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COPPER METABOLISM IN RATS FED ASCORBIC ACID OR RESTRICTED AMOUNTS OF COPPER

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STELLINGEN

behorende bij het proefschrift

"Copper metabolism in rats fed ascorbic acid or restricted amounts of copper"

1. Het is weinig zinvol om meer vitamine C in te nemen dan de aanbevolen dagelijkse hoeveelheid.

2. Een verantwoord gebruik van proefdieren kan wel leiden tot vermindering, verfijning en/of vervanging van een aantal proefdieren, maar zal nooit leiden tot een totale afschaffing van het gebruik van dieren.

3. De elementaire samenstelling van de aardkorst, met name het hoge Si-gehalte, vormt een (grote) bedreiging voor het leven.

4. Het nieuws rondom Chernobyl heeft een kortere "halfwaarde tijd" dan de gevolgen van deze ramp.

5. De uitspraak in de pers: "1993 wordt vast (weer) een goed wijnjaar" zal zich na het persen moeten bewijzen.

6. De kreet: "Ze hebben 'm al", waarmee hardlopers vaak nageroepen worden, werkt demotiverend op beginners.

7. De uitspraak dat vakantie in Nederland beter is voor "onze" economie, verdient bijstelling.

8. Mensen die denken dat ze het goed doen, behalen goede resultaten.

9. Perfectie is geen criterium, maar een streven.

10. "Everybody needs some time to be alone, to dream and to wonder".

G.J. van den Berg — 25 november 1993

COPPER METABOLISM IN RATS FED ASCORBIC ACID OR RESTRICTED AMOUNTS OF COPPER

KOPERHUISHOUDING BIJ RATTEN NA VERSTREKKING VAN ASCORBINEZUUR-RIJKE OF KOPER-ARME VOEDERS

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doctorandus in de wiskunde en natuurwetenschappen

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GENERAL INTRODUCTION AND OUTLINE OF EXPERIMENTAL WORK

Copper as an essential element. Copper (Cu) was established as an essential nutrient in the 1920s and 1930s for rats [1]. The acceptance of copper as a nutrient essential for animal life, including humans and probably for most (or all) living systems, was followed by the recognition of the fundamental need for copper in various metabolic pathways. Copper expresses its biological activity while bound to specific protein ligands, and these cuproenzymes mediate the biochemical functions of the trace element Cu.

Interest in copper as an essential trace element has led to an intensive literature. Reviews of aspects of copper biochemistry, metabolism, function, and nutrition are those by Sass-Kortsak [2], Evans [3], Mason [4], Bremner [5], Frieden [6,7], Owen [8-10], Cousins [11], Davis and Mertz [12], Camakaris [13], Danks [14], Prohaska [15] and Lindner [16], as well as volumes on *Copper Proteins* [17], *Metal Ions in Biological Systems* [18], *Copper in Animals and Man* [19] and *Copper Bioavailability and Metabolism* [20].

Dietary copper intake is the primary factor to cover the body's copper requirement. In addition to copper concentrations in food and the amount of food that is eaten, another factor is involved in determining the value for food as a source of copper, that is (bio)availability of copper [20,21]. In other words, the nutritional value of foods and diets for Cu is not synonymous with their copper amounts as determined by chemical analysis. Generally, it is not the ingested amount of copper that is important to maintain balance, but the amount that is (bio)available (available for biological and biochemical processes in the body) [cf. 22,23]. This view implicates that availability can be assessed by changes in functional status of the organism. Indicators or so-called "biological markers" are needed to quantify the response of the organism to food and diets in nutritional studies.

Various factors may affect copper availability during the so-called *pre-absorptive stage* (**Table 1**); [cf. 16], during intestinal digestion and absorption into the body's copper pools. On the other hand, in the *post-absorptive stage* endogenous factors control copper transport and daily copper excretion. During digestion, peptides (ligands of alimentary or enzymatic origin) are released which may influence the availability by changing the solubility of copper. Another factor, the transit time through the gastrointestinal tract, i.e., gastric emptying, partly influenced by the composition of the food ingested, may also influence copper solubility and copper availability. Cupric Cu(II) species show a greater solubility at the pH of the intestinal contents than do cuprous Cu(I) species. Among the dietary factors that can influence the oxidation state, and hence the solubility of copper, is ascorbic acid (vitamin C). It is assumed [24,25], but not proven, that ascorbic acid may decrease copper availability because of the reduction to cuprous species.

Table 1. *Factors effecting whole body copper balance.*

Pre-absorptive stage	Post-absorptive stage
Intestinal absorption:	Storage/transport:
– amount eaten	– pool size
– gastrointestinal factors:	– chemical form
* interactions in lumen	
* pH	
* chemical form	
* solubility	
– rate of transit	Excretion:
– mucosal regulation	– hepatic/biliary function
* absorption efficiency depends on pool size	– kidney function
	– intestinal secretion function
* copper-zinc interaction	– skin turnover

The prediction to which extent a copper species is formed in gastrointestinal contents is difficult from a theoretical point of view because of so many interactions. And, the ultimate copper species formed will not necessarily be absorbed or, when absorbed, it may not release copper as a biologically active form. In vitro techniques could be used to estimate the relative importance of the factors mentioned for copper availability and to determine solubility, but predictions of copper availability through in vitro data must be checked by animal experiments, either by direct analysis of the gastrointestinal content or by controlled balance studies, including the use of radioisotopes [26). However, one must realize that intestinal absorption *per se*, or true absorption, is not always studied but that apparent absorption or overall retention of copper is measured instead. In convential balance studies it is far from clear whether alterations in absorption or alterations in excretion, of both, are responsible for the retentions observed. Copper absorption will be underestimated with the fecal monitoring method, due to excretion of absorbed copper into the gastrointestinal tract.

The amount of daily copper loss from the body is some function of the size of the body copper pool. A positive or negative balance indicates that the intake and/or bioavailability of copper from the diet is greater or smaller than the daily loss. Intakes that result in an excess of absorbed copper over the prevailing daily excretion will increase the pool until the daily loss equals the daily absorbed amount. At that time a steady state is reached. Should the copper intake now be diminished, the daily loss would initially exceed the inflow, resulting

in a diminution of the body copper pool until at some time the smaller daily outflow would again equal the inflow and a new equilibrium would be established. Thus, the amount of available copper in the diet determines the body pool size with time, but does not predict *a priori* deficiency or toxicity. In other words, there are factors on the pre- and post-absorptive level affecting the rate of copper inflow into the body pool and daily copper loss. The physiological implication of the foregoing discussion is that a copper balance can be achieved with a variety of intakes and pool sizes. When the daily intake is small relative to the total pool size, it will take some time to establish a new equilibrium or so-called "steady state" after changes of intake, in comparison with larger intakes. Balance studies may help to measure the availability of copper for absorption, but in the interpretation of data from balance studies the factor time has to be taken in consideration.

Metabolic copper-ascorbic acid interactions. It was recognized that high intakes of ascorbic acid cause reduced Cu status in various animal species, including humans (**Table 2**). It was hypothesized that ascorbic acid impairs copper utilization through decreased copper absorption [27-29] or through increased turnover of copper [30,31]. An animal model would help to study the role of ascorbic acid in copper metabolism in more detail.

It is clear from **Table 2** that ascorbic acid supplementation has qualitatively similar effects on copper status in species that require ascorbic acid, such as men, monkeys and guinea pigs [32–35], and those that can synthesize ascorbic acid, such as rabbits and rats [29,36]. High intakes of ascorbic acid in humans and monkeys have shown to increase plasma ascorbate levels several-fold [32-34,37] and to reduce serum copper levels [34], and serum ceruloplasmin oxidase activity [33,34]. In one study with guinea-pigs, ascorbic acid injections resulted in significantly increased plasma ascorbate concentrations and lower serum copper concentrations [38]. This result support also the idea for a post-absorptive effect of ascorbate. These data suggest that in species that require ascorbic acid there is an effect of ascorbate both at the level of absorption, during the *pre-absorptive stage*, and during *post-absorptive stage*, by increased circulating plasma ascorbate concentrations.

When mutant Wistar rats, unable to synthesize ascorbic acid, were fed diets with increasing levels of ascorbic acid, an gradual increase in plasma ascorbate concentrations was seen [39], showing that at least in this rat strain plasma ascorbate levels can be manipulated by dietary ascorbic acid supplementation. In a preliminary experiment, however, we have also demonstrated that Wistar rats (Hsd/Cpb:Wu) fed on diets supplemented with ascorbic acid produced marked increases in plasma concentrations of ascorbate [40]. Moreover, increasing dietary concentrations of ascorbic acid above 1 g/kg diet (comparable with a daily intake of about 70 mg/kg body weight) did not further increase plasma ascorbate concentrations.

Table 2. *Effects of high intakes of ascorbic acid on indicators of copper status in various species.*

	Ascorbic acid supplementation		Percentage change in copper status			Ref.
Species	Amount (mg/kg body weight/d)	Duration (days)	Plasma copper	Liver copper	Plasma ceruloplasmin	
Man	20	64	–5%		–25%	[32]
Man	10	21	+5%		–20%	[33]
Monkey	25	168	–20%		–20%	[34]
Guinea pig	900	21	–40%	–50%		[35]
Rabbit	400	84		–15%		[36]
Rat	1000	28		–10%	–10%	[29]

In any event, ascorbate status in the rat can also be modulated by ascorbic acid intake, which implies that the rat is a suitable model to study the effects of ascorbic acid supplementation on copper metabolism. Therefore, we have taken Wistar rats (Hsd/Cpb:Wu) as animal model. Factors that complicate interpretation of ascorbic acid-copper interactions in vivo can be eliminated by in vitro studies where conditions are precisely known and controlled. In order to investigate possible ascorbic acid-copper interactions at the cellular level, i.e., at the cell membrane, also in vitro studies are performed with parenchymal cells and plasma membrane vesicles isolated from rat liver. However, the validity of an in vitro system depends on the extent to which conditions selected occur in vivo.

Copper metabolism in the rat. Below, Figure 1 presents an overview of copper metabolism in the rat. Traces of dietary Cu may be absorbed through the wall of the stomach and the rest in the small intestine, across the mucosal cells. Dietary Cu reaching the small intestine is mixed with Cu secreted into the gastrointestinal tract [41]. Bile is assumed the major (or perhaps the only major) source of endogenously secreted copper that is largely unavailable for reabsorption [42], and is excreted with the feces [42], along with dietary Cu that is not absorbed. Absorbed Cu first enters the cells of the intestinal mucosa and then the blood. Some regulation of entry occurs at the serosal (basolateral) surface [43-45], i.e., by a specific Cu carrier and via the amounts and rates of synthesis of intestinal metallothionein [41]. The concentrations of the membrane carriers may be regulated by copper need, which is increased in dietary copper deficiency for instance.

The distribution of dietary copper after its absorption from the intestinal tract appears to occur in two stages. In the first stage, copper moves from the intestinal mucosa to the liver and kidney, and in the second stage, it moves from the liver to peripheral tissues. Evidence for these two stages of distribution comes from tracing the path of the copper radioisotope after its intraduodenal or intragastric intubation (or direct injection) into rats. Immediately after administration, radioactivity is found in the portal blood, where it is attached to albumin [46], and transcuprein [47]. The involvement of albumin in the initial transport of absorbed copper is well documented [8,9,46].

The liver has a key role in the copper metabolism since it is the primary storage organ, and it is also the site for the synthesis of copper-containing proteins, viz. ceruloplasmin. In addition, the liver provides, via the bile, the major excretory pathway of copper, since very little is eliminated via the urine. Liver copper probably reflects intake and the copper status of the organism.

The mechanism of copper transport and delivery into liver cells has been extensively studied in vivo and in vitro [48,49]. Recently, a single (unified) hypothesis for copper uptake into cells was proposed [49], in which the membrane carrier recognizes only the Cu-binding complex, allowing to utilize Cu from a variety of ligands, such as albumin, histidine, ternary complex (CuHisAlb) or ceruloplasmin, viz. carriers of copper to hepatocytes and extrahepatic tissues.

Mechanisms by which ascorbate may influence copper metabolism. The effects of high intakes of ascorbic acid have usually been studied during copper deficiency [28,29,35,36]. Laboratory animals fed copper-deficient diets supplemented with 1 to 5% ascorbic acid have shown lower body weights, lower hematocrit and decreased blood hemoglobin concentrations, depressed plasma ceruloplasmin and organ copper levels than control animals [28,29]. However, Cu deficiency *per se* as induced by copper-deficient diets is associated with a lower Cu status and decreased blood hemoglobin concentrations and lowered hematocrit [29,35,36].

Elevated levels of ascorbic acid in copper-adequate diets also resulted in anemia, depressed ceruloplasmin and organ copper levels in rats (**Table 2**), and a tendency toward decreased apparent absorption of Cu [28,29]. This suggests that reduced Cu status in rats fed ascorbic acid is caused, at least partly, by diminished Cu uptake from the gut. Moreover, in short-term experiments with orally administered radiolabeled copper, ascorbate decreased the Cu absorption from the lumen of ligated intestinal segments [27]. Thus, it is plausible that ascorbate impairs Cu absorption.

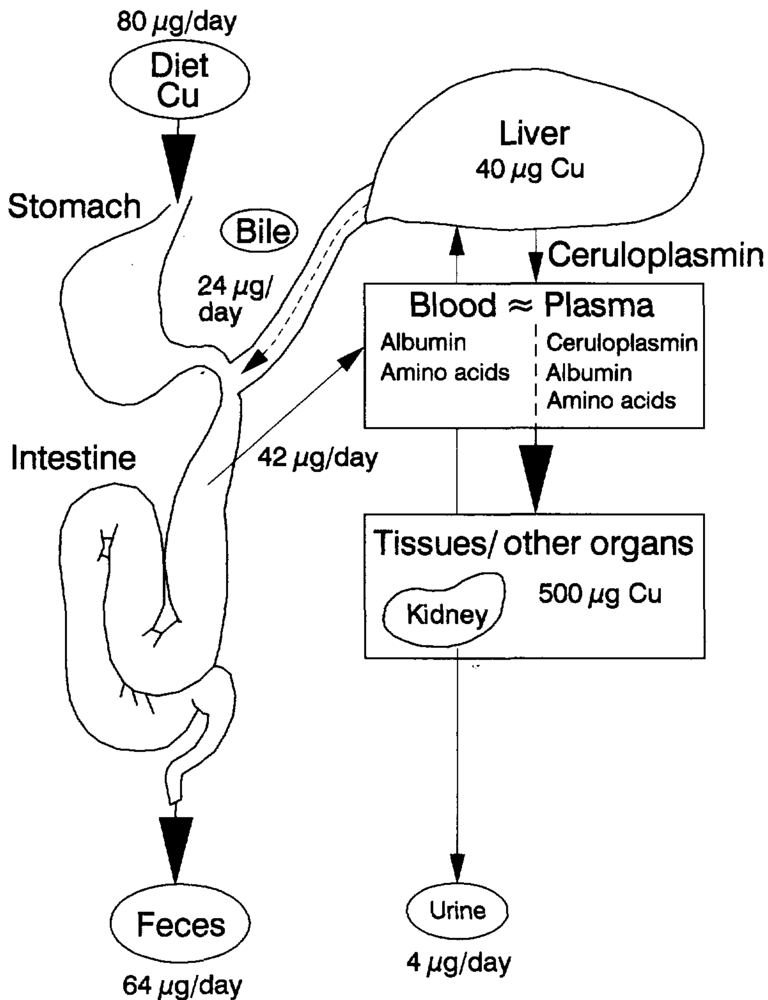

Figure 1. *Overview of copper metabolism. Approximate rates and organ copper pool sizes in rats (about 300 g body weight) fed a diet containing 5 mg Cu/kg are indicated.*

More recent studies have indicated a possible post-absorption role of ascorbate, i.e., the transfer of copper into cells [30,31]. It is suggested that ascorbic acid reacts directly or indirectly with ceruloplasmin, specifically labilizing the bound copper atoms and facilitating their cross-membrane transport. The release of copper from the complex can be accomplished by reducing the cupric ion to cuprous ion, because cuprous chelators have shown to block the ascorbate promoting copper uptake [50]. Similar effects have been obtained in mouse hepatocytes (HJ McArdle, personal communication) and a single (unified) hypothesis for Cu uptake in mammalian cells has been proposed [49]. It was speculated that vitamin C (as a

reducing agent) or a membrane reductase, will reduce cupric ions to cuprous ions in the Cu-ligand complex after binding to the membrane, and the dissociated copper is transferred across the cell membrane. Ascorbate may stimulate copper uptake, i.e. by the liver in vivo or by hepatocytes and plasma membranes vesicles isolated from hepatocytes. A higher hepatic Cu uptake due to ascorbate, may lead to a higher biliary Cu excretion to achieve homeostasis at the level of the hepatocyte. Previous studies [3,8,51] have concluded that copper homeostasis is controlled by variations in biliary copper excretion.

At least three possible explanations for the effect of dietary ascorbic acid on Cu metabolism are evident (cf. **Figure 1**): 1) interference with the absorption of copper from the intestine; 2) interference with transport of and function of copper at the cellular level, viz. the liver and other tissues; 3) interference with endogenously excreted Cu, i.e. the bile. The effects of ascorbic acid-copper interactions can be divided arbitrarily into those at the so-called *pre-absorptive stage* (absorption), and those at the *post-absorptive stage* (copper transport, storage and excretion).

Use of radiotracers in metabolic studies. The conventional balance technique, whereby the difference between dietary intake and fecal excretion gives the extent of apparent absorption, has its limitations of demarcation of feces in periods corresponding to intake periods. Moreover, experimental errors, e.g. in collecting excrements, may have a large influence, particularly when the level of absorption is low. This problem can be circumvented by a radiotracer technique, using a diet labeled with a radioisotope and direct measurement of the absorption via whole-body counting.

Absorption studies performed with the balance technique determine only apparent absorption and not the true absorption. If the true intestinal absorption is to be determined, the unabsorbed fraction of the oral dose needs to be known. However, part of the initially absorbed dose may be excreted into the intestine (endogenous excretion). The higher this endogenous excretion in relation to dietary intake, the greater is the difference between apparent and true absorption in the balance technique. Also in the radiotracer method a fraction of the absorbed radioactivity is endogenously excreted and leaves the intestinal lumen with the feces. Consequently, a correction for endogenous losses has to be introduced. This can be done by an intraperitoneal administration of the radiotracer, in addition to an oral administration, performed in a cross-over fashion. Comparison of the retention curves for the two methods of administration, results in the true percentage of absorption of orally administered radioactivity.

The radiotracer technique is a powerful tool in metabolic studies, particularly when using radiotracers with high specific activities, enabling experiments with sufficient amounts of radioactivity and still very low amounts (down to ng and pg) of the element of interest, thus not disturbing physiological processes. On the other hand, when larger amounts of the

element have to be administered, the radioactive tracer can be diluted by addition of non-radioactive amounts of the element. Another advantage of the radiotracer method is the subsequent measurements in the same rat in a time course. The animal serves as its own reference, avoiding the problem of biological variability, in comparison with only one measurement point per animal. Thus, the technique may also contributes to minimize the number of experimental animals required.

Part of these studies described in this thesis are not performed with whole rats, but with primary cultures of liver parenchymal cells or plasma membrane vesicles isolated from rat liver, entailing small amounts of material. This implies that tiny amounts (μg to ng) of the element of interest are involved in influx, efflux, and accumulation to be studied. Net uptake in cultured cells can eventually be determined via differential measuremments, although this approach may lead to substantial problems due to losses or contamination at such low levels. Further, at low uptakes even small errors in the differential measurement may have a large impact. Moreover, net influxes and effluxes at equilibrium (steady state) condition cannot be measured by differential measurement. Using a radiotracer, which has proven to be very useful to determine influx, efflux and/or net uptake in in vitro studies, accurate and sensitive measurements are possible when high specific activities are used.

As mentioned above, the rationale for the radiotracer technique with a copper radioisotope is the isotopic exchange between radioactive copper and (stable) copper offered, viz. in the diet of the rats or in the medium of the cultured cells. Generally radioactive copper is supplied to rats or cells, containing normal levels of copper. This latter copper which will gradually exchange with the radioactive tracer taken up, lowering the specific activity. Thus, the radioactivity signal cannot always be directly translated into an overall copper transport. Dilution of the radiotracer taken up with stable copper from the system may tracked down by measuring the amount of total copper in parts of rats or cells for the calculation of the specific activity. Also mathematical modelling, in particular compartment analysis, may be useful. In conclusion, it can be said that results obtained for stable copper (measured by atomic absorption spectrometry) and those obtained for radioactive copper sometimes may differ, and that the interpretation of this difference may give more insight in particular aspects of the copper metabolism, e.g., in kinetics.

