 1968). In retrospect, it is unfortunate that we did not analyse the amount of Cu in bile fluid samples.

Whole-body retention of ^{64}Cu

There was a discrepancy in the results for whole-body retention of intraperitoneally injected ^{64}Cu in rats fed on recommended Cu diets containing either 10 or 1 g ascorbic acid/kg. In rats fed on a 10 g ascorbic acid/kg diet, the whole-body retention of ^{64}Cu was slightly increased (Fig. 1), whereas in rats fed on a 1 g ascorbic acid/kg diet it was slightly decreased (Fig. 2). This may relate to the somewhat different tissue Cu concentrations in rats fed on the two ascorbate diets. Retention of intraperitoneally injected ^{64}Cu essentially refers to ^{64}Cu accumulated by cells minus urinary excretion of ^{64}Cu and non-reabsorbed ^{64}Cu excreted in bile. As shown earlier (Van den Berg *et al.* 1990), and in the present study (Fig. 2), low Cu intake increases ^{64}Cu retention, probably by stimulating cellular uptake of Cu (Van den Berg *et al.* 1990, 1991). Ascorbate, on the other hand, tends to decrease ^{64}Cu retention through increased biliary excretion of ^{64}Cu. Apparently, in rats fed on the diet containing 10 g ascorbic acid/kg for 42 d (Fig. 1) the net effect of ascorbate-induced lowering of tissue Cu concentrations and increased biliary excretion results in enhanced ^{64}Cu retention. In other words, the effect of reduced tissue Cu concentrations overrules that of circulating ascorbate. The opposite may hold for Cu retention (Fig. 2) in rats fed on the recommended Cu diet containing 1 g ascorbic acid/kg.

Conclusions

It has been shown that dietary ascorbic acid concentrations of 1 and 10 g/kg reduce tissue Cu concentrations in rats. Ascorbate interfered with intestinal Cu absorption. Ascorbate also stimulated hepatic uptake of ^{64}Cu. This may be responsible for the observed ascorbate-induced enhancement of biliary excretion of intravenously administered ^{64}Cu, although this should not necessarily be associated with an increased biliary excretion of Cu mass. Evidence is presented that, when studying the mechanism underlying the lowering of tissue Cu concentrations by ascorbic acid feeding, the primary effects of ascorbate itself and the secondary effects of the ascorbate-induced reduced tissue Cu concentrations should be distinguished.

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