OUTLINE OF THE EXPERIMENTAL WORK

The effects of feeding supplements of ascorbic acid on dietary copper availability and copper status were studied in rats. Decreases in copper absorption have the potential to influence Cu status of the blood, including hematocrit and hemoglobin levels, and ceruloplasmin oxidase activity, liver and other organs, biological half-life as determined by ^{64}Cu retention and therefore these variables are possible "markers" to monitor Cu status.

Male outbred Wistar (Hsd/Cpb:Wu) rats were used fed on purified diets adequate in copper (5 mg/kg) without or with 1 or 10 g ascorbic acid/kg diet. Rats fed on a copper-deficient diet (< 1 mg Cu/kg) were included as positive control and reference groups, to compare and contrast the effects of copper deficiency with those of ascorbate on copper metabolism.

In Chapters 1 and 2 the mechanisms are described by which dietary ascorbic acid influences copper metabolism; the effects of a very high concentration (10 g ascorbic acid/kg diet) are compared with those of a pharmacological one (1 g/kg diet). The third Chapter describes a whole-body counting technique and the use of radiotracers which allow determination of true copper absorption in vivo. Chapter 4 describes the influence of ascorbic acid on true copper absorption and biliary excretion. Chapter 6 deals with the effects of ascorbic acid on intestinal copper solubility. Intestinal solubility of copper was also studied in rats fed fructose instead of glucose in their diet (Chapter 5). In Chapter 7 copper uptake and retention by primary cultures of liver parenchymal cells isolated from nutritionally copper-deficient rats are studied. The effect of nutritional copper deficiency on copper membrane transport is illustrated by using plasma membrane vesicles isolated from livers of copper-deficient rats (Chapter 8). A model for copper uptake is proposed in which reducing agents, ascorbic acid or a membrane bound NADH reductase, are involved (Chapter 9). Chapter 10, shows that the effects described for ascorbic acid on copper metabolism are specific for this vitamin as they do not occur with other vitamins that have antioxidant properties. Finally, in the general discussion the results presented are summarized and integrated.

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ASCORBIC ACID SUPPLEMENTATION AND COPPER STATUS IN RATS

ABSTRACT

The effect of a high concentration (1%, w/w) of ascorbic acid in a Cu-adequate (150 µmol/kg) purified diet was studied in rats. After 6 wk, ascorbic acid had significantly reduced Cu concentrations in muscle and bone. The estimated whole body content of Cu in rats fed ascorbic acid was reduced by 20%. Within 1 d after oral administration of ^{64}Cu, the recovery of the dose in feces was increased in rats fed ascorbic acid, suggesting that the vitamin depresses intestinal absorption of Cu.

After intraperitoneal (ip) administration of ^{64}Cu, the rate of loss of the dose from the body was decreased in rats fed ascorbic acid. This study suggests that the ascorbic acid induces a decreased efficiency of intestinal Cu absorption, which in turn triggers mechanisms to preserve Cu in the body stores. This is supported by the observation that the feeding of a Cu-deficient diet (5 µmol/kg) had similar effects, although more pronounced.

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INTRODUCTION

High intakes of ascorbic acid may be antagonistic to Cu status in humans. Ascorbic acid has been shown [1,2] to decrease serum activities of ceruloplasmin, which is the main copper-containing protein in plasma. Rats fed a diet containing high concentrations of ascorbic acid showed a reduced whole body retention of orally administered ^{64}Cu [3]. Fecal Cu excretion, expressed as a percentage of Cu intake, is increased in rats fed ascorbic acid [4]. These studies with rats suggest that ascorbic acid depresses the intestinal absorption of Cu. However, the effect of dietary ascorbic acid on the concentration of Cu in the liver, which is the major location of storage Cu, is not clear [3,4]. This prompted us to study the effects of high ascorbic acid loads on Cu concentrations in various tissues and on ^{64}Cu retention in rats. The effects of the high ascorbic acid diets were compared with those of a low Cu diet.

MATERIALS AND METHODS

Experiment 1. Male Wistar rats of the Hsd/Cpb:WU strain (Harlan-CPB, Zeist, The Netherlands) were used. The animals weighing about 200 g, were housed individually in Makrolon type III cages with raised, wire mesh floors. The cages were located in a room with controlled lighting (light: 06.00–18.00 h), temperature (19–21 °C), and relative humidity (50–60%). A commercial pelleted diet (SRMA^R, Hope Farms, The Netherlands) and demineralized water were supplied *ad libitum.*

The recovery of orally administered radioactive Cu in feces was determined with a dose of ^{64}Cu. Each nonstarved rat was given by stomach tube 0.8 μmol/kg of ^{64}Cu (specific activity 20.2 TBq/mol, HOR, Interfaculty Reactor Institute, Delft, The Netherlands) in a total volume of 0.25 mL 50 mM sodium acetate buffer, pH 5.4, without or with 140 μmol ascorbic acid. Feces of each rat was collected quantitativity for the measurement of ^{64}Cu activity.

Experiment 2. Male rats (Hsd/Cpb:WU), weighing about 80 g, were housed as described above. On d 0 of this experiment, the animals were randomly divided into three groups consisting of four or eight animals each. Two groups were fed a Cu-adequate diet without or with 56.8 mmol/kg (w/w) of ascorbic acid (L-ascorbic acid, Merck, Darmstadt, FRG). A third group served as a positive control and was fed a Cu-deficient diet. The rats were fed a purified diet that consisted of (g/100 g): glucose, 50.65; corn starch, 15.0; ovalbumin, 20.0; sunflower seed oil, 4.0; cellulose, 5.0; choline chloride, 0.3; mineral mix, 3.95; trace element mix, 0.1; and vitamin mix, 1.0. The composition of the mixes has been described elsewhere [5]. Cu was added in the form of $CuSO_4.5H_2O$ to give concentrations of 150 and 5 μmol/kg (by analysis) in the Cu-adequate and Cu-deficient diet. Ascorbic acid was added to the diet at the expense of glucose. Previous work has shown that dietary Cu

concentration does not affect feed intake [6]. Therefore, rats in the present study were fed *ad libitum*. Diets were stored at 4 °C, and fresh food was supplied daily. The rats had free access to demineralized water.

On d 35 of this experiment, each non-starved rat was injected intraperitoneally with a dose of ^{64}Cu (Cu acetate, 0.8 µmol/kg body wt; specific activity 20.2 TBq/mol). Whole body counting of animals was performed by placing a container with the animal into a tank filled with a scintillation liquid (pseudocumene), equipped with a photomultiplier connected to a multichannel analyzer [7]. The efficiency of this counter for ^{64}Cu was 14%. Whole body counting of the animals was performed 2 h postinjection and then daily for 4 d. The measured whole body ^{64}Cu activities at any time, R(t), and those measured 2 h postinjection, R_0, were used to calculate the log% of the administered dose:

$$\text{log\% dose, retained at time t, } R_t = R_0 . 10^{-mt}$$

The log% dose was plotted vs time, t, and a linear relation from d 2–4 was found. By a least square fit, the slope (m) and the intercept, the % apparent retention were computed. The biological half-life (T_b) was calculated as:

$$T_b = \log 2/m$$

On d 41 of this experiment, the nonstarved rats were again injected intraperitoneally with ^{64}Cu (0.8 µmol/kg body wt; specific activity 10.6 TBq/mol), and the next day, they were anesthetized by exposure to CO_2. Blood was collected in heparinized syringes by heart puncture, and the animals were killed by further exposure to CO_2. Plasma was collected by low speed centrifugation. Liver, left tibia, and left flexor digitorum longus muscle were removed for the determination of radioactivity and Cu concentration. Whole body contents of Cu were assessed with the use of analyzed Cu in liver, plasma, muscle, and bone and values for the mass of these tissues according to Owen [8], whereas Cu in such locations as the gastrointestinal tract, brain, and fur were not accounted for. The specific activity was calculated from the ^{64}Cu activity in Bq divided by Cu content in nmol.

Analyses. ^{64}Cu activity in tissues was measured in a gamma counter. Corrections were made for decay and background. Total Cu in diets and tissues was determined in duplicate after wet digestion with 1.0 mL of 65% nitric acid (Suprapur, Merck, Darmstadt, FRG) and 0.5 mL of 30% hydrogen peroxide (Aristar, BDH Chemicals, Poole, UK) by atomic absorption spectrophotometry (Perkin Elmer Model 2380, Norwalk, CT, USA). The results are given as means ± SD. Statistical analysis was performed by the two-tailed Student's t-test and 0.05 was considered the maximum value for the type I error. Ascorbic acid in plasma was measured as described [9]. Ceruloplasmin in rat plasma was assayed as p-phenylenediamine oxidase activity, as described by Sunderman [10].

RESULTS

Experiment 1. Table 1 shows that ascorbic acid administered by stomach tube simultaneously with ^{64}Cu significantly increased the recovery of the dose in the feces: within 1 d after administration, about 80% of the dose was found in feces of ascorbic acid-treated animals and about 60% for the nontreated animals.

Table 1. *Effect of ascorbic acid on the recovery of orally-administered ^{64}Cu in feces (Experiment 1).*

	Supplement	
	None n= 5	Ascorbic Acid n= 5
	% of dose	
Day 1	62.9 ± 4.0	77.4 ± 4.0*
Day 2	3.0 ± 3.4	4.8 ± 5.9
Day 3	1.5 ± 0.3	2.1 ± 1.2
Days 1–3	67.4 ± 5.7	84.3 ± 2.5*

* $p < 0.05$.

Experiment 2. Growth and feed intake were similar in rats fed the purified diets either with adequate Cu without or with ascorbic acid, or the Cu-deficient diet (Table 2). In rats fed the Cu-deficient diet, Cu status was drastically impaired when compared with rats fed the Cu-adequate diets. In the former animals, Cu concentrations in all tissues were significantly reduced at d 42. Likewise, plasma activity of ceruloplasmin was markedly decreased in the rats fed the Cu-deficient diet (Table 2).

The addition of ascorbic acid to the diet significantly increased plasma concentrations of this vitamin (Table 2). Ascorbic acid also significantly reduced Cu concentrations in muscle and bone. The estimated whole body content of Cu, therefore, was decreased by 20% in rats that ingested ascorbic acid (Table 2).

Table 2. *Growth performance, tissue copper concentrations, plasma ascorbate, and ceruloplasmin activities (Experiment 2)*[1].

Measure	Diet		
	Cu adequate n=4	Cu adequate + ascorbic acid n=8	Cu deficient n=4
Cu-intake, nmol/d	2250	2250	80
Ascorbic acid intake, μmol/d	–	850	–
Body wt, g, d 0	$77^a \pm 2$	$78^a \pm 3$	$76^a \pm 3$
Body wt, g, d 42	$290^a \pm 1$	$289^a \pm 17$	$287^a \pm 17$
Plasma Cu, μM	$20^a \pm 3$	$20^a \pm 3$	$2^b \pm 1$
Liver Cu, nmol/g wet wt	$69^a \pm 9$	$66^a \pm 11$	$18^b \pm 2$
Muscle Cu, nmol/g wet wt	$22^a \pm 3$	$17^b \pm 2$	$10^c \pm 4$
Bone Cu, nmol/g wet wt	$15^a \pm 4$	$10^b \pm 3$	$4^c \pm 1$
Estimated whole body Cu, μmol	$4.0^a \pm 0.4$	$3.3^b \pm 0.4$	$1.5^c \pm 0.5$
Plasma ceruloplasmin, μM	$3.6^a \pm 0.5$	$3.8^a \pm 0.7$	$0.7^b \pm 0.1$
Plasma ascorbate, μM	$71^a \pm 22$	$150^b \pm 28$	$90^a \pm 18$

[1] $\bar{x} \pm SD$ in each horizontal line common superscripts indicate $p > 0.05$.

One day after the intraperitoneal administration of ^{64}Cu, the distribution of the dose between the tissues studied was different in rats fed diets containing either adequate or deficient amounts of Cu (Table 3). The Cu-deficient diet caused the dose to accumulate preferentially in liver and muscle, whereas its excretion was depressed. In rats fed ascorbic acid, when compared with their controls, plasma and liver contained more and bone less radioactivity. Ascorbic acid inhibited the excretion of radioactivity. The pattern of specific activities of rats fed ascorbic acid and Cu-deficient rats was similar, but differ with their controls.

Figure 1 illustrates the time course of body retention of the dose. In rats fed the Cu-deficient diet, the loss of the dose was diminished when compared with their counterparts fed the Cu-adequate diet. Dietary ascorbic acid also reduced the rate of Cu loss from the body although this effect was not as pronounced as that induced by the deficient intake of Cu. The biological half-life, as determined from body retention between d 2 and 4, was markedly prolonged in the Cu-deficient animals ($T_b = 13 \pm 2$ d) when compared with the control animals ($T_b = 5 \pm 2$ d) and the rats fed ascorbic acid ($T_b = 6 \pm 1$ d). Apparent Cu retention was significantly higher both in Cu-deficient animals ($85 \pm 7\%$) and rats fed ascorbic acid ($88 \pm 4\%$), in comparison with control animals ($73 \pm 12\%$).

Table 3. *^{64}Cu distribution (% Dose/tissue) and specific activities (SA) on day 42, one day after the intraperitoneal administration of ^{64}Cu (Experiment 2)*[1].

Measure	Diet: Cu-adequate n=4	Cu-adequate + Ascorbic acid n=8	Cu-deficient n=4
Plasma, % Dose/tissue	$11^a \pm 2$	$17^b \pm 3$	$8^c \pm 2$
Plasma, SA Bq/nmol	$782^a \pm 103$	$1164^b \pm 192$	$8094^c \pm 1258$
Liver, % Dose/tissue	$11^a \pm 1$	$15^b \pm 2$	$16^b \pm 1$
Liver, SA Bq/nmol	$267^a \pm 46$	$419^b \pm 44$	$1655^c \pm 196$
Bone, % Dose/tissue	$15^a \pm 1$	$9^b \pm 1$	$15^a \pm 1$
Bone, SA Bq/nmol	$616^a \pm 137$	$440^b \pm 122$	$1684^c \pm 315$
Muscle, % Dose/tissue	$10^a \pm 1$	$10^a \pm 2$	$13^b \pm 1$
Muscle, SA Bq/nmol	$65^a \pm 8$	$84^b \pm 9$	$294^c \pm 45$
Carcass, % Dose/tissue	$25^a \pm 3$	$31^b \pm 5$	$30^b \pm 3$
Carcass, SA Bq/nmol	$297^a \pm 20$	$418^b \pm 39$	$897^c \pm 47$
Excretion[2], % Dose	$28^a \pm 2$	$18^b \pm 2$	$18^b \pm 2$

1 $\bar{x} \pm$ SD in each horizontal line common superscripts indicate $p>0.05$.

2 Calculated difference between the dose administered and the whole body activity 24 h later.

DISCUSSION

This study shows that high amounts of dietary ascorbic acid impair the Cu status of rats. In rats fed ascorbic acid, Cu concentrations of muscle and bone were significantly decreased. The estimated content of whole body Cu was lowered by 20%. It is clear, however, that a decrease in the dietary Cu concentration from 150 to 5 µmol/kg had a more drastic effect on Cu status than the addition of 1% (w/w) of ascorbic acid to the diet. Ascorbic acid did not influence liver Cu, and this agrees with the work of Van Campen and Gross [3] using deficient- or adequate-Cu (150 µmol/kg) diets. In a study of Johnson and Murphy [4], liver Cu was decreased by ascorbic acid only if the diet contained sufficient Cu (90 µmol/kg). With lower dietary Cu levels (7 µmol/kg) ascorbic acid did not influence liver Cu concentrations. Perhaps other components of the background diet can modify effects of ascorbic acid on liver Cu concentrations.

It seems likey that ascorbic acid inhibits the intestinal absorption of Cu. The vitamin has been shown to inhibit the disappearance of ^{64}Cu from ligated duodenal segments of rats [3]. Moreover, orally-administered ^{64}Cu was retained less efficiently in the body of rats fed ascorbic acid than their controls [3]. Johnson and Murphy [4] reported that ascorbic acid

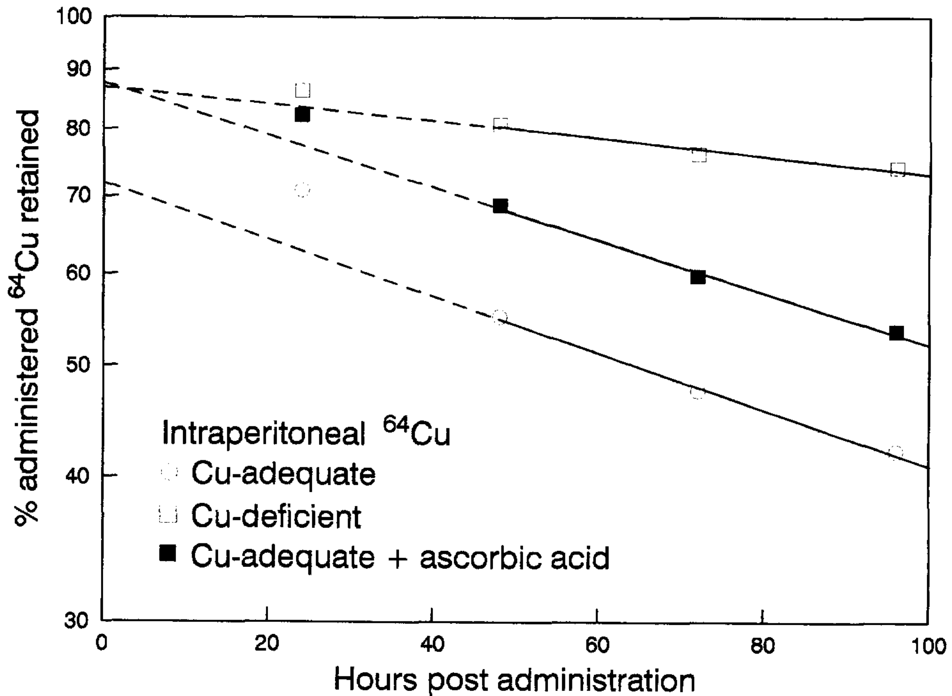

Figure 1. *Time course of whole body retention of injected ^{64}Cu. Symbols: O, rats fed Cu-adequate diet (n = 4); □, rats fed Cu-deficient diet (n = 4); ■, rats fed Cu-adequate diet supplemented with ascorbic acid (n = 8).*

increased fecal output, expressed as a percentage of intake. The present data would support these observations. Ascorbic acid significantly increased the recovery in feces of orally-administered ^{64}Cu (Table 1). Since this effect was seen within one day after administration of the dose, it may be concluded that ascorbic acid depressed the intestinal absorption of Cu.

If ascorbic acid reduces the efficiency of Cu absorption in the intestine, then this vitamin and low intakes of Cu should have similar effects on Cu homeostasis within the body, viz. postabsorptively. When gastric and intestinal absorption were circumvented by intraperitoneal administration of ^{64}Cu, tissue distribution and excretion of ^{64}Cu were altered similarly in Cu-deficient rats and animals fed ascorbic acid. However, in Cu-deficient animals, the biological half-life of ^{64}Cu was about twice that of controls, whereas in animals fed ascorbic acid, no increase in biological half-life was found. Careful inspection of Figure 1 suggests that ascorbic acid induced an increased initial retention of Cu. Thus, although the kinetics are different, both Cu-deficiency and ascorbic acid trigger mechanisms to reduce Cu loss from body stores to a minimum. This is illustrated by the inhibition of loss of radioactivity after intraperitoneal administration of ^{64}Cu (Table 3). It could be suggested that Cu excretion in urine and/or bile, and thus eventually feces, is inhibited after feeding a Cu-deficient diet or a diet supplemented with ascorbic acid.

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INFLUENCE OF ASCORBIC ACID SUPPLEMENTATION ON COPPER METABOLISM IN RATS

ABSTRACT

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 copper absorption from the intestine. Ascorbate, intravenously administered together with ^{64}Cu, caused an increase of ^{64}Cu in 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.

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INTRODUCTION

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 copper 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, 1968). Simultaneous oral administration of ^{64}Cu and ascorbate increased the recovery of ^{64}Cu in faeces within one day 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 liver were increased in rats fed on ascorbate (Van den Berg et al. 1990). This effect may relate to the reduced tissue copper 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 of ascorbic acid/kg, which is equivalent to intakes of about 1 g of ascorbic acid per 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 a low-Cu diet the effects of ascorbic acid feeding may be more pronounced.

MATERIALS AND METHODS

Experiment 1. *Cu uptake by isolated rat hepatocytes.* Male Wistar rats of the Hsd/Cpb:WU strain (Harlan-CPB, Zeist, The Netherlands) were used as hepatocyte donors. The rats were aged 10 weeks and had been fed on a commercial pelleted diet (SRMA^R , Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. 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 °C 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] Cu-acetate (15 μmol Cu/l), were added to the medium. After various incubation periods at 37 °C, the radioactive medium was aspirated and the cells were harvested and washed twice with Ham's F-10 prior to 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 and/or uptake by subtracting cellular radioactivity determined after incubation of hepatocytes at 4 °C in parallel experiments. To test whether ascorbate interacts specifically with Cu uptake, [^{65}Zn]$ZnCl_2$ 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.

Experiment 2. *Hepatic uptake and biliary excretion of ^{64}Cu after intravenous administration of ^{64}Cu without or with ascorbate.* Male Wistar rats, aged 10 wk and weighing on average 250 g, were used. The rats had been fed on commercial pelleted diet and tap water *ad libitum*. The animals were anaesthetized with pentobarbital sodium (60

mg/kg body wt, intraperitoneally; Nembutal[R], Sanofi Sante Animale SA, Paris, France). The bile duct was cannulated as described elsewhere (Villalon et al. 1987). Body temperature was kept at 37 °C with the use of a thermostatically-controlled heating lamp. At 15 min after collection of the first bile, [^{64}Cu] Cu-acetate (0.5 μg Cu) without or with 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.

Experiment 3. *Effect of a diet containing 10 g ascorbic acid/kg on Cu metabolism.* Male, specified-pathogen-free Wistar rats, aged about 3 wk, 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, The Netherlands) with a layer of sawdust as bedding. For 10 d, they were fed *ad libitum* on a purified diet containing 5 mg of 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] Cu-acetate (5 μg Cu) and on day 35 an intraperitoneal injection of [^{64}Cu] 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] 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 carbon dioxide 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.

Experiment 4. *Effects of diets containing 1 g ascorbic acid/kg on Cu metabolism.* Male Wistar rats, aged about 3 wk, were used. All animals were fed on the purified diet containing 5 mg of 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

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

	Expt 3		Expt 4			
Copper[1], mg/kg	5.0	5.0	5.0	5.0	1.0	1.0
Ascorbic acid[1], 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[2], g	297.4	297.4	297.4	297.4	297.4	297.4
Chemical analysis						
Copper[3], mg/kg	5.2	5.2	5.3	5.1	0.8	0.8
Ascorbate[3], g/kg	–	9.1	–	1.0	–	0.9

[1] Calculated values.

[2] The constant components consisted of (g): ovalbumin, 151; corn 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; niacinamide, 20; DL-calcium panthothenate, 17.8; choline chloride, 2000; pyridoxine, 6; cyanocobalamine, 50; folic acid, 1; biotin, 2; menadione, 0.05; DL-alpha tocopheryl acetate, 60; retinyl acetate and retinyl palmitate, 8 (4000 IU); cholecalciferol, 2 (1000 IU); maize meal, 9826.15.

[3] Average values of six measurements.

similar. Each group was randomly assigned to one of four experimental diets. The diets contained 5 mg of Cu/kg without or with 1 g ascorbic acid/kg or 1 mg of Cu/kg without or with 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 wk, and that between the second and third was 16 wk. During the experimental period (days 0–28), the rats were housed individually in metabolic cages (Tecniplast Gazzada, Buguggiate, Italy). One batch of diet was used for the three cohorts. The diets, which were in powdered form, were stored at –20 °C until feeding. The concentration of ascorbic acid was checked prior to 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 HCl/l. Urinary and faecal Cu were analysed.

On day 24, each non-starved rat was injected intraperitoneally with [^{64}Cu] 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, animals were killed by exposure to CO_2. Blood samples were taken by aortic puncture. Tissues were collected, weighed, and frozen at –20 °C 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 copper wire (purity 99.999%, Ventron, Karlsruhe, Germany) in a thermal neutron flux of 10^{17} m^{-2}. s^{-1} 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 HNO_3 (undiluted) and diluted with sodium acetate 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 TBq ^{64}Cu/kg 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 NaI 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, 1984). 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 pre-treated 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 °C for 18 h and dissolved in 6 mol HCl/l. Tissues were freeze-dried and then digested with nitric acid (Suprapur; Merck, Darmstadt, Germany) and hydrogen peroxide (Aristar; BDH Chemicals, Poole, UK). The mixture consisted of 1 g tissue/l of nitric acid and hydrogen peroxide (13:6, v/v). Feed samples were pre-treated 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 (EC 1.16.3.1) 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 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 and Cherian (1981).

Statistical analyses. The Kolmogorov-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 above for normally distributed data.

RESULTS

Experiment 1. *Cu uptake by isolated hepatocytes.* Hepatocytes accumulated ^{64}Cu (Table 2) with time and this was a temperature-dependent process because at 4 °C copper uptake rates were less than 5% of those measured at 37 °C (data 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.

Experiment 2. *Hepatic uptake and biliary excretion of ^{64}Cu after intravenous administration of ^{64}Cu without and 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.

Experiment 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

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

[1] For details of procedures, see pp 11.
There were significant effects of ascorbate, incubation period and interaction (two-way analysis of variance; $P < 0.001$).

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)*[1].
(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*	1.2

[1] For details of procedures, see pp. 11–12.
Mean values were significantly different from those of animals not given ascorbate (two-tailed Student's t-test): * $P < 0.05$.

body weight gain. Packed cell volume and blood haemoglobin concentrations were significantly reduced by ascorbate intake. Rats fed on ascorbic acid showed significantly decreased activities of plasma ceruloplasmin. Control values of ceruloplasmin-protein concentration correspond well with those reported by others (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.

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*[1].
(Mean values with their standard errors for six rats per group)

Dietary ascorbic acid (g/kg)...	None		10	
	Mean	SE	Mean	SE
Body weight, 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[2], g/l [*EC* 1.16.3.1]	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[3], 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

1 For details of procedures, see p. 12.

2 As measured by its oxidase acivity.

3 As μg Cu/g dry weight.

Mean values were significantly different from those of animals not given ascorbic acid (two-tailed Student t-test): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Whole-body retention of ^{64}Cu after oral and intraperitoneal administration of ^{64}Cu is shown in Figure 1a and 1b. 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 as 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. 1b). Cu absorption, as calculated from the y intercept values after extrapolation of the linear part of the retention curves (42–96 h)

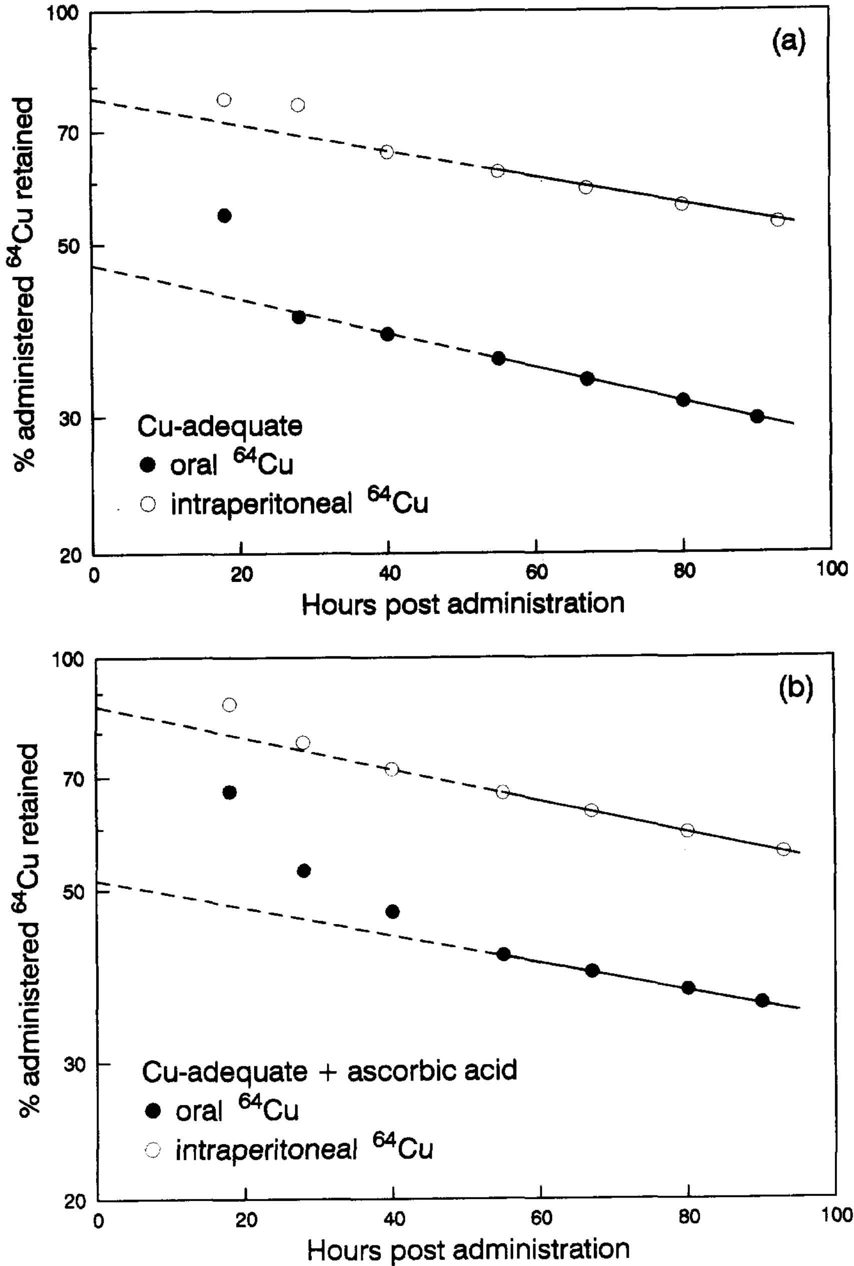

Figure 1. *Expt. 3 Whole-body retention of orally (●) and intraperitoneally (○) administered ^{64}Cu in rats fed on diets with recommended copper concentrations without (a) or with (b) 10 g of ascorbic acid/kg. Results are means for five animals per dietary group. Linear fits were calculated over the time period 42–96 h after ^{64}Cu administration. For details, see p. 12.*

Table 5. *Expt 3. Recovery of administered ^{64}Cu (% dose) in urine, faeces, liver and bile of rats fed a diet containing 10 g ascorbic acid/kg*[1].

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

Dietary ascorbic acid (g/kg)...		None		10	
Route of ^{64}Cu administration[a]	Site of recovery	Mean	SE	Mean	SE
Oral	Urine[b]	3	0.5	3	0.6
	Faeces[b]	60	1.2	59	3.7
Intraperitoneal	Urine[b]	7	0.7	5	0.6
	Faeces[b]	34	1.9	27*	2.0
Intravenous	Liver[c]	43	3.1	45	3.0
	Bile[c]	10	0.9	13*	0.9
	Carcass[c]	40	2.7	35	1.8

[1] For details of procedures, see p. 12.

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

[b] ^{64}Cu accumulated for 3 d after administration.

[c] ^{64}Cu accumulated for 150 min after administration.

Mean values were significantly different from those of animals given the diet without ascorbic acid (two-tailed Student's t-test): $^{*}P < 0.05$.

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

Experiment 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 and blood haemoglobin concentrations both in rats given diets with recommended and low Cu concentrations, the effect being somewhat more

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*[1].
(Mean values for nine rats per dietary group)

						Statistical significance of effect of[3]:	
Cu[2] (mg/kg)...	5.0	5.0	1.0	1.0	Pooled	Ascorbic	
Ascorbic acid[2] (g/kg)	–	1.0	–	1.0	SE	acid	Cu
Body-weight, g							
Initial	79	79	80	81	0.1		
Final	201	197	194	183	1.1	–	P< 0.001
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, g/l [*EC* 1.16.3.1]	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[4]							
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

[1] For details of procedures, see pp. 12–14.

[2] Calculated values.

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

[4] As μg Cu/g dry weight.

pronounced in the latter. Plasma ceruloplasmin (activity) was not influenced by ascorbate, but almost completely suppressed by low copper intake.

In rats fed the diets with 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 diets containing 1 g ascorbic acid/kg*[1]. (Mean values for nine rats per dietary group)

						Statistical significance of effect of [3]:	
Cu^2(mg/kg)...	5.0	5.0	1.0	1.0	Pooled	Ascorbic	
Ascorbic acid[2](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[4]							
μg/d	31	16	8	7	0.7	$P< 0.01$	$P< 0.001$
% of intake	42	23	70	59	2.1	$P< 0.01$	$P< 0.001$

1 Values for days 11–13 and days 24–26 of the experiment. For details of procedures, see pp. 12–14.

2 Calculated values.

3 All data were subjected to ANOVA after log transformation.

4 Apparent absorption is expressed in absolute (intake-faecal output) and relative terms [100× (intake-faecal output)/intake].

Dietary ascorbic acid increased faecal loss of Cu both in rats fed diets with recommended and low Cu concentrations (Table 7). Copper in urine was not detectable. The absolute and percentage apparent absorption 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 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 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 Figure 2. A significantly higher retention was found in rats fed the low-Cu diets, when compared with the rats fed diets with recommended Cu concentration. Biological half-lives of the 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 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 copper concentration, but this effect was very small.

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

(Mean values for nine rats per dietary group)

						Statistical significance of effect of[3]:	
Cu[2](mg/kg)...	5.0	5.0	1.0	1.0	Pooled	Ascorbic	
Ascorbic acid[2] (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

[1] Values for days 24–26 of the experiment. For details of procedures, see pp. 12–14.

[2] Calculated values.

[3] All data were subjected to ANOVA after log transformation.

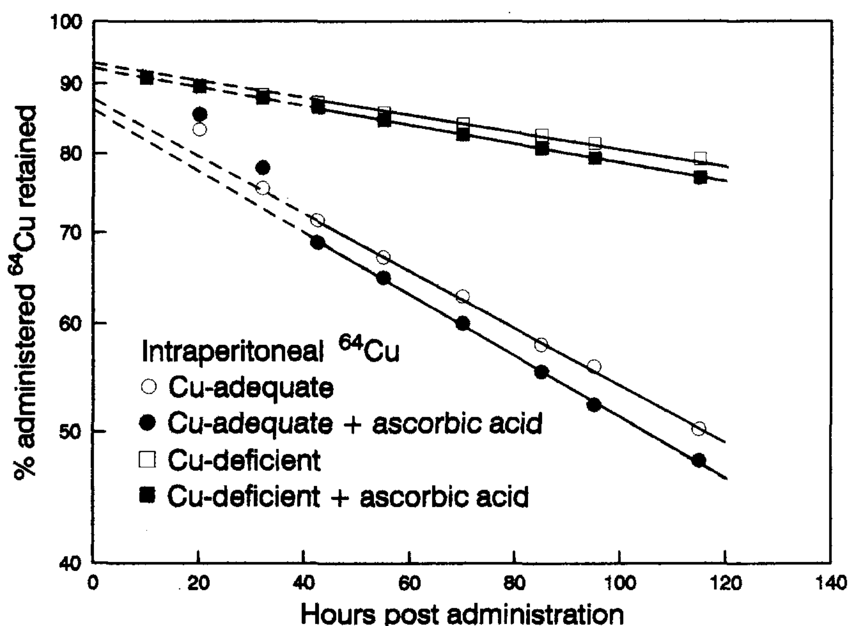

Figure 2. *Expt. 4 Whole-body retention of intraperitoneally administered ^{64}Cu in rats fed on diets containing either recommended (○,●) or low (□,■) amounts of Cu without (○, □) or with (●, ■) 1 g ascorbic acid/kg. Results are means for nine animals per dietary group. Linear fits were calculated over the time period 42–96 h after ^{64}Cu administration. For details of the procedure, see pp. 12–14.*

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 copper concentrations resulted in depressed plasma ceruloplasmin activity and decreased Cu concentrations in plasma and various tissues, especially liver. This agrees with 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 per 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 of 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 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 ascorbic acid (Fig. 1b), 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 copper 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 of 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 ascorbic acid and given ^{64}Cu intravenously (Table 5). This could be related to the reduced tissue Cu concentrations of rats fed 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 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 a diet containing 10 g ascorbic acid/kg, more intravenously administered ^{64}Cu was recovered in bile than in rats fed 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 a diet containing 10 g of ascorbic acid/kg (Table 5). This may be explained by the Cu-retaining effect of ascorbate-induced reduced tissue Cu concentrations, an effect being more pronounced in rats fed 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 copper 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 copper excretion. This is supported by the observation that low copper 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 recommended copper diets containing either 10 or 1 g ascorbic acid/kg. In rats fed a 10 g ascorbic acid/kg diet, whole-body retention of ^{64}Cu was slightly increased (Fig. 1b) whereas in rats fed 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 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 copper ^{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 the diet containing 10 g ascorbic acid/kg for 42 d (Fig. 1b) 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 copper 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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3

ABSORPTION AND RETENTION STUDIES OF TRACE ELEMENTS AND MINERALS IN RATS USING RADIO-TRACERS AND WHOLE-BODY COUNTING

ABSTRACT

A description is given of a whole-body counting technique using radiotracers, permitting the determination of true absorption and endogenous excretion of trace elements and minerals in the rat in vivo. This non-invasive counting method involves oral and intraperitoneal administration of tracer doses of a radioisotope in a cross-over fashion and subsequent measurement of the whole-body retention in a whole-body counter. Thus, true absorption can be determined in one animal which contributes to the reduction of animal use. To study the variations in counting response due to radioisotope distribution, to size or shape of the animal body, the influence of the position of a point source and distribution over different phantoms to simulate various body sizes was experimentally evaluated for ^{64}Cu, ^{65}Zn, ^{59}Fe and ^{28}Mg. Results from two studies, with ^{64}Cu and ^{28}Mg, as an example for a trace element and a mineral respectively, are presented and illustrate that absorption as measured by apparent absorption does not necessarily reflect true absorption. True absorption as determined by the whole-body retention method using radioisotopes corrects for faecal losses of endogenous origin.

submitted

INTRODUCTION

The mechanism by which the mammalian organism maintains a relatively constant internal environment, including optimal concentrations of essential metal ions, may involve regulation at entry (intestinal absorption) and/or exit from the body, by endogenous losses, viz., biliary, pancreatic or intestinal secretions and by urinary losses. Various studies on trace elements and minerals involve the measurements of apparent absorption (intake minus total faecal excretion) and/or true absorption (intake minus exogenous faecal excretion).

The conventional balance technique, whereby the difference between dietary intake and faecal excretion gives the extent of apparent absorption, has its limitation of demarcation of faeces in periods corresponding to the intake periods. Moreover, because the degree of absorption is calculated as the difference between two measurements, the precision will be decreased by at least $\sqrt{2}$ times the average precision of the two measurements.

Tracer techniques with radioisotopes can be used for absorption studies. The rationale for these methods is an isotopic exchange between an extrinsic tag and the stable form of the element present in the diet. Determination of the whole-body radioactivity can generally be done accurately if the detector response is not sensitive to the distribution of the radioactivity within the body.

Absorption studies performed with the conventional balance technique determine only apparent absorption and not the true absorption. If the true intestinal absorption is to be determined, the unabsorbed fraction of the oral dose needs to be quantified. However, during measurements part of the absorbed dose may be excreted into the intestine (endogenous excretion). The higher this endogenous excretion in relation to dietary intake, the greater is the difference between apparent and true absorption in the convential balance technique. Also in the radiotracer method a fraction of the absorbed radioactivity is endogenously excreted and leaves the intestinal lumen with the faeces.

Consequently, a correction for endogenous losses has to be introduced. This may be done by comparison of the whole-body retention curves for two methods of administration, viz. an oral and an intraperitoneal or intravenous gift, resulting directly in the actual percentage absorption of orally administered radioactivity. The procedure and rationale for the calculation of the true absorption has been described for ^{65}Zn [Becker and Hoekstra, 1967].

The whole-body counting technique for determining intestinal absorption requires suitable radioisotopes and a whole-body counter. During the entire experimental period the whole-body counter must be insensitive to: 1) changes in the spatial distribution of the radioisotope within the body, particularly when the radioisotope is initially concentrated into a small volume followed gradual distributing throughout the body; 2) changes in body size and shape, and thus in radiation absorption effects of the body. Possible effects of these

variations may be dependent on the energies of the radioisotope. The accuracy of total retention measurements determined by whole-body counting depends on the degree, with which variations arising from the above-mentioned factors are absent or can be predicted and thus corrected for.

The aim of this study was to show the potentials of the radiotracer technique using whole-body counting, and to identify possible sources of errors. The response of the counter has been experimentally investigated for the radioisotopes: ^{64}Cu, ^{65}Zn, ^{59}Fe and ^{28}Mg. We describe a scintillation whole-body counter for small experimental animals (rats) for absorption and retention studies. The method allows to make estimates of endogenous excretion rates. The usefulness of the method for copper and magnesium absorption and excretion in rats will be illustrated.

MATERIALS AND METHODS

Whole-body counter. The liquid-scintillation counter used to measure the activities of small animals was constructed in the Interfaculty Reactor Institute. A schematic representation of the detector and the electronics are shown in Figure 1. The tank containing the scintillation solution is almost entirely surrounded by lead bricks which provide shielding (not shown in the figure). The scintillation solution is composed of terphenyl and 2,2-p-phenylene bis(5-phenyloxazole) (POPOP) in pseudocumene (1,2,4-trimethyl benzene). A coating of TiO_2 in an epoxy resin protects the inner walls of the scintillation tank from the chemical action of the scintillator solution and provides light reflectance. One photomultiplier is mounted at the lower end of the tank.

The total set-up functions as a single channel gamma-ray spectrometer with a lower level discriminator (threshold). The threshold was set to achieve a maximum signal to background ratio. Due to the low Z-value of the scintillation solution, the compton effect dominates strongly over the photo-electric effect, and thus, actually no gamma-ray spectrometry can be performed. Thus, additional qualitative measurements were carried out with a high-resolution gamma detector to verify radionuclic purity. The radioisotopes ^{64}Cu and ^{28}Mg used in this study were shown to be pure.

The counter was stable in background counting rate over a period of several hours, i.e., the counting period for all animals within a series. The background rate was 14924 counts in a counting period of 1 min, with a standard deviation of 130, being largely due to (natural) statistical fluctuations. A 2–3% random deviation about the mean of the background value was measured over a 14 day period, i.e., the entire experimental period, and include fluctuations due to external influences. Significant coincidence losses were not observed until counting rates exceeded 100,000 counts per second (cps), as experimentally determined with increasing activities of radioisotope solutions (results not shown). Overall counting

Table 1. *Nuclear decay properties of radioisotopes and initial dose administered to conduct whole-body counting.*

Radioisotope	Physical half-life	Gamma-energies in MeV (Abundancies)	Whole-Body Counter Efficiency	Dose Adminis-tered
^{64}Cu	12.8 h	0.511 (2×19%) 1.340 (0.5%)	14%	1.5 MBq
^{28}Mg	20.9 h	0.400 (30%) 0.942 (30%) 1.342 (70%) 1.780 (100%)	65%	20 kBq
^{59}Fe	45 d	0.142 (0.8%) 0.191 (2.8%) 1.100 (56%) 1.290 (44%)	61%	10 kBq
^{65}Zn	244 d	0.511 (3.4%) 1.115 (49%)	31%	20 kBq

efficiencies ranged from 14 to 65% (Table 1). In practice, all rats were routinely counted for 30 seconds unless the radioactivity levels (depending on combined physical- and biological half-life) were less than net 1500 cps. At 1500 cps as net signal, 14294 cpm as background and a counting time of 30 seconds, the precision (expressed as the coefficient of variation) amounts to 0.5%. Below 1500 cps the counting time was increased, but not longer than 300 seconds. For relatively low whole-body activities, the background counting rate contributes appreciably to the statistical error. For instance, measurement of a net signal of 100 Bq (viz. 14 cps) of ^{64}Cu for 300 seconds yields a precision of only 9.3%.

The variation in the response of the counter to various animal body sizes and shapes was investigated by the use of various polyethylene bottles filled with water (up to 250 mL) serving as phantom, approximately tissue-equivalent to the animals and containing size-independent levels of radioactivity. The phantoms were placed in the well of the whole-body counter with the use of the rat holder (cf. Fig. 1). The phantoms imitate a homogeneous distribution over the animal. The radioisotopes ^{64}Cu, ^{65}Zn, ^{59}Fe and ^{28}Mg were chosen, because of their applications in nutritional studies.

When the distribution of the radionuclide is varying rapidly with time [Gupta et al. 1976], sources of errors may be introduced. Since the variation in the response of the counter to the body size was the largest for ^{64}Cu, the variation of counting efficiency with the positions of

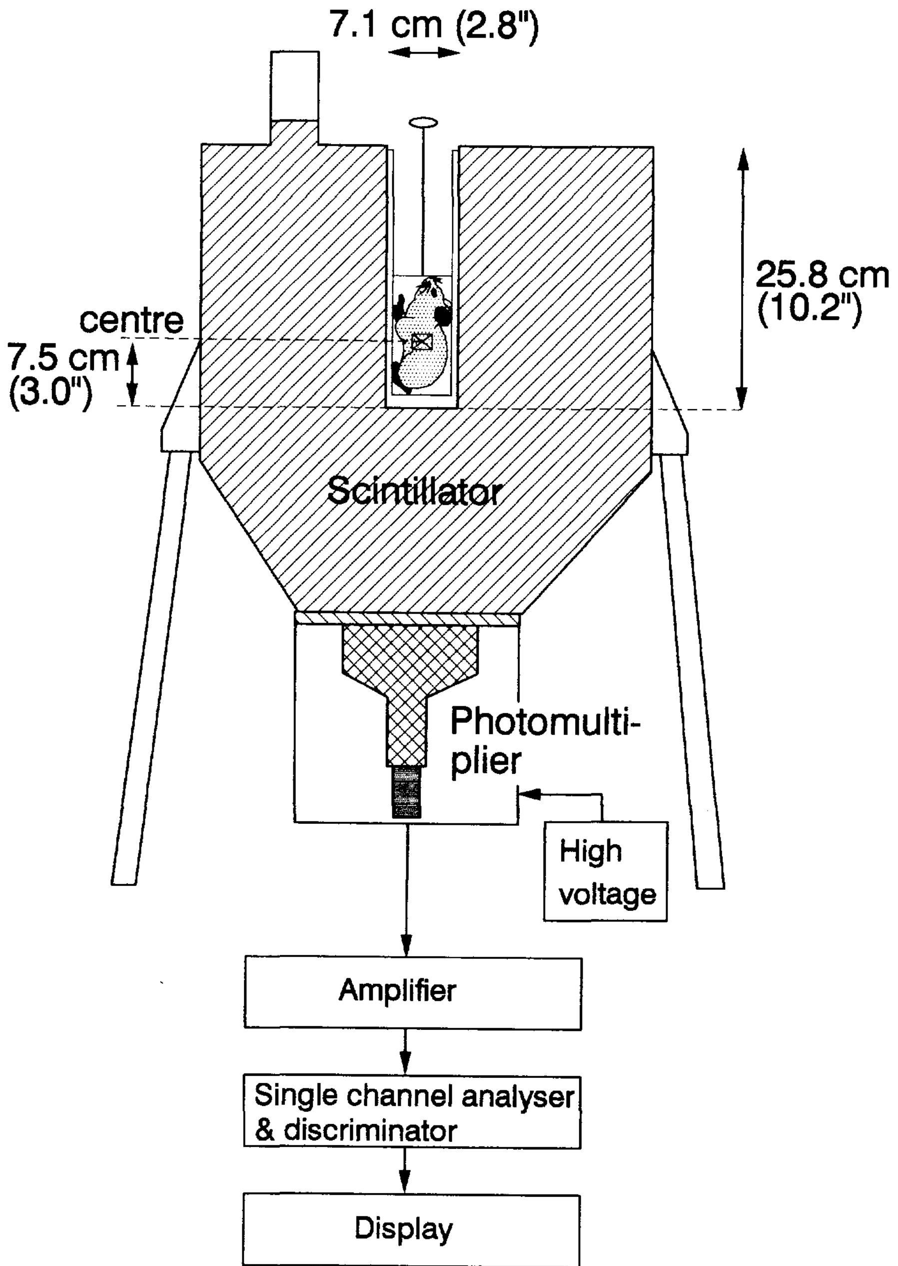


Figure 1. *Diagram of whole body-counter and rat holder in the well used for whole-body counting. Lead bricks, providing shielding not shown.*

the radioisotope in the body was assessed for this radioisotope only. To simulate heterogeneous distribution of ^{64}Cu in an animal, an irradiated copper wire was positioned at various vertical and horizontal places at the inside and outside of a 250-mL phantom.

Radioisotopes. ^{64}Cu was prepared by irradiating a copper wire (purity 99.999%; Ventron, Karlsruhe, Germany) in a thermal neutron flux of $10^{17}/m^2.s$ for 36 h in the nuclear reactor of the Interfaculty Reactor Institute. Following irradiation, the wire was dissolved in 25 µl concentrated nitric acid and diluted with acetate buffer (0.05 mol/L, pH 5.4) resulting in a final copper concentration of 1 mg/mL. The specific activity of the [^{64}Cu]Cu solution at the start of the experiments was about 19 TBq/mol Cu.

^{28}Mg was prepared as described elsewhere [Kolar et al., 1991], resulting in a solution of 160 mmol/L [^{28}Mg]$MgCl_2$ with a specific activity of 1.2 GBq/mol Mg.

^{65}Zn (as $ZnCl_2$ in 0.1 M HCl) with a specific activity of 200 TBq/mol and ^{59}Fe (as $FeCl_2$ in 0.1 M HCl) with a specific activity of 4400 TBq/mol, were purchased from Amersham (Little Chalfont, Buckinghamshire, UK).

Application of the method. The experiments reported here were derived from two separate rat studies [Van den Berg & Beynen, 1993; Van der Heijden et al., 1993]. The experimental protocol was approved by the animal experiments committee of the Erasmus University at Rotterdam, also serving as such for the Delft University of Technology.

Animals, Housing and Diets. Outbred, male Wistar rats (Hsd/Cpb:WU, Harlan, Zeist, The Netherlands) aged 3 weeks (Expt 1) or 6 weeks (Expt 2) were used. The rats had been fed a commercial, pelleted diet (SRMA^R, Hope Farms, Woerden, The Netherlands) and tap water *ad libitum.*

Expt 1 (^{64}Cu study). On arrival, the rats were housed in groups of 4 animals in polycarbonate cages (37.5*22.5*15.0 cm) with inlaid wire mesh floors with filter paper underneath. After a pre-experimental period of 10 d (day 0 of the experiment), during which they received a purified diet (Cu-adequate, control diet) containing 5 mg Cu/kg, the rats were divided into 2 groups of 6 rats each, which were stratified for body weight. One group remained on the control diet, and another group was transferred to a diet containing 1 mg Cu/kg (Cu-deficient). The composition of the purified diets has been described [Van den Berg & Beynen, 1992]. The rats were housed in metabolism cages (Tecniplast Gazzada, Buguggiata, Italy). The experiment lasted 35 d.

Expt 2 (^{28}Mg study). On arrival, the rats were housed as described above, and had *ad libitum* access to the commercial diet and tap water. After 2 d (day 0) the rats were

housed individually in metabolic cages (Tecniplast Gazzada, Buguggiata, Italy), and were transferred to a purified diet and demineralized water for another 35 d. The composition of the diet has been described [Bergstra et al., 1993].

Apart from the Cu concentration (Expt 1), the powdered diets were formulated according to the nutrient requirements of rats [National Research Council, 1978], and stored at 4 °C until feeding. Food and demineralized water were provided *ad libitum.* The cages were placed in randomized position in a room with controlled temperature (20–22 °C), relative humidity (40–65%) and light cycle (light, 6.00–18.00 h). Feed consumption and body weight were recorded weekly.

Experimental design radioisotope study. ^{64}Cu administrations (Expt 1) were performed on days 21 and 28, after overnight fasting. Half of the animals in each dietary group received ^{64}Cu in the extrinsically labeled meal. The remaining three animals of each group were injected with the radioisotope intraperitoneally. On day 28, the route of administration of radiotracer for each animal was alternated. The radioactive meals were prepared by adding 5 μg radiolabeled Cu in 100 μl acetate buffer (0.05 mol/L, pH 5.4) to 2 g of the experimental diet. For intraperitoneal administration, 100 μl of radioisotope solution, also in acetate buffer, was injected.

^{28}Mg administrations (Expt 2) were performed on days 22 and 29. On day 22, after overnight fasting, five animals received [^{28}Mg]$MgCl_2$ with an extrinsically labeled meal. The remaining four animals were injected with the radioisotope intraperitoneally. On day 29, the route of administration of radioisotope for each animal was alternated. The radioactive meals were prepared by adding 100 μl of 163 mmol/L [^{28}Mg]$MgCl_2$ in distilled water to 2 g of the experimental diet. For intraperitoneal administration, 100 μl of radioisotope solution, also in distilled water, was injected.

To equalize handling and treatment of each rat, the rats receiving the radioisotope orally were injected intraperitoneally with distilled water or phosphate buffered saline and the rats that were injected with the radioisotope were given the meal without the radioisotope. On the days of radioisotope administration, treatment order of the rats was randomized. The meals without or with radioisotope were presented to the rats after a 16 h fast. The meals were consumed within 5 min. Subsequently, the intraperitoneal injection with and without radioistope, respectively, was given. Radioactivity in individual rats was counted in the whole-body counter within 5 min after administration of the radioisotope. Thereafter, the animals received their normal diets. For another 4 d, the animals were counted every 8 h. All animals were also measured one day before the second administration of the radioisotope; whole-body activity was found not to differ from background measurements.

Calculation. Apparent absorption was calculated according to the conventional balance method, viz. intake minus faecal excretion and expressed as percentage of intake.

True absorption was calculated according to Heth & Hoekstra (1965) and Becker & Hoekstra (1967). Countings (corrected for background and radioisotope decay) were expressed as percentage of administered dose. Plots of the logarithm of the percentage radioactivity retention after intraperitoneal and oral administration of the radioisotope versus time were constructed. The zero-time intercepts were determined by extrapolating the linear parts of the curves to t= 0 (intersection with the Y-axis). Ideally the slope of the terminal component after oral administration will parallel the slope of the curve after intraperitoneal administration, indicating that at that time interval the radioisotope is handled independently of the route of administration. Percentage true absorption was calculated by dividing the intercept of the retention curve for the orally administered radioisotope by that of the retention curve for intraperitoneally administered radioisotope and multiplying by 100. This calculation was executed for each animal.

The absolute amount of true copper absorption was calculated by multiplying intake and percentage absorption. Faecal excretion of endogenous losses was calculated as absolute true absorption minus absolute apparent absorption.

If we consider the whole-body as a single compartment system, after clearance of the radioactive faeces from the intestine, the decrease of the amount of radioisotope (corrected for decay) can be described by an exponential equation of the form:

$$\log\% \text{ dose retained at time t, } R_t = R_0 .10^{-mt}.$$

The biological half-life (T_b) can be calculated as: $T_b = \log 2/m$, in which m is the slope of the linear part of the curve.

Collection of samples. In Expt 1, during d 21–35 faeces of each rat was collected quantitatively, concurrently after the administration of ^{64}Cu for 5 days, and used for total Cu determination. In Expt 2, from d 22-26 and d 29–33, urine and faeces of each rat were collected quantitatively, and used for total Mg determination.

Chemical analysis. Faeces was freeze-dried overnight, homogenized and weighed. A sample of 150 mg was taken and ashed at 500 °C for 17 h and dissolved in 6 mol/L HCl. Feed samples were processed in the same way. Magnesium was determined in feed samples, plasma and faeces in the presence of 41 mmol/L lanthanum chloride with a Varian Atomic Absorption Spectrophotometer (AAS) type AA-475. Copper was also determined by AAS.

Statistical analyses. Within the dietary groups in Expt's 1 and 2, the results of radioisotope administration on day 21 versus 28 (Expt 1) or on day 22 versus 29 (Expt 2),

including the slopes of the retention curves for the same administration route, were not significantly different (Student's t-test), and thus the data were pooled. The same held for the results of the two balance periods in Expt 1 and 2. All values are presented as means ± SEM. Differences between group means were evaluated with Student's t-test as the data were normally distributed (Kolmogorov-Smirnov test). The level of significance was preset at $p < 0.05$. Data were analyzed by computer using the SPSS/PC$^+$ statistical package [SPSS, Inc, 1988].

RESULTS AND DISCUSSION

Effects of radioactivity distribution on counting efficiency. The variation of the response of the whole-body counter to animals of various sizes and shapes was assessed by the use of phantoms (polyethylene bottles filled with water), having the same amount of radioactivity but different volumes: 20 mL (imitating roughly the distribution of an intraperitoneally or orally dose immediately after administration), 50 mL, 100 mL and 250 mL (simulating roughly the size of the rats under study), respectively, for a number of radioisotopes with different gamma energies: ^{64}Cu, ^{65}Zn, ^{59}Fe and ^{28}Mg (Table 2a-d).

These results show that the variations due to the factors imitated (body size, inhomogenity) are small, although depending on the emitted gamma-ray energies. Initial measurements (after administration, $t = 0$) and measurements at later time points (assuming homogeneous distribution) in a 250-g rat may differ up to 2.5% (cf. Fig. 2). Thus, a phantom of 250 mL as an approach for the whole animal seems to be useful within an error margin of a few percent.

Now, to investigate in more detail the effect of a nonhomogeneous distribution of the radioactivity as a "spot" geometry, count rates were measured with a ^{64}Cu point source along the vertical axis within a phantom filled with 250 mL water; the points were in the phantom at heights of 2.5 cm, 7.5 cm and 12.5 cm from the bottom of the well (Fig. 3). Count rates were also measured in horizontal direction. When the ^{64}Cu source was uniformly distributed in a water phantom of 250 mL, the count rate was only 2.1% less than the count rate obtained for a point source of equal activity at the centre of the well (Fig. 2; Fig. 3a, viz. reference point 2), and corresponds with earlier results for a 20 mL geometry (cf. Table 2a). The variations in response for other positions in the same horizontal plane were very small (Fig. 3a). The variation in response was greatest for source movements in the vertical direction (i.e. corresponding to the anterior-posterior axis of the animal), resulting in a maximum difference of about 10% for ^{64}Cu between highest and lowest position (Fig. 3a).

Assuming a nonhomogeneous distribution of ^{64}Cu and all radioactivity as a point source in the animal, for example in the head or in the tail, this would result in an error of –5.6% and +3.7% respectively compared to a homogeneous distribution over 250 mL (Fig. 3a).

Table 2a. *Geometrical counting response for ^{64}Cu.*

Height of phantom centre relative to the centre of the well[2] (cm)	Relative Counting Efficiency[1]			
	20 mL	50 mL	100 mL	250 mL
4	96.3			
3	98.1	97.5		
2	99.5	99.9	99.9	
1	101.0	100.6	100.1	
0	102.6	102.0	101.5	100
–1	103.7	104.3	104.1	
–2	104.8	105.6	106.6	
–3	106.3	107.4		
–4	107.7			

1 Counts expressed as a percentage of the counts obtained relative to a 250 mL water phantom.

2 Centre position of the well is the point along the central axis at 5 cm above the bottom of the rat holder (7.5 cm above the bottom of the well), representing the centre of the animal of about 250 g.

Table 2b. *Geometrical counting response for ^{28}Mg.*

Height of phantom centre relative to the centre of the well[2] (cm)	Relative Counting Efficiency[1]			
	20 mL	50 mL	100 mL	250 mL
4	97.4			
3	98.8	99.9		
2	99.5	100.4	100.2	
1	100.5	100.6	101.1	
0	101.2	101.1	100.4	100
–1	101.4	101.8	102.2	
–2	102.8	103.2	103.1	
–3	103.1	103.7		
–4	103.7			

1 Counts expressed as a percentage of the counts obtained relative to a 250 mL water phantom.

2 Centre position of the well of the point along the central axis at 5 cm above the bottom of the rat holder (7.5 cm above the bottom of the well), representing the centre of the animal of about 250 g.

Table 2c. *Geometrical counting response for ^{59}Fe.*

Height of phantom centre relative to the centre of the well[2] (cm)	Relative Counting Efficiency[1]			
	20 mL	50 mL	100 mL	250 mL
4	94.9			
3	96.6	96.4		
2	97.5	97.5	97.7	
1	98.9	99.0	98.6	
0	100.3	100.3	100.1	100
–1	101.6	101.6	101.6	
–2	102.1	102.4	102.1	
–3	103.7	103.2		
–4	104.5			

[1] Counts expressed as a percentage of the counts obtained relative to a 250 mL water phantom.

[2] Centre position of the well is the point along the central axis at 5 cm above the bottom of the rat holder (7.5 cm above the bottom of the well), representing the centre of the animal of about 250 g.

Table 2d. *Geometrical counting response for ^{65}Zn.*

Height of phantom centre relative to the centre of the well[2] (cm)	Relative Counting Efficiency[1]			
	20 mL	50 mL	100 mL	250 mL
4	96.7			
3	98.0	97.4		
2	99.2	99.1	98.8	
1	100.4	100.7	100.0	
0	102.0	101.0	100.4	100
–1	103.0	103.3	103.1	
–2	103.5	104.3	103.6	
–3	103.9	105.0		
–4	104.1			

[1] Counts expressed as a percentage of the counts obtained relative to a 250 mL water phantom.

[2] Centre position of the well is the point along the central axis at 5 cm above the bottom of the rat holder (7.5 cm above the bottom of the well), representing the centre of the animal of about 250 g.

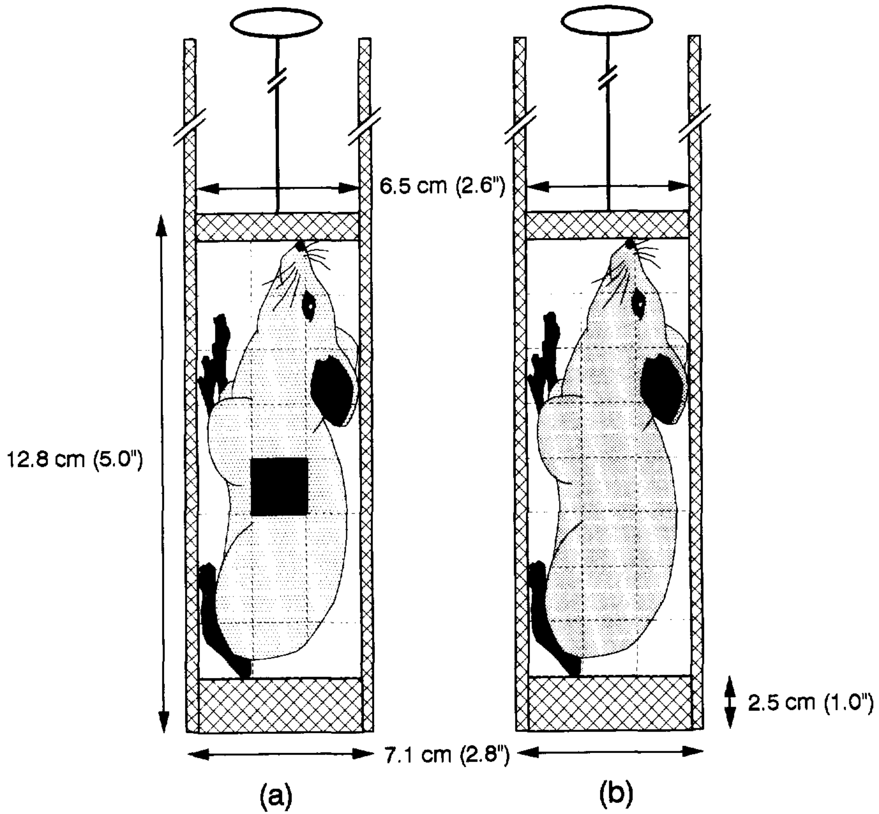
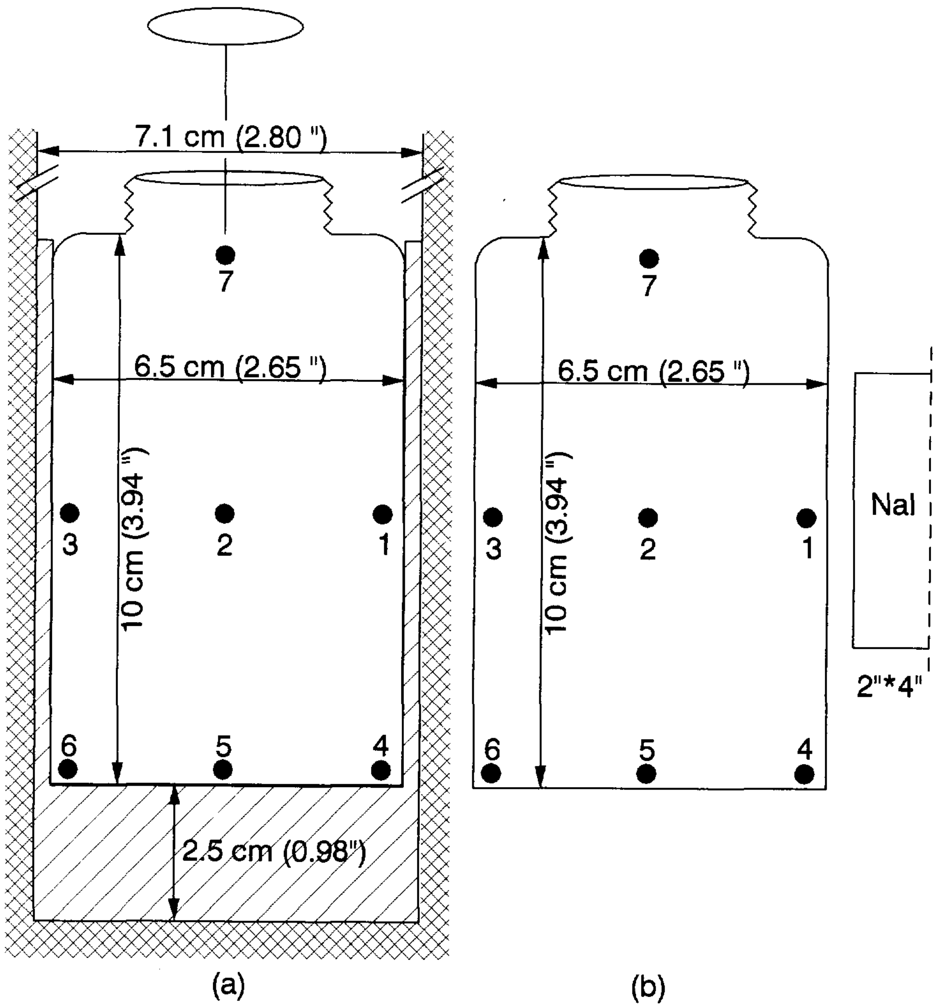


Figure 2. *Rat (weighing about 250 g) in rat holder (dimensions: width 6.5 cm (inside), adjustable length between 10–20 cm) and schematic radioactivity distribution (a) initially, "spot" (t= 0) after oral or intraperitoneal administration, or (b) a few hours after administration and lateron during retention measurements, "homogeneous". Centre of the animal along axis at 5.0 cm above bottom of holder, viz. 7.5 cm above bottom of the well.*

However, head and tail comprise relatively few mass [Owen, 1964; Owen, 1965], and moreover do not generally concentrate trace elements and minerals. Thus, the maximum error due to inhomogenity of radioactivity in a rat is only a few percent at most.

Other sources of error. Two other main sources of errors in whole-body counting are counting statististics and contaminating radionuclides. Errors due to counting statistics are determined by the efficiency of the counter and the background count rate. Due to the relatively short physical half-life of the radioisotopes ^{64}Cu and ^{28}Mg and the relatively rapid loss of the radioisotope from the body (biological half-life), it is not opportune to measure animals 4-5 days beyond administration, especially for ^{64}Cu. The dose of ^{64}Cu (1.5 MBq) was the largest dose that did not swamp the detector directly after administration and was just



reference (250 ml phantom)	100%
points 1,2,3	102.1%
points 4,5,6	103.7%
point 7	94.4%

reference (250 ml phantom)	100%
point 1	311%
point 2	100%
point 3	41%
points 5,7	66%

Figure 3. *Variation in counting response of the whole-body counter for a point source of ^{64}Cu along the vertical axis (3a: points 2, 5 and 7) within a phantom filled with 250 water and in horizontal directions (3a: points 1, 2 and 3; and points 4, 5 and 6), compared with (b) calculated counting response using a NaI(Tl) detector.*

detectable at about twice background levels 4 days after administration (counting time, 300 seconds). For ^{65}Zn and ^{59}Fe initial doses are up to a factor 100 less (Table 1) to conduct absorption and retention studies.

Radiation from possible "contaminating" radionuclides, for example ^{64}Cu in a ^{28}Mg preparation [MJC Bijvelds, personal communication], or "daughters", for example ^{47}Sc (a ^{47}Ca daughter, cf. Welch and House, 1980) in the body cannot be discriminated by the whole-body counter, thus leading to erroneous results. It is therefore recommended to carry out an additional qualitative measurement with a high-resolution gamma detector to verify radionuclidic purity.

When gamma-energy resolution is required in the whole-body counting of the animal (e.g., when dealing with impure radionuclides, or performing a measurement for two elements using two radioisotopes at the same time), NaI(Tl) detectors or even better HPGe/Ge(Li) detectors should be used. This has been reported by others. However, when only one detector is used [cf. McElroy et al., 1991], the counting result is strongly dependent on the geometry of the animal and the activity distribution in it. This is demonstrated in Figure 3b for the geometry of the activity spots in the 250 mL phantom and a 2×4 inch NaI(Tl) scintillation detector. Using the Monte Carlo model of Overwater [RMW Overwater, personal communication], differences of a factor 5 to 7 were found. This variability may be reduced by using several detectors approaching a 4π geometry, or by a large well-type detector. However, both solutions are rather expensive, particularly when dealing with HPGe/Ge(Li) detectors. Another solution is to stick to one detector, but to reduce the differences in measurement geometry for the various spots in the animal. This can be done by rotating the animal or the detector in respect to the anterior-posterior axis of the animal and at the same time moving the animal or the detector along the anterior-posterior axis parallel with detector surface. When in addition a collimated detector is used, so that any moment only a slice (perpendicular to the anterior-posterior axis) of the animal is seen by the detector, the variability may further be reduced. However, this solution leads to a considerable loss of the detection efficiency and increased technical complexibility.

Application of the method. *Expt 1 (^{64}Cu study).* The average body weight of the rats at the end of the study was 270 ± 20 g (mean $\pm$ SD, n = 12), being not different for the two treatment groups. Whole-body retentions of ^{64}Cu after injection and oral administration are shown in Figure 4. Early in the experiment (zero to about 50 hours post-administration) there was a rapid loss of whole-body ^{64}Cu after oral administration. Analyses of excreta showed that this rapid loss is largely via faeces and is thus related to passage of unabsorbed ^{64}Cu. After 50 hours, the loss of ^{64}Cu was at a much slower rate representing excretion (endogenous losses) of ^{64}Cu which had been absorbed from the intestine into the body.

Table 3. *Apparent absorption and true absorption of copper and loss of endogenous copper in rats fed either a copper-adequate or copper-deficient diet*[1].

	Cu-adequate 5.2 Cu mg/kg diet	Cu-deficient 1.1 Cu mg/kg diet
Intake, ug/d	88 ± 3	17 ± 1*
Apparent absorption, %	31 ± 2	48 ± 3*
True absorption, %	56 ± 10	70 ± 5*
Total faecal loss, μg/d	60 ± 3	9 ± 2*
Endogenous loss, μg/d	22 ± 8	3 ± 1*

[1] Means ± SEM for five rats per dietary group.

* Significant difference ($p < 0.05$) versus control.

For the control rats, whole-body ^{64}Cu turnover was not different after oral or intraperitoneal administration. ^{64}Cu retentions plotted on a semi-log scale against time gave straight lines between 50–100 hours post-administration; the slopes of the linear lines were not different, i.e. $m = -(0.0022 \pm 0.0007)\ h^{-1}$, $r = -0.981$ ($n = 10$); biological half-life ($T_{1/2}$ = –log2/slope) of 5.7 ± 1.9 days. This value is comparable with reported values after injection of ^{67}Cu [Lindner & Roboz, 1986], and very similar to values obtained in our earlier studies [Van den Berg et al., 1991; Van den Berg & Beynen, 1992].

^{64}Cu retention curves for control rats revealed higher values for true than for apparent Cu absorption (Table 3). This is because endogenous Cu excretion made up a considerable amount of the total faecal Cu excretion (Table 3) in rats fed Cu-adequate diets.

Rats fed on a low Cu-diet retained more of the radioactivity administered when compared to animals fed the Cu-adequate diet. Cu-deficient rats had significantly higher apparent and true copper absorption (Table 3). Endogenous copper losses were significantly lower compared to animals fed on the Cu-adequate diet (18% vs 25% of the intake). This result is very similar to recently reported values using an isotope dilution method [Johnson and Lee, 1988]. Thus, the body accomodates to a low Cu intake by enhancing the efficiency of Cu absorption and reducing biliary Cu excretion [Owen & Hazelrig 1968; Van den Berg & Beynen, 1992; Van den Berg et al., 1993], so that Cu homeostasis can be better approached. Cu-deficient rats had a significantly increased biological half-life, 12.0 ± 3.9 days versus 5.7 ± 1.9 days ($n = 5$ per dietary group). Therefore, whole-body counting also provides a method to assess the Cu status of the animal as indicated by its biological half-life.

Apparent copper absorption determined by whole-body retention of ^{64}Cu (viz. after orally administration) almost equals the true copper absorption as measured with the whole-body

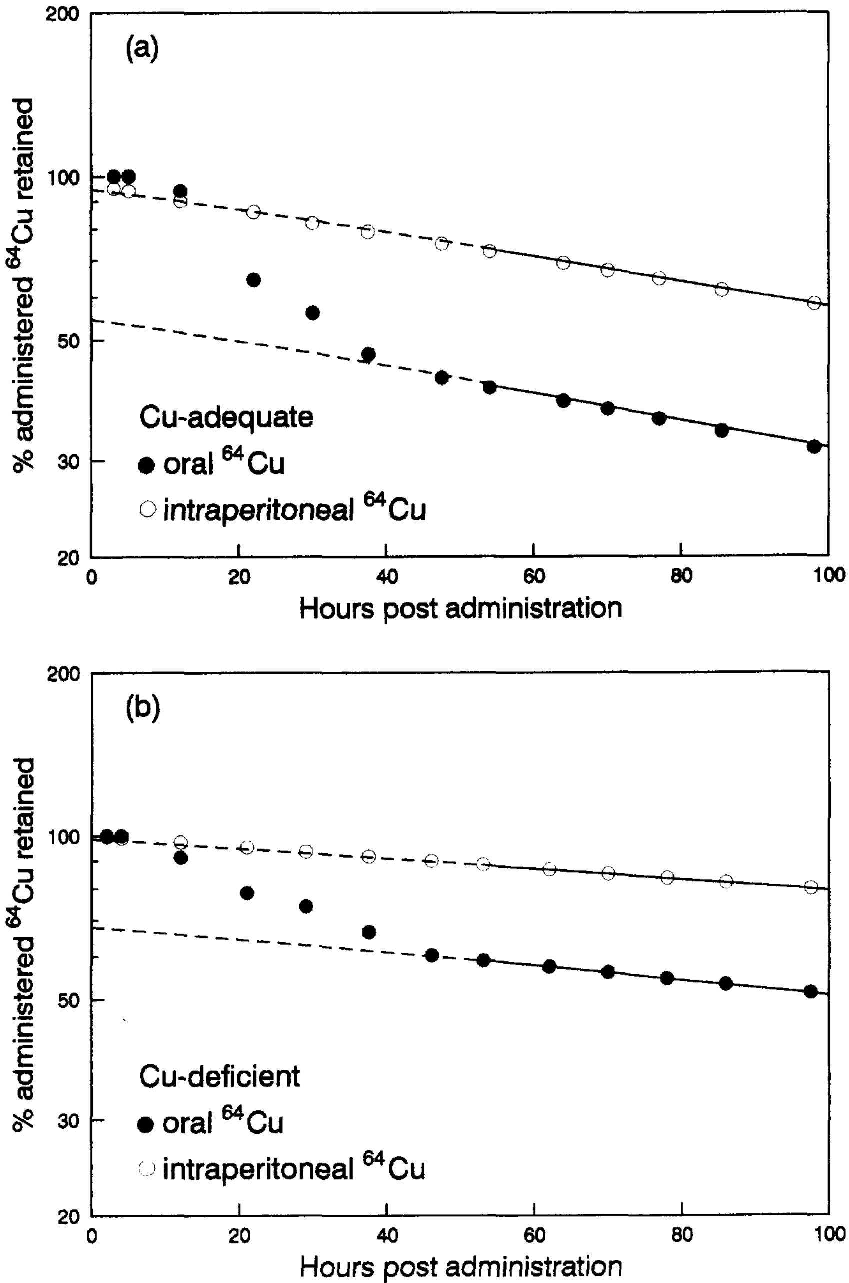


Figure 4. *Retention curves for radioactivity in rats fed a Cu-adequate diet (a) or Cu-deficient diet (b) for 5 weeks after oral and intraperitoneal administration of ^{64}Cu. Data are presented as the mean for 5 rats per dietary group. Cu-adequate diet, oral: log y = −0.0021x + 1.73; intraperitoneal: log y = −0.0022x + 1.98. Cu-deficient diet, oral: log y = −0.0013x + 1.85; intraperitoneal: log y = −0.0009x + 2.00 (y = % retention, x = time in h).*

Table 4. *Apparent- and true absorption of magnesium and loss of endogenous magnesium in rats fed a purified diet*[1].

Intake, mg/d	5.2 ± 0.1
Apparent absorption, %	36 ± 5
True absorption, %	67 ± 2
Total faecal loss, mg/d	3.2 ± 0.6
Endogenous loss, mg/d	1.8 ± 0.2

[1] Means ± SEM for nine rats per dietary group.

method (viz. after orally and intraperitoneally gifts). An explanation of this phenomenon is discussed in the Appendix.

Expt 2 (^{28}Mg study). Absorption and retention studies for magnesium are scarce, in part resulting from lack of the availability of adequate amounts of ^{28}Mg with sufficient specific activity. With the use of ^{28}Mg, made available by the Interfaculty Reactor Institute and the whole-body counting method we explored magnesium absorption and excretion, i.e., to quantify magnesium from endogenous origin. It is known that a significant portion of faecal magnesium is of endogenous origin [Brink & Beynen, 1992], and therefore apparent absorption will differ from true absorption of magnesium [Brink et al. 1992]. We compared apparent absorption and true absorption of magnesium in rats fed on a purified diet. The final average body weight of the rats in this study was 289 ± 16 g (mean ± SD, n = 9).

The semi-logarithmic retention curves after orally and intraperitoneally administered ^{28}Mg are shown in Figure 5. The true Mg absorption was significantly higher than the measured apparent absorption (Table 4). This study also indicates that apparent absorption does not necessarily reflect true absorption of Mg depending on influences on endogenous excretion [Brink et al., 1992].

CONCLUSIONS

The absorption and retention of dietary trace elements and minerals can be determined by a simple radiotracer technique when suitable radioisotopes and an adequate whole-body counter are available. The whole-body counter described here requires rather unsophisticated instrumentation and is simple to use. The whole-body counter has the following proporties: an approximation of a 4π counting geometry and a well (25 cm deep and 7.1 cm wide) allowing measurements of animals (dimensions: 6.5 cm width and about 10 cm height; about 250 mL volume) with a maximum error due to inhomogeneous radioactivity distribution of only a few percent.

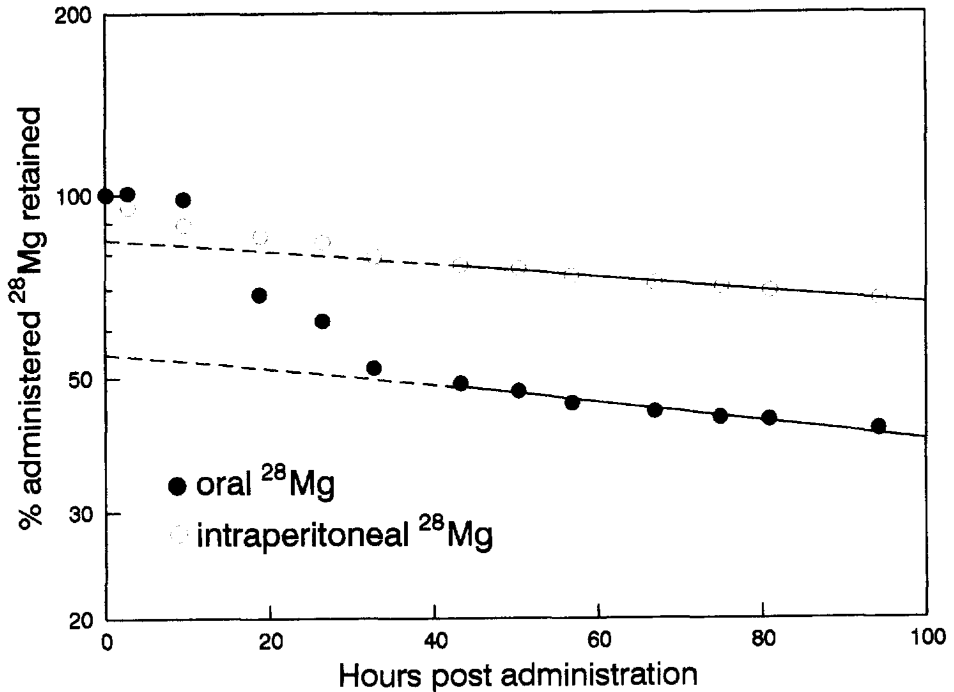


Figure 5. *Retention curves for radioactivity in rats fed a purified diet after oral and intraperitoneal administration of ^{28}Mg. Data are presented as the mean for 9 rats. Linear regression equations for each curve were established for 7 time points beyond 33 h post-administration. Intraperitoneal: log y= –0.0012x + 1.93; oral: log y= –0.0016x + 1.76 (y= % retention, x = time in h).*

For the true absorption measurement a correction for faecal losses of endogenous origin is applied by comparison of the retention of orally and intraperitoneally administered radioisotopes. Because apparent absorption does not always reflect true absorption, combined measurement of true absorption and apparent absorption gives a better knowledge of minimum dietary requirements.

The radioactivity counting method described is non-invasive and allows measurements of true absorption in one animal. Thus, the whole-body counting technique contributes to a reduction in the use of animals.

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APPENDIX

The conventional balance method gives the apparent absorption of trace elements and minerals calculated as intake minus total faecal excretion and is identical to true absorption minus the endogenous faecal excretion. The apparent absorption as measured in this study with the radiotracer method (determined by extrapolating the linear part of the oral retention curve to t = o, viz. intersection with Y-axis) shows to be significantly higher compared to the result for the conventaial balance method, and approaches almost the true absorption. For instance, for the Cu-adequate and the Cu-deficient rats the average apparent absorption via the convential balance method was 31% and 48% respectively, the apparent absorption via the radiotracer method was 54% and 71% respectively, and the true absorption via the radiotracer method was 56% and 70% respectively.

This phenomenon that the apparent absorption as measured with the radiotracer method almost equals the true absorption as measured with the same method, can be explained as follows. The radioactive copper absorbed will exchange with non-radioactive copper in the readily exchangeable copper pool in the rat, thus lowering the specific activity. Assuming a steady-state situation in a Cu-adequate rat (no change in exchangeable copper pool size, e.g., due to growth), an urinary excretion of 5% of the intake (viz. 0.05 x 88 μg Cu/day= 4.4 μg Cu/day), an endogenous loss of 22 μg Cu/day, and a half-life for the administered ^{64}Cu of 5.9 days, then a pool size of 224 μg exchangeable Cu may be derived. This is substantially larger than the amount of radioactive copper absorbed from the intestine, being 56% of 2 grams of diet (test-dose) with a level of 5.1 mg Cu/kg diet = 5.7 μg of radioactively labeled copper vs 224 μg Cu in the total copper pool. Thus, in the endogenous loss the radioactivity is reduced by a factor of 39 to a neglectible level. This implies that the apparent absorption measured with the radiotracer method essentially represents true absorption.

Coincident with the similarity of true and apparent absorption measured is the fact that the intraperitoneal lines intersect the vertical axis at almost 100% (for the Cu-adequate and Cu-deficient rats at 100% and at 96% respectively), indicating that within the error margins and the limited time interval studied, the exchangeable copper pool in the rats studied may be described with only a single compartment.

From the actual measurements and calculations above, it follows that for the assessment of true copper absorption in rats the intraperitoneal injection with ^{64}Cu is not strictly necessary. With the use of an oral dose of ^{64}Cu alone a reasonably accurate determination of true copper absorption can be carried out.

4

ASCORBIC ACID FEEDING OF RATS REDUCES COPPER ABSORPTION CAUSING IMPAIRED COPPER STATUS AND BILIARY COPPER EXCRETION

ABSTRACT

The feeding of diets enriched with ascorbic acid (10 g/kg) to rats has previously been shown to lower plasma and liver copper concentrations. The present studies corroborate this. We hypothesized that ascorbic acid initially reduces copper absorption, this effect being masked later by the stimulatory effect on copper absorption of the impaired copper status. We also hypothesized that the impaired copper status as induced by ascorbic acid feeding is followed by a diminished biliary excretion of copper in an attempt to preserve copper homeostasis. Both hypotheses are supported by the present studies. Ascorbic acid feeding initially reduced apparent copper absorption, and in the course of the experiment this effect tended to turn over into a stimulatory effect. Copper deficiency, as induced by feeding a diet containing 1 mg Cu/kg instead of 5 mg Cu/kg, systematically increased copper absorption. Biliary excretion of copper in rats given ascorbic acid was unaffected initially, but became depressed after prolonged ascorbic acid feeding. A similar time course was seen for fecal endogenous copper excretion which was calculated as the difference between true and apparent copper absorption. Copper deficiency systematically reduced biliary copper excretion and fecal endogenous copper loss.

Biological Trace Element Research in press

INTRODUCTION

High amounts of ascorbic acid in the diet reduce plasma and tissue copper concentrations in rats (1), which is most likely due to a reduction of copper absorption (1–5). However, in rats fed ascorbic acid we could not demonstrate a reduced efficiency of true copper absorption (1). We speculated that the ascorbate-induced lowering of copper status had masked the reduction in copper absorption (1) because copper deficiency, as produced by the feeding of a diet deficient in copper, stimulates the efficiency of copper absorption (1,6,7). Thus, in the present studies with rats we addressed the question whether dietary ascorbic acid influences true copper absorption differently after short- versus long-term challenge. The feeding of ascorbic acid for a shorter period, which may not depress copper status to a large extent, is expected to lower true copper absorption. After a long-term feeding period, ascorbic acid will lower copper status significantly which in turn enhances copper absorption so that the direct effect of ascorbic acid on copper absorption is diminished or even becomes masked. We determined true copper absorption with a whole-body counting technique (8) and ^{64}Cu administered either orally or intraperitoneally. From the two retention curves of the radiocopper, true copper absorption was calculated according to the method of Heth and Hoekstra (9).

Diminished copper status as induced by a copper-deficient diet has been shown to be associated with low rates of biliary copper excretion (10). Earlier we hypothesized that biliary excretion of copper is depressed in rats fed on ascorbic acid, this effect being secondary to the ascorbate-induced lowered tissue copper concentrations (1). To test our hypothesis, biliary copper excretion was determined in rats given ascorbic acid by collection of bile using a bile duct cannulation while the rats were under anesthesia. If the effect of ascorbic acid on biliary copper excretion is secondary to the reduced copper status, then the effect should be greater after long-term than after short-term feeding of ascorbic acid. Thus, the time course of biliary copper excretion was measured to check this reasoning.

MATERIALS AND METHODS

The experimental protocols were approved by the animal welfare officer of the Wageningen Agricultural University and the animal experiments committee of the Rotterdam Erasmus University, The Netherlands, the latter also serving for the Delft University of Technology.

Experimental designs. Two experiments were performed concurrently. Each experiment had three dietary groups. We used purified diets with an adequate amount of copper (5 mg Cu/kg) without or with added ascorbic acid (10 g/kg) or a diet that was deficient in copper (1 mg Cu/kg). The latter diet was incorporated into the experimental design for comparison and to assess which effects of supplemental ascorbic acid could be

secondary to a reduced copper status. Except for the variable components, the diets were formulated according to the nutrient requirements of rats (11). The purified diets, which were in powdered form, were stored at 4 °C until feeding. The concentration of ascorbic acid was checked before and after each study. The purified diets, as well as demineralized water, were provided *ad libitum.* Feed consumption and body weights were recorded.

For Expt 1, male Wistar rats (Hsd/Cpb:WU), aged 3 wk, were purchased from Harlan, Zeist, The Netherlands. For Expt 2, outbred, male Wistar rats (Cpb:WU), aged about 7 wk, were derived from the colony of the Laboratory Animals Center of the Wageningen Agricultural University. The rats had been fed a commercial, pelleted diet (RMH-B, Hope Farms, Woerden, The Netherlands) and tap water *ad libitum.*

Experiment 1. In this experiment true copper absorption was measured. On arrival, the rats were housed in groups of 4 animals in polycarbonate cages (37.5*22.5*15.0 cm) with inlaid wire mesh bases above filter paper. After a pre-experimental period of 10 d (day 0 of the experiment), during which they received the copper-sufficient, purified diet, the rats were divided into 3 groups of 18 rats, which were stratified for body weight. Each group was assigned randomly to one of the three diets (Table 1). As from d 0, 6 rats of each dietary group were housed individually in metabolism cages (314 cm^2 * 12 cm). The cages were placed in randomized position in racks in a room with controlled temperature (20–22 °C), relative humidity (40–65%) and light cycle (light, 06:00–18:00 h). Six other rats of each dietary group were transferred to metabolism cages on day 14 and the remaining animals on day 35.

Experiment 2. In this experiment biliary excretion of copper was measured. All rats went through a pre-experimental period of 10 d, during which they received the control diet containing 5 mg Cu/kg. They were individually housed in metabolism cages. At the end of the pre-experimental period (day 0 of the experiment) from 6 randomly chosen rats the bile duct was cannulated. The remaining animals were divided into 3 groups of 24 rats each so that body weight distributions of the groups were similar. Each group was randomly assigned to one of the purified diets given in Table 1.

True copper absorption. ^{64}Cu was obtained by irradiating a copper 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. Following irradiation, the wire was dissolved in 25 µl of nitric acid (17 mol/L) and diluted with sodium acetate buffer (0.05 mol/L, pH 5.4), resulting in a final copper concentration of 1 mg/mL. The specific activity of the ^{64}Cu solution at the start of the experiments was about 300 GBq ^{64}Cu/g Cu.

Table 1. *Composition of the purified diets used.*

Copper[1] (mg/kg)	5.0	5.0	1.0
Ascorbic acid[1] (g/kg)	–	10.0	–
Components (kg diet)			
Glucose, g	702.6	692.6	702.6
Ascorbic acid, g	–	10.0	–
$CuSO_4.5H_2O$, mg	15.7	15.7	–
Constant components[2], g	297.4	297.4	297.4
Chemical analysis			
Cu[3] (mg/kg)	5.2	5.2	1.1
Ascorbate[3] (g/kg)	–	10.0	–

[1] Calculated values.

[2] 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-a-tocopheryl acetate 60, retinyl acetate and retinyl palmitate 8 (4000 IU), cholecalciferol 2 (1000 IU), maize meal 9826.15.

[3] Average values of four measurements.

^{64}Cu administrations in Expt 1 were performed after overnight fasting of the rats on days 0 and 7, d 21 and 28, d 42 and 49. On day 0, 21 and 42, 3 animals of each dietary group received ^{64}Cu with an extrinsically labeled meal. The remaining three animals of each group were injected with the radiotracer intraperitoneally. On days 7, 28 and 49 the route of administration of radiotracer for each animal was alternated. On the days of radiotracer administration, treatment order of rats was randomized.

The radioactive meals were prepared by adding 5 μg radiolabeled Cu (1.5 MBq) in 100 μl acetate buffer to 2 g of experimental diet. For intraperitoneal administration, also 100 μl of radiotracer solution was injected. The meals were presented to the rats after a 18 h fast and consumed within 5 min. After the first measurement in the whole-body counter all rats received their normal diets.

Radioactivity in the rats was determined in a small animal whole-body counter (8,12) immediately after administration of ^{64}Cu, and at regular intervals for another 96 h. The overall efficiency of this counter for ^{64}Cu was 14%, and its stability was monitored by counting a ^{65}Zn source.

On days 14, 35 and 56, six animals of each dietary group were killed by exposure to CO_2. Blood samples were taken by aortic puncture. The liver was excised and weighed, and frozen at –20 °C until analysis. During d 0–14, d 21–35, and d 42–56, feces of these rats had been collected quantitatively.

Bile cannulation. Bile cannulations in Expt 2 were executed in six rats at baseline (day 0) and in six rats of each dietary group on days 7, 14, 28 and 56. Feces of these rats had been collected quantitatively from d (–7) to d 0, d 0–7, d 7–14, d 21–28, and d 48–55. Bile collections were performed between 9:00 h and 13:00 h to prevent effects of circadian variations in bile flow (13). The rats were anesthetized with ketamine (60 mg/kg) and xylazine (8 mg/kg) administered i.m. (14). Polyethylene tubing (PE-10, Clay Adams, USA) was inserted into the common bile duct via a small incision and ligated. Body temperature was maintained at 36–38 °C by placing the rats on an electric heating pad. Bile samples were collected into preweighed vials for a period of 60 min. Bile production was determined gravimetrically, assuming a density of 1.0 g/ml for the bile fluid. At the end of the bile collection blood samples were taken by abdominal aorta puncture, and the rats were killed by decapitation. The liver was excised and weighed, and frozen at –20 °C until further use.

Chemical analyses. 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 Sunderman & Nomoto (15).

Feces were freeze-dried, ashed at 500 °C 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 treated as described for feces. Copper analyses in feed samples, plasma and liver were performed by flame atomic absorption spectrometry with the use of a Varian AA-475 (Varian Techtron, Springvale, Australia).

Copper in bile was measured directly by using flameless atomic absorption spectrometry with the autosampler Varian SpectrAA-300 (Varian Techtron).

Bovine liver (SRM #1577a, National Institute of Standards and Technology, Gaithersburg, MD, USA) was analyzed with each batch of samples. Liver standards were determined to contain copper concentrations that were on average 95% (n = 8) of the certified value. Precinorm (Boehringer, Mannheim, Germany) was analyzed along with the bile samples, and results equalled 90% (n = 3) of the certified value for copper.

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 (16). 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 (17). Ascorbate was then determined by a HPLC method applying pre-column derivatizaton and spectrofluorometry (18).

Calculations. Apparent absorption was calculated as copper intake minus fecal copper and expressed as percentage of intake.

True copper absorption was calculated according to Heth and Hoekstra (9). 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 ^{64}Cu administration versus time were constructed. The zero-time intercepts were determined by extrapolating the linear parts of the curves. Percentage true absorption was calculated by dividing the intercept of the retention curve for oral ^{64}Cu by that of the retention curve for intraperitoneal ^{64}Cu and multiplying by 100. This calculation was executed for each animal. Absolute amounts of true copper absorption was calculated by multiplying copper intake and percentage true copper absorption. Slopes of orally administered ^{64}Cu retention curves in some rats were steeper than those for the intraperitoneal ^{64}Cu dose. While blind to treatment modality, we selected rats that showed obstensibly identical slopes for the linear parts of the two retention curves and used their data to calculate true copper absorption.

Fecal excretion of endogenous copper was calculated as absolute true absorption minus absolute apparent absorption of copper.

Biological half-life, $T_{1/2}$, of ^{64}Cu was calculated for the injected rats using the slopes of the whole-body retention plots between 60 and 100 hours after dosing: $T_{1/2} = \log 2/\text{slope}$.

Statistical analyses. Data are presented as mean $\pm$ standard error of the mean. Differences between group means were evaluated for statistical significance with Student's t-test; the data were normally distributed as based on the Kolmogorov-Smirnov one-sample test. The level of significance was preset at $P < 0.05$. Data were analyzed by computer using the SPSS/PC$^+$ statistical package (20).

RESULTS

Experiment 1. The dietary treatments did not significantly affect feed intake and body weights of the rats; average weights (means $\pm$ SEM, n = 18) at the end of each true absorption measurement were 178 $\pm$ 7 g (day 14), 270 $\pm$ 4 g (day 35) and 308 $\pm$ 6 g (day 56).

Table 2. *Selected indices of Cu and ascorbate status for the rats in Expt 1*[1].

Dietary variables						
Cu (mg/kg)	Ascorbate (g/kg)	Days	Plasma ascorbate (µmol/L)	Plasma Cu (µg/mL)	Plasma ceruloplasmin (g/L)	Liver Cu (µg/g dry wt)
5	–	14	71 ± 10	0.93 ± 0.04	0.31 ± 0.01	13.8 ± 0.4
5	10	14	186 ± 22*	0.81 ± 0.07	0.25 ± 0.02*	12.1 ± 0.5
1	–	14	47 ± 6	0.07 ± 0.04*	0.03 ± 0.02*	8.3 ± 0.7
5	–	35	58 ± 4	0.96 ± 0.04	0.32 ± 0.02	12.2 ± 0.1
5	10	35	103 ± 7*	0.85 ± 0.02*	0.25 ± 0.01*	11.8 ± 0.3
1	–	35	28 ± 6	0.24 ± 0.10*	0.12 ± 0.04*	9.9 ± 0.5*
5	–	56	38 ± 6	0.96 ± 0.04	0.31 ± 0.01	12.7 ± 0.4
5	10	56	144 ± 11*	0.85 ± 0.03	0.24 ± 0.01*	11.7 ± 0.4
1	–	56	34 ± 9	0.61 ± 0.11*	0.18 ± 0.05*	10.8 ± 0.5*

[1] Means ± SEM for 6 rats per dietary group.

* Significant ($p < 0.05$) difference versus control group fed the copper-adequate diet without supplemental ascorbic acid.

The biochemical indices of copper and ascorbate status are given in Table 2. The copper-deficient diet significantly reduced plasma and liver copper concentrations and plasma ceruloplasmin levels, but the effect became lesser in the course of the experiment. Ascorbic acid loading markedly raised plasma ascorbate concentrations. The diet rich in ascorbic acid lowered group mean concentrations of plasma copper and ceruloplasmin and liver copper, but only the latter effect did not reach statistical significance.

Addition of ascorbic acid to the copper-adequate diet did not significantly affect apparent copper absorption whereas during copper deficiency apparent absorption was significantly increased (Table 3). True absorption of copper tended to be reduced by ascorbic acid feeding after 8 weeks but not after 2 and 5 weeks. Copper deficiency raised true copper absorption within two weeks after administration of the copper deficient diet, but the increase became gradually less lateron.

Endogenous fecal copper excretion had decreased substantially in rats fed the copper deficient diet. Supplemental ascorbic acid reduced endogenous copper excretion after 5 and 8 weeks but not after 2 weeks (Table 3).

The biological half-life of ^{64}Cu administered intraperitoneally for rats fed the copper adequate diets without or with ascorbic acid did not differ significantly (5.6 ± 0.4) days

Table 3. *Apparent and true copper absorption and fecal endogenous copper excretion in Expt I*[1].

Dietary variables				Cu absorption			Fecal endogenous
Cu (mg/kg)	Ascorbate (g/kg)	Days	Cu intake (µg/day)	Apparent (%)	True (%)	True (µg/day)	Cu (µg/day)
5	–	0–14	74 ± 3	32 ± 2	55 ± 5	41 ± 4	16 ± 3
5	10	0–14	75 ± 2	31 ± 3	53 ± 8	40 ± 6	16 ± 2
1	–	0–14	15 ± 1	46 ± 4*	72 ± 6*	11 ± 1*	3 ± 1*
5	–	21–35	88 ± 3	31 ± 2	56 ± 13	49 ± 10	22 ± 10
5	10	21–35	89 ± 3	41 ± 3	56 ± 7	50 ± 6	12 ± 4
1	–	21–35	17 ± 1	48 ± 3*	66 ± 3	11 ± 1*	3 ± 1*
5	–	42–56	80 ± 1	22 ± 3	52 ± 10	42 ± 8	24 ± 7
5	10	42–56	79 ± 2	27 ± 2	37 ± 7	29 ± 5	11 ± 3
1	–	42–56	16 ± 1	53 ± 3*	58 ± 7	9 ± 1*	3 ± 1*

[1] Means ± SEM for 3–6 rats per dietary group.

* Significant ($p < 0.05$) difference versus control group fed the copper-adequate diet without supplemental ascorbic acid.

versus (6.4 ± 0.4) days, means ± SEM, n = 18). The biological half-life was significantly increased (18.8 ± 1.1 days, means ± SEM, n = 18) in rats fed the low-copper diet. The values for the control rats agree with those reported by Lindner & Roboz (19), who also used the whole-body counting method, but they are somewhat greater than those reported by Johnson & Lee (6) on the basis of isotope dilution measurements.

Experiment 2. The three dietary treatments did not differently affect feed intake and body weights of the rats during the course of the experiment; the average weights (means ± SEM) were 148 ± 5 g (day 0; n = 6), 261 ± 3 g (day 7; n = 18), 284 ± 4 g (day 14; n = 18), 345 ± 6 g (day 28; n = 18) and 407 ± 9 g (day 56; n = 18).

The biochemical indices of copper status show that supplemental ascorbic acid significantly depressed ceruloplasmin levels as from day 14, and copper concentrations in liver and plasma after day 28 (Table 4). Copper deficiency resulted in lower plasma copper and ceruloplasmin levels and lower liver copper concentrations within one week.

Apparent copper absorption was significantly increased after 14 days in the copper-deficient rats (Table 4). After 7 days, rats fed on the ascorbic acid diet had a significantly lower apparent absorption of copper, but after 28 days it was significantly raised.

Table 4. *Apparent copper absorption and selected indices of copper status in Expt 2*[1].

Dietary variables							
Cu (mg/kg)	Ascorbate (g/kg)	Days	Plasma ascorbate (μmol/l)	Plasma Cu (μg/mL)	Plasma ceruloplasmin (g/L)	Liver Cu (μg/g dry wt)	Apparent Cu absorption (%)
5	–	7	64± 11	0.92± 0.02	0.35± 0.01	13.3±0.4	40±2
5	10	7	143± 17*	0.92± 0.02	0.33± 0.01	13.2±0.6	31±2*
1	–	7	49± 5	0.42± 0.09*	0.06± 0.03*	10.6±0.6*	35±5
5	–	14	67± 4	0.92± 0.04	0.30± 0.04	13.0±0.7	32±3
5	10	14	160± 16*	0.83± 0.09	0.24± 0.03*	11.7±0.5	29±2
1	–	14	53± 8	0.62± 0.08*	0.20± 0.04*	11.2±0.6*	51±8*
5	–	28	61± 3	1.02± 0.02	0.31± 0.01	13.9±0.4	28±2
5	10	28	151± 5*	0.93± 0.04*	0.19± 0.02*	12.1±0.3*	35±2*
1	–	28	58± 8	0.31± 0.11*	0.08± 0.04*	8.7±1.1*	45±4*
5	–	56	75± 4	1.06± 0.03	0.34± 0.01	14.8±0.5	28±1
5	10	56	162± 12*	0.92± 0.03*	0.26± 0.02*	12.4±0.4*	35±2*
1	–	56	57± 7	0.68± 0.13*	0.18± 0.04*	10.5±0.9*	44±4*

[1] Means ± SEM for 6 rats per dietary group.

* Significant ($p < 0.05$) difference versus control group fed the copper-adequate diet without supplemental ascorbic acid.

Table 5 shows that rats fed on ascorbic acid had a decreased biliary copper output after 14 days whereas in the copper-deficient rats there was a reduction from at least 7 days after beginning the restriction. No difference in bile flow between the three dietary groups (average rate: 0.30 ml/100 g body weight) was observed.

DISCUSSION

The present studies corroborate earlier work (1) in that ascorbic acid feeding impairs copper status in rats. After 8 weeks of supplying a purified diet with 10 g ascorbic acid/kg, plasma copper was reduced by about 12%, plasma ceruloplasmin levels by 23%, and liver copper by on average 12%. The effects of dietary copper restriction, i.e. 1 mg Cu/kg feed instead of 5 mg Cu/kg, were more pronounced.

In previous studies we have shown that dietary ascorbic acid reduces copper absorption from the intestine (1,5). Apparent copper absorption in the present studies was found to be decreased in rats fed on ascorbic acid for 7 days, but thereafter apparent absorption gradually

Table 5. *Biliary copper excretion in Expt 2*[1].

Dietary variables			
Cu (mg/kg)	Ascorbate (g/kg)	Days	μg Cu/100 g body weight per h
5	–	0	0.29 ± 0.12
5	–	7	0.11 ± 0.06
5	10	7	0.23 ± 0.07
1	–	7	0.02 ± 0.01*
5	–	14	0.24 ± 0.07
5	10	14	0.06 ± 0.02*
1	–	14	0.06 ± 0.04*
5	–	28	0.16 ± 0.06
5	10	28	0.09 ± 0.02
1	–	28	0.03 ± 0.02*
5	–	56	0.19 ± 0.07
5	10	56	0.08 ± 0.03
1	–	56	0.03 ± 0.02*

[1] Means ± SEM for 5–6 rats per dietary group.

* Significant ($p < 0.05$) difference versus control group fed the copper-adequate diet without supplemental ascorbic acid.

increased above control values. The reduced copper status as induced by ascorbic acid feeding may have masked the effects of ascorbate on intestinal copper absorption. A decrease in copper status, as induced by dietary copper restriction, was associated with a rise in copper absorption. This may explain why true copper absorption measured after 14 days was not depressed by ascorbic acid feeding.

Intestinal copper absorption and biliary excretion of copper are the major determinants of copper homeostasis (6,7,10,19). Thus, the efficiency of copper absorption was increased and biliary copper excretion decreased in the rats fed the diet deficient in copper, which agrees with reported data (6). This explains the observed longer biological half-life of ^{64}Cu when a low-copper diet was fed. Because biliary copper is poorly absorbed (21,22), fecal endogenous copper excretion may mirror biliary excretion. As based on acute bile drainage, biliary copper excretion in the rats fed the copper-adequate diet was about 0.2 μg Cu/100 g body weight/h. Fecal excretion of endogenous copper was calculated as absolute true absorption minus absolute apparent absorption of copper, and in the control rats was found

to be about 0.3 μg Cu/100 g body weight/h. This supports the view that copper excreted with bile forms a large portion of fecal endogenous copper.

Ascorbic acid loading did not clearly affect biliary copper excretion after 7 days, but reduced it significantly after 14 days and thereafter. Likewise, fecal endogenous copper excretion was reduced by ascorbic acid in the diet only after about 4 weeks. The copper-deficient diet had a marked lowering effect on both biliary copper excretion and fecal endogenous copper loss as from 7 days after administration. Since copper status was reduced in the rats fed the low-copper diet, the reduction in biliary copper excretion must be a secondary, compensatory mechanism which is necessary to reach a new steady state of copper metabolism. This also implies that in the rats given ascorbic acid the reduced body copper status had caused the depressed excretion of endogenous copper in feces.

We conclude that high amounts of ascorbic acid in the diet of rats inhibit intestinal copper absorption, causing impaired copper status. After longer feeding periods, the ascorbic acid-induced depression of copper absorption may not be observed because the reduced copper status raises the efficiency of copper absorption. Secondary to the impaired copper status, biliary copper excretion will be dampened in rats fed ascorbic acid. This effect, which is a compensatory mechanism in an attempt to maintain copper homeostasis, is reflected by a decrease in fecal endogenous copper excretion.

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5

DIETARY FRUCTOSE VERSUS GLUCOSE LOWERS COPPER SOLUBILITY IN THE DIGESTA IN THE SMALL INTESTINE OF RATS

ABSTRACT

The hypothesis was tested that dietary fructose versus glucose lowers copper solubility in the digesta in the small intestine of rats, which in turn causes a decreased copper absorption. Male rats were fed adequate-copper (5 mg Cu/kg) diets containing either fructose or glucose (709.4 g monosaccharide/kg) for a period of 5 wk. Fructose versus glucose significantly lowered copper concentrations in plasma and the liver, but did not alter hepatic copper mass. Fructose feeding resulted in a significantly lesser intestinal solubility of copper as based on either a smaller soluble fraction of copper in the liquid phase of small intestinal contents or a lower copper concentration in the liquid phase. The latter fructose effect can be explained by the observed fructose-induced increase in volume of liquid phase of intestinal digesta. After administration of a restricted amount of diet extrinsically labeled with ^{64}Cu, rats fed fructose also had significantly lower soluble ^{64}Cu fraction in the digesta of the small intestine. Although this study shows that fructose lowered intestinal copper solubility, only a slight reduction of apparent copper absorption was observed. It is suggested that the fructose-induced lowering of copper status in part counteracted the fructose effect on copper absorption at the level of the intestinal lumen.

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INTRODUCTION

Dietary fructose or sucrose, when compared with glucose or starch in the diet, lowers plasma and liver copper concentrations in rats (1–10). So far, there is no satisfactory explanation for this effect of the type of dietary carbohydrate. Conflicting results as to fructose effects on copper absorption have been published. Dietary fructose versus glucose either enhanced (6), reduced (3,7,8) or did not influence (11) apparent absorption of copper in rats. After feeding a fructose diet extrinsically labeled with ^{64}Cu, impaired absorption of copper was found (12). The conflicting results may relate to secondary effects of copper status on copper absorption (13). An impaired copper status by itself is associated with increased efficiency of copper absorption (3,6-8,13). Thus, it could be hypothesized that fructose lowers copper absorption, at least initially, but that after longer periods of fructose feeding the degree of fructose-induced impairment of copper status determines whether copper absorption is still inhibited or even is stimulated.

Fructose in the gut could reduce cupric ions to cuprous ions, which may (co)precipitate as cuprous oxide (14), which in turn reduces the availability of copper for intestinal absorption. In the present experiment with rats, we investigated the effect of dietary fructose versus glucose on copper concentrations in the liquid phase of digesta in the small intestine. On the assumption that only soluble copper can be absorbed, we hypothesized that a depressing effect of fructose feeding on copper solubility may be reflected by impairment of apparent copper absorption, depending on the degree of fructose-induced lowering of copper status.

MATERIALS AND METHODS

Animals, Housing and Diets. Male, Wistar rats of the Hsd/Cpb:WU strain (Harlan, Zeist, The Netherlands) were used. The rats, aged about 6 wk, had been fed a commercial, pelleted diet (RMH-B^{R}, Hope Farms, Woerden, The Netherlands) and tap water *ad libitum.* All rats went through a pre-experimental period of 10 d during which they received the 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 4 animals in wire-topped, polycarbonate cages (37.5×22.5×15.0 cm) with wire mesh bases. The cages were placed in a room with controlled temperature (20–22 °C), relative humidity (40–65%) and light cycle (light, 06.00-18.00 h).

At the end of the pre-experimental period (d 0 of the experiment), the rats were divided into 2 groups of 9 animals each, so that body weight distributions of the groups were similar. One group remained on the control diet, and the other was switched to the test diet containing fructose instead of glucose as sole source of carbohydrate. Apart from the carbohydrate source, the diets were formulated according to the nutrient requirements of rats (15).

Table 1. *Composition of the experimental diets.*

	Glucose	Fructose
Ingredients, g/kg		
Glucose[1]	709.4	–
Fructose[2]	–	709.4
Constant components[3]	290.6	290.6
Chemical analysis		
Cu, mg/kg	5.0	5.0

[1] Morsweet 01934 (Cerestar, Haubourdin, France).

[2] Fruchtzucker Art. no. 0781 (Sudzucker AG, Mannheim/Ochsenfurt,Germany).

[3] The constant components consisted of (g/kg diet): casein, 151; corn oil, 25; coconut fat, 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; vitamin premix, 12. The mineral premix consisted of the following (mg): 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; $SnCl_2.2H_3O$, 1.9; NH_4VO_3, 0.2; KI, 0.2; $Na_2SeO_3.5H_2O$, 0.3; $CuSO_4.5H_2O$, 15.7; corn meal 9679.2. The vitamin premix consisted of the following (mg): thiamin, 4; riboflavin, 3; niacinamide, 20; D,L-calcium panthothenate, 17.8; pyridoxine, 6; cyanocobalamine, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-alpha tocopheryl acetate, 60; retinyl acetate and retinyl palmitate, 8 (4000 IU); cholecalciferol, 2 (1000 IU); corn meal, 9826.15.

The composition of the diets is shown in Table 1. The purified diets, which were in powdered form, were stored at 4 °C until feeding. Food and demineralized water were provided *ad libitum*. The experiment lasted 35 d. During the experimental period, the rats were housed individually in metabolic cages (Tecniplast Gazzada, Buguggiata, Italy). From d 21–25, feces of each rat was collected quantitatively.

Collection of intestinal digesta. On d 35, after 16 h without access to food, 6 animals of each dietary group received 3 g of their diet. Three hours later, the rats were anesthetized by intraperitoneal administration of 15 mg of pentobarbital (Nembutal, Sanofi Sante Animale SA, Paris, France), and blood was obtained from the abdominal aorta and collected in heparinized tubes. The entire small intestine, between stomach and cecum, was removed. It was divided into a proximal and distal half. The digesta of both halves of the intestine were collected separately in preweighed centrifuge tubes by gently squeezing the intestine between finger and thumb. The digesta were immediately centrifuged (10 min, 10,000×g), and supernatant and pellet were separated. Weight of pellet and supernatant were determined.

Radiotracer study. On d 35, after 16 h without access to food, 3 animals of each dietary group received [^{64}Cu]copper-acetate (Interfaculty Reactor Institute, University of Technology, Delft, The Netherlands) in the form of an extrinsically labeled meal; 150 µl of

16.5 μmol/L [^{64}Cu]copper-acetate (about 150 MBq/mg Cu) in sodium acetate buffer (0.05 mol/L; pH 5.4) was added to 3 g of each of the experimental diets. The 3-g meals were consumed within 5 min. Three hours later, the rats were anesthetized and blood obtained as described above. The stomach and small intestine, including contents, and liver were removed, and radioactivity assessed in a Packard 5000 γ (Canberra Packard, Brussels, Belgium). Digesta of the proximal and distal intestine were collected as described above.

Chemical analyses. Feed and feces samples, and pellets of contents of proximal and distal part of the intestine were freeze-dried, homogenized and weighed, and subsequently ashed at 500 °C for 18 h and dissolved in 6 mol/L HCl. The supernatants of intestinal contents were used without further pretreatment. Copper in the samples was determined by atomic absorption spectroscopy using either an acetylene-air flame or graphite furnace (Varian AA-475 and Varian Spectra AA-3300; Varian Techtron, Springvale, Australia).

Plasma was collected immediately from the heparinized blood samples by low-speed centrifugation. Plasma ceruloplasmin was estimated by measurement of its p-phenylenediamine oxidase activity (16).

Calculations. Solubility of copper was determined as copper present in the liquid phase of the intestinal contents. The pellet obtained after centrifugation comprises the solid phase contaminated with liquid phase. Weight of the solid phase was obtained after freeze-drying the pellet. Weight of the liquid phase was calculated as the sum of weight of liquid phase in the pellet (= total pellet weight minus solid-phase weight) and that of supernatant. The concentration of copper in the supernatant was assumed to be identical to that of the liquid phase. The amount of copper in the liquid phase was obtained after multiplying copper concentration (mg/L) in the supernatant with the weight of the liquid phase. The amount of copper in the solid phase was calculated as that in total pellet minus that in liquid phase of the pellet.

Apparent absorption of copper was calculated as copper intake minus fecal excretion and expressed as percentage of intake.

Statistical analyses. All values are presented as means ± SD. Data within groups were found to be normally distributed (Kolmogorov-Smirnov test). Differences between group means were evaluated with Student's t-test. The level of significance was preset at $P < 0.05$. Data were analyzed by computer using the SPSS/PC$^+$ statistical package.

RESULTS

Body weight and feed consumption did not differ significantly between the two dietary groups (Table 2). Fructose significantly raised the weight of liver. Fructose versus glucose in

Table 2. *Effect of dietary fructose on growth performance and indicators of copper status*[1].

	Glucose	Fructose
Feed intake, g/d (d 21–25)	16.0±2.0	16.9± 1.0
Body weight, g	300±25	306± 15
Liver weight, g wet wt	8.1±1.0	10.1±0.9*
Liver Cu, µg/g dry wt	16.3±1.2	13.5± 1.3*
Liver Cu, µg /organ	42.2±4.5	43.5± 4.8
Plasma Cu, µg/mL	1.05±0.21	0.89±0.06*
Ceruloplasmin, g/L	0.31±0.07	0.28±0.05
Apparent Cu absorption, % of intake (d 21–25)	52±9	45± 8

1 Data are expressed as means ± SD for 9 rats per dietary group and refer to d 35 of the experiment, except for feed intake and copper absorption.

* Significantly different (two-tailed Student's t-test, $P < 0.05$) from the glucose group.

the diet significantly reduced liver copper concentration, but total amount of copper in liver was unaltered. Plasma copper was significantly lowered in rats fed the fructose diet. Plasma ceruloplasmin and apparent absorption of copper tended to be somewhat depressed in rats fed fructose.

Fructose significantly raised the volume of the liquid phase of the proximal and distal part of the small intestine (Table 3). Fructose versus glucose increased the absolute amount of copper in the solid as well as liquid phase of the distal intestine. However, copper in the liquid phase of the distal intestine, expressed as percentage of total copper, was significantly lowered by fructose. Fructose feeding resulted in a significantly lower concentration of copper in the liquid phase of the small intestine. In the rats fed a restricted amount of their diet extrinsically labeled with ^{64}Cu, radioactivity in the contents of the distal part of the small intestine was significantly increased by fructose (Table 4). The activity of ^{64}Cu in the liquid phase of the proximal and distal part of the small intestine, expressed as percentage of activity in whole intestinal contents, was significantly lowered in rats fed fructose. Fructose significantly increased the amount of ^{64}Cu recovered in the stomach. Plasma and hepatic levels of ^{64}Cu were significantly lower in animals fed fructose compared with those given glucose. The amount of radioactivity recovered in the carcass minus liver, stomach and small intestinal contents, was significantly reduced by fructose when compared with glucose.

DISCUSSION

Based on plasma copper concentration as an indicator, it is suggested that fructose versus glucose in the diet slightly impaired copper status of the rats. However, total hepatic copper

Table 3. *Distribution of copper between the liquid and solid phase of digesta in the intestine of rats fed the experimental diets*[1].

	Glucose	Fructose
Proximal intestine		
Liquid phase weight, g	0.129 ± 0.060	0.261 ± 0.127*
Solid phase weight, g	0.004 ± 0.005	0.005 ± 0.003
Copper		
Amount in liquid phase, µg	0.177 ± 0.095	0.121 ± 0.065
Amount in solid phase, µg	not detectable	not detectable
Concentration in liquid phase, µg/mL	1.380 ± 0.477	0.448 ± 0.135*
Distal intestine		
Liquid phase weight, g	0.310 ± 0.092	0.751 ± 0.195*
Solid phase weight, g	0.074 ± 0.022	0.090 ± 0.044
Copper		
Amount in liquid phase, µg	0.438 ± 0.159	0.757 ± 0.268*
Amount in solid phase, µg	0.124 ± 0.107	1.492 ± 1.008*
Fraction in liquid phase, %	82 ± 13	40 ± 21*
Concentration in liquid phase, µg/mL	1.406 ± 0.356	0.994 ± 0.195*

1 Data are expressed as means ± SD for 9 rats per dietary group.

* Significantly different (two-tailed Student's t-test, $P < 0.05$) from the glucose group.

content was not affected, and plasma ceruloplasmin activity was not significantly reduced by fructose feeding. The significantly lower copper concentration of the liver of animals receiving the fructose diet may relate to their increased liver weight. Thus, fructose feeding causes a "dilution" of copper in the liver (17). It can be concluded that in this study fructose only slightly reduced copper status in the rats, which generally agrees with literature data (1–10).

We found that fructose feeding diminished the soluble fraction of copper in the distal part of the small intestine. In the light of the idea that only soluble copper may cross the intestinal epithelium (18), copper absorption may be reduced in rats fed fructose. Indeed, apparent absorption of copper was somewhat lower after feeding the fructose diet, but the effect failed to reach statistical significance ($P = 0.10$). Perhaps, the primary effect of fructose on copper absorption had become invisible because the reduced copper status in the rats fed fructose, which can be considered a secondary effect (13), caused an increase in the efficiency of copper absorption.

Table 4. *Recovery of radioactivity in selected tissues and distribution of ^{64}Cu between the liquid and solid phase of digesta in the intestine of rats given a restricted amount of their diet extrinsically labeled with ^{64}Cu*[1].

	Glucose	Fructose
^{64}Cu recovery		
Plasma, % dose/mL	0.6±0.2	0.3±0.1*
Liver, % dose/organ	22±8	9±3*
Stomach + contents, % dose	29±7	62±4*
Small intestinal contents		
–Proximal intestine, % dose	0.5±0.2	0.5±0.1
–Distal intestine, % dose	13.5±0.2	22.4±4.8*
Carcass, % dose	36±6	5±3*
Sum of tissues, % dose	102±12	99±7
^{64}Cu distribution		
Proximal intestine:		
Fraction in liquid phase, %	97±5	80±5*
Fraction in solid phase, %	3±5	20±5*
Distal intestine:		
Fraction in liquid phase, %	73±22	35±16*
Fraction in solid phase, %	27±22	65±16*

1 Data are expressed as means ± SD for 3 rats per dietary group.

* Significantly different (two-tailed Student's t-test; $P < 0.05$) from the glucose group.

Fructose versus glucose reduced the fraction [percentage] of ^{64}Cu in the liquid phase of the digesta, for both proximal and distal intestine. This is in line with the data concerning the distribution of stable copper between the liquid and solid phase of the small intestinal contents. The two approaches, i.e., measurement of the distribution of stable copper and ^{64}Cu, indicated that the fraction of soluble copper in the distal intestine was about 40% for rats fed fructose, and about 80% for rats fed glucose.

Fructose reduced copper concentrations in the liquid phase of digesta in the distal part of the small intestine, but raised the absolute amount of copper in both liquid and solid phase. Fructose feeding was associated with increased recovery of orally administered ^{64}Cu in the stomach, while ^{64}Cu levels in plasma, liver and carcass were lowered. This points to a delayed passage of digesta through the gastrointestinal tract of rats fed fructose. Such a delay has also been suggested by others (3,12). Thus, the characteristics of intestinal contents from rats fed either fructose or glucose feeding may simply reflect different stages in the passage process of digesta.

The radiotracer experiment indicates that fructose caused a slower gastric emptying of copper when compared with glucose. However, there is evidence that fructose stimulates general gastric emptying (19). Perhaps, fructose lowers copper solubility not only in the small intestine but also in the stomach. This could lead to a temporarily enhanced retention of copper in the stomach despite an increased rate of gastric emptying.

The major objective of the present study was to examine the intestinal solubility of copper in rats fed diets containing either fructose or glucose. Fructose versus glucose lowered the soluble fraction of copper in the liquid phase of intestinal digesta. We suggest that the somewhat lowered apparent copper absorption in rats fed fructose is related with the fructose-induced lowered copper solubility in intestinal digesta and/or a lowered copper concentration in the liquid phase. The latter effect is explained by an increase in volume of the liquid phase in rats fed fructose, which may be caused by an increased osmolarity brought about by fructose which may not be absorbed by the small intestine as rapidly as glucose.

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6

DIETARY ASCORBIC ACID LOWERS THE CONCENTRATION OF SOLUBLE COPPER IN THE SMALL INTESTINAL LUMEN OF RATS

ABSTRACT

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

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INTRODUCTION

In rats fed on ascorbic acid, the apparent intestinal absorption of copper is reduced (Johnson & Murphy, 1988; Van Campen & Gross, 1968; 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 cupric to cuprous ions (Harris & Percival, 1991; McArdle, 1992), which may lower copper solubility (Gollan et al. 1971). If this effect of ascorbic acid also occurs in the lumen of the intestine, the inhibition of copper absorption caused by ascorbic acid intake can be explained because the efficiency of copper absorption probably depends on the concentration of copper 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 copper absorption in rats is associated with a decrease in the concentration of copper in the liquid phase of small intestinal digesta. Any effect of dietary ascorbic acid on intestinal copper solubility may be independent of the duration of feeding with this vitamin. In contrast, ascorbic-acid-induced inhibition of copper absorption may become smaller with longer feeding periods due to compensatory effects of the reduced tissue copper concentrations as produced by ascorbic acid feeding (Van den Berg & Beynen, 1992). Thus, the effect of ascorbic acid (10 g/kg diet) on apparent copper absorption and concentration of soluble, intestinal copper in rats was determined after short-term (6 days) and long-term (42 days) 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 wk, were used. On arrival, the rats had *ad libitum* 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 4 animals in wire-topped, polycarbonate cages ($37.5 \times 22.5 \times 15.0$ cm) with inlaid wire mesh floors above filter paper. The cages were placed in a room with controlled temperature (20–22 °C), relative humidity (40–65%) and light cycle (light, 06.00–18.00 h).

At the end of the pre-experimental period (d 0 of the experiment), the rats were divided into 3 groups of 8 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 °C until feeding. Food and demineralized water were provided *ad libitum*. 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 (d 37–41), faeces of each rat was collected quantitatively.

Table 1. *Composition of the purified diets.*

Dietary supplement...	None	Ascorbic acid
Components (/kg)		
Glucose (g)	709.4	699.4
Ascorbic acid (g)	–	10.0
Constant components (g)[1]	290.6	290.6
Chemical analysis		
Cu[2] (mg/kg)	5.1	5.1
Ascorbate[2] (g/kg)	0	10.5

[1] The constant components consisted of (g): casein, 151; corn oil, 25; coconut fat, 25; cellulose, 30; calcium carbonate, 12.4; magnesium carbonate, 1.4; potassium chloride, 1.0; potassium bicarbonate, 7.7; sodium dihydrogenphosphate, 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; D,L-calcium panthothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; pteroylmonoglutamic acid, 1; biotin, 2; menadione, 0.05; D,L-alpha tocopheryl acetate, 60; retinyl acetate and retinyl palmitate, 8 (1200 retinol equivalents); cholecalciferol, 0.025; maize meal, 9828.125.

[2] Average values of four measurements.

Collection of samples. On d 42, as from 9.00 h, the animals received 3 g of their food in random order with 5-min intervals. The rats had been fasted for 16 h, and each rat consumed its meal within 5 min. Three hours after feeding, each rat was anaesthetized by intraperitoneal administration of 15 mg of 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 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 10,000×g and supernatant and pellet were separated. Weight of pellet and supernatant 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 °C until analysis.

Chemical analyses. Food and faeces samples, and pellets from digesta were freeze-dried, homogenized and weighed, and subsequently ashed at 500 °C for 18 h and dissolved in 6 mol HCl/L. The supernatants of intestinal contents were used without further pre-treatment. Plasma and tissues were freeze-dried and then digested in 14 mol HNO_3/L (Suprapur; Merck, Darmstadt, Germany). Copper 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 copper analysis was checked with a reference sample (Standard Reference Material 1577 Bovine Liver; U.S. National Institute of Standards and Technology, Gaithersburg, MD, U.S.A.); the bias was always less than 5%. Precinorm (Boehringer, Mannheim, Germany) was analysed along with the plasma and soluble digesta and analysed copper concentration differed less than 10% from the target. The combined between- and within-run variation of analysed copper concentrations in the reference samples was less than 5% (coefficient of variation).

Ascorbic acid in diet samples was quantified after extraction with 0.68 mol meta-phosphoric acid/L by high-performance liquid chromatography (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 mol metaphosphoric acid/L (1:4, v/v) in order to precipitate proteins and to prevent oxidation of ascorbate (Parviainen et al. 1986). Hundred µl of clear supernatant was injected into a reversed phase column (Merck Lichrospher 100–5RP18 cat

no 50943; 125*4 mm id.; Merck, Darmstadt, Germany) and eluted with 0.08 mol metaphosphoric acid/L 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 copper was estimated as copper present in the liquid phase of the intestinal contents. The pellet obtained after centrifugation comprises the solid phase contaminated with liquid phase. Weight of the solid phase was obtained after freeze-drying the pellet. Weight of the liquid phase was calculated as the sum of weight of liquid phase in the pellet (=total pellet weight minus solid phase) and that of supernatant. The concentration of copper in the supernatant was assumed to be identical to that of the liquid phase. The amount of copper in the liquid phase was obtained after multiplying copper concentration (µmol/L) in the supernatant with the weight of the liquid phase. The amount of copper in the solid phase was calculated as that in total pellet minus that in liquid phase of the pellet.

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

Statistical anlalyses. The data are summarized as group means and pooled SE. One-way analysis of variance was used to calculate pooled SE's; 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 $P < 0.025$ 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 of 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.

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

(Mean values for eight rats per dietary group)

Dietary supplement	None	Ascorbic acid		Ascorbic acid		
Feeding period	d 0–42	d 37–42		d 0–42		
	Mean	Mean	P value[1]	Mean	P value[1]	Pooled SE[2]
Body weight (g)						
d 0	111	111	0.92	109	0.61	2.2
d 42	319	312	0.37	319	1.00	7.1
Feed intake, g/d (d 37–41)	18.9	18.4	0.26	18.7	0.81	0.46
Faecal output, g wet wt/d (d 37–41)	8.1	8.1	1.00	8.5	0.34	0.35
Faecal dry wt, g/100 g	58.2	57.6	0.72	56.5	0.25	1.13
Plasma ascorbate (µmol/L)	53	86	0.00	81	0.00	4.9

[1] Significance probability of the difference between the control and the supplemented groups.

[2] Degrees of freedom = 23.

Indicators of copper status and copper absorption. Copper concentrations in plasma and kidney were significantly lowered by ascorbic acid feeding for 42 days, but not when fed for 6 days (Table 3). In rats given ascorbic acid for 42 days, copper concentrations in liver, heart, spleen and muscle tended to be reduced. Faecal copper 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 copper absorption after 6 days, but not after 42 days.

Intestinal, soluble copper concentration. The amounts of copper in liquid and solid phase 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 copper in the liquid phase and slightly raised the group mean liquid phase weight. Thus, ascorbic acid in the diet significantly decreased the concentration of copper in the liquid phase of the proximal part of the small intestine. In the distal part of the small intestine, the amount of copper in the liquid phase was not significantly affected by dietary ascorbic acid, but the group mean was reduced by about 15 percent. In addition, in rats given ascorbic acid the volume of the liquid phase was significantly raised so that copper concentration in the

Table 3. *Effect of feeding ascorbic acid for either 6 or 42 days on indicators of copper status and apparent copper absorption.*

(Mean values for eight rats per dietary group)

Dietary supplement	None	Ascorbic acid		Ascorbic acid		
Feeding period	d 0–42	d 37–42		d 0–42		
	Mean	Mean	P value[1]	Mean	P value[1]	Pooled SE[2]
Copper concentrations[3]						
Plasma, μmol/L	15.99	14.99	0.21	14.26	0.02	0.496
Liver, nmol/g	234	232	0.99	209	0.04	7.0
Kidney, nmol/g	396	347	0.05	316	0.00	16.1
Heart, nmol/g	310	316	0.61	271	0.03	10.6
Spleen, nmol/g	79	78	0.77	66	0.04	4.0
Muscle, nmol/g	52	55	0.44	43	0.05	2.5
Faecal Cu output, μg/d (d 37–41)	65	72	0.06	67	0.57	2.7
Apparent Cu absorption, % of intake (d 37–41)	32.5	23.4	0.00	29.3	0.31	2.07

[1] Significance probability of the difference between the control and the supplemented groups.

[2] Degrees of freedom = 23.

[3] For tissues as nmol Cu/g dry weight.

liquid phase of the distal part of the small intestine was significantly decreased after ascorbic acid feeding. pH in the liquid phase of the distal part of the small intestine was significantly lower in rats fed on ascorbic acid. Irrespective of the feeding period, ascorbic acid in the diet produced an increase in the amount of copper in the solid phase of the distal intestine.

DISCUSSION

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

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

(Mean values for eight rats per dietary group)

Dietary supplement	None	Ascorbic acid		Ascorbic acid		
Feeding period	d 0–42	d 37–42		d 0–42		
	Mean	Mean	P value[1]	Mean	P value[1]	Pooled SE[2]
Proximal intestine						
Liquid phase weight, mg	191	208	0.79	208	0.77	38.6
Solid phase weight, mg	40	40	0.99	36	0.73	8.5
Copper						
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 weight, mg	433	614	0.04	628	0.01	49.3
Solid phase weight, mg	98	93	0.72	98	1.00	11.2
pH	7.03	6.57	0.02	6.69	0.03	0.104
Copper						
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

[1] Significance probability of the difference between the control and supplemented groups.

[2] Degrees of freedom = 20–23.

6 days. Thus, when studying effects of ascorbic acid on copper metabolism, rebound effects of impaired copper status should be reckoned with.

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

It is not known why ascorbic acid in the diet reduced the concentration of soluble, intestinal copper. Ascorbic acid may reduce Cu(II) to Cu(I) (Harris & Percival, 1991; McArdle, 1992) which could impair copper binding to physiological ligands so that it becomes less soluble (Gollan, 1971). The observed, systematic tendency for the amount of copper 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 copper 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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7

COPPER UPTAKE AND RETENTION IN LIVER PARENCHYMAL CELLS ISOLATED FROM NUTRITIONALLY COPPER-DEFICIENT RATS

ABSTRACT

Copper uptake and retention were studied in primary cultures of liver parenchymal cells isolated from copper-deficient rats. Male Sprague-Dawley rats were fed a copper-deficient diet (< 1 mg Cu/kg) for 10 wk. Copper-deficient rats were characterized by low copper concentrations in plasma and liver, anemia, low plasma ceruloplasmin oxidase activity and increased ^{64}Cu whole-body retention. Freshly isolated liver parenchymal cells from copper-deficient rats showed a higher ^{64}Cu influx, which was associated with a higher apparent V_{max} of 45 ± 4 pmol Cu.mg protein^{-1}.min^{-1} as compared to 30 ± 3 pmol Cu.mg protein^{-1}.min^{-1} for cells isolated from copper-sufficient rats. No significant difference in the apparent K_m (about 30 µmol/L) was observed. Relative ^{64}Cu efflux from cells from copper-deficient rats was significantly smaller than the efflux from cells from copper-sufficient rats after prelabeling as determined by 2-h efflux experiments. Analysis of the medium after efflux from cells from copper-deficient rats showed elevated protein-associated ^{64}Cu, suggesting a higher incorporation of radioactive copper during metalloprotein synthesis. Effects of copper deficiency persists in primary cultures of parenchymal cells derived from copper-deficient rats, and short-term cultures of these cells offer a prospect for the study of cell biological aspects of the metabolic adaptation of the liver to copper deficiency.

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INTRODUCTION

Dietary copper is transported from the intestine in portal plasma, mainly bound to albumin, and possibly as amino acid complexes (1–4). The liver plays a central role in the uptake and homeostasis of copper (5). Copper is taken up by the liver parenchymal cells and subsequently released in plasma as a constituent of ceruloplasmin (6). Cultured liver parenchymal cells are widely used as a model to study the kinetics and other characteristics of copper uptake and processing by the liver (review in ref. 7). No such experiments have been reported with isolated cells from copper-deficient animals.

Nutritional copper deficiency in vivo induces decreased copper concentrations, especially in plasma and liver (8,9), and decreased activities of copper-dependent enzymes, e.g., ceruloplasmin and superoxide dismutase (10). In vivo, a higher ^{64}Cu whole-body retention in copper deficiency has been reported as has an altered tissue radiolabeled copper distribution, including an increase in the liver (9,11,12). One study (13) showed that the uptake of ^{64}Cu in liver parenchymal cells isolated from nutritionally copper-deficient mice was higher than that of cells from copper-sufficient mice, but this was based on experiments with only one copper concentration.

The purpose of this study was to investigate the effect of dietary copper deficiency using short-term cultured liver parenchymal cells isolated from a well-characterized copper-deficient rat model. We examined uptake and efflux of ^{64}Cu and determined the kinetic parameters of copper uptake, i.e., K_m and V_{max}. In efflux experiments ^{64}Cu in the medium was separated into protein-bound and nonprotein-bound fraction using an ultrafiltration technique. Because zinc is believed to be transported by the same system (7), zinc uptake also was measured to assess the metal specificity of the copper deficiency effects. Cells were incubated in a culture medium containing copper and albumin and amino acids as copper ligands. All the in vitro studies were performed using short-term cultured liver parenchymal cells isolated from copper-sufficient or copper-deficient rats.

MATERIALS AND METHODS

Animals and Diets. Weanling male Sprague-Dawley rats weighing about 40 g were used. These specific pathogen-free rats (TNO, Rijswijk, The Netherlands) were maintained under clean conventional conditions (14). Rats were housed individually in stainless steel mesh-bottom Macrolon cages (UNO, Zevenaar, The Netherlands), at 22 °C with 12-h light:dark cycle. The animals were randomly allotted to two treatment groups: a copper-sufficient and a copper-deficient group. Copper-deficient rats were fed a low copper purified diet that consisted of (g/kg): glucose monohydrate, 655; casein, 200; corn oil, 50; cellulose, 50; vitamin mix, 10 and mineral mix without copper, 35. The vitamin mix and

mineral mix were prepared according to specifications recommended by the American Institute of Nutrition (15). The copper-sufficient rats were fed the same diet supplemented with cupric carbonate. Diet and demineralized water were provided *ad libitum* during the 10 week period. Food intake and body weight were recorded weekly for all rats.

The two diets were analyzed for their copper content. The copper-deficient diet contained 0.3 ± 0.1 mg Cu/kg and the copper-sufficient diet contained 8.1 ± 0.3 mg Cu/kg. For the analysis of the diet, 1-g portions were digested with 65% nitric acid (Suprapur, Merck, Darmstadt, FRG) and 30% hydrogen peroxide (Aristar, BDH Chemicals, Poole, U.K.) then analyzed for total copper by furnace atomic absorption spectroscopy (AAS) (Model 2340 with HGA-400 graphite furnace, Perkin-Elmer, Norwalk, CT). Analyses were checked with the Standard Reference Material 1577 Bovine Liver (U.S. National Bureau of Standards and Technology, Gaithersburg, MD). Copper levels were found within the confidence limits of the certified value.

Radioactive materials. Radioactive copper (^{64}Cu) was obtained by irradiating a 5 mg copper wire (purity 99.999%; Ventron, Karlsruhe, Germany) in a thermal neutron flux of $10^{17} m^{-2}.s^{-1}$ for 36 h in the research reactor of the Interfaculty Reactor Institute of the Delft University of Technology. Following irradiation, the wire was dissolved in 25 μL 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. The specific activity of the resulting ^{64}Cu solution at the start of the experiments was 320 TBq/kg Cu (8.5 Ci/g). Radioactive zinc ($^{65}ZnCl_2$ 35 TBq/g Zn) was purchased from the Radiochemical Centre, Amersham, U.K. and diluted in acetate buffer before use.

Parenchymal cell isolation procedure. Parenchymal cells were isolated at 37 °C as described previously (16) from overnight-fasted animals. The liver was perfused with perfusion medium (17) without Ca^{2++} and collagenase, followed by a perfusion and an incubation with medium containing 1 mmol Ca^{2++}/L and collagenase. The isolation of parenchymal cells from copper-deficient rats was optimized by reducing the collagenase concentration by 10%. Parenchymal cells were further purified by centrifugal elutriation in a JE-6 elutriator rotor at 4 °C, using a Sanderson chamber (Beckmann Instruments, Palo Alto, CA) and a perfusion medium containing 0.5% bovine serum albumin (BSA). Contaminating debris and cell types were elutriated at a rotor speed of 3250 rpm and a flow of 70 mL per min. The purified parenchymal cells were harvested from the chamber and resuspended in perfusion medium without BSA. The yield and purity of all cell preparations were checked by light microscopy. Cell counts were performed in a hemocytometer. Viability was determined by a trypan blue exclusion test using 0.25% (wt/v) trypan blue in Gey's balanced salt solution (18).

Cell culture and cell incubation conditions. Cells were preincubated at 37 °C in a 5% CO_2– H_2O saturated atmosphere in Ham's F-10 medium (19), supplemented with 12% fetal calf serum (FCS), glutamic acid (2 mmol/L), penicillin (100,000 U/L), streptomycin (100 mg/L), insulin (0.5 mg/L) and 20 mmol/L HEPES at pH 7.4. Cells were cultured in collagen-coated dishes (diam. 60 mm), 1–2×10^6 cells per 5 mL. More than 85% of the cells were attached after 2 h. Then the medium was removed and the cells were washed to remove debris and dead cells. Subsequently, 5.0 mL of fresh medium containing ^{64}Cu or ^{65}Zn were added. The cells were incubated with increasing total copper concentrations, 1.5–48 µmol Cu/L at a constant ^{64}Cu specific activity for 2 h (concentration dependence of ^{64}Cu uptake), with 24 µmol Cu/L up to 16 h (time dependence of ^{64}Cu uptake) or with 12 µmol Zn/L up to 16 h (time dependence of ^{65}Zn uptake) at 37 °C. Parallel experiments were conducted at 4 °C but only for incubation periods up to 2 h. These experiments provided cell surface binding values, which were subtracted from the values obtained at 37 °C, to obtain net copper uptake values. Binding at 4 °C ranged between 5% and 10% of the values obtained at 37 °C for low copper and high copper concentrations, respectively. After the incubations, the radioactive medium was aspirated and the cells were washed twice with Ham's F-10 medium, supplemented with 12% FCS. Cells were harvested using a rubber policeman. Thereafter, the cells were washed once using PBS to remove albumin. Final pellets were resuspended in 1 mL PBS. A 0.8-mL aliquot was assayed for ^{64}Cu or ^{65}Zn by gamma counting (Philips Model PW4800, Philips, Eindhoven, The Netherlands, 3×3 inch NaI crystal detector, efficiency 6% for both radionuclides).

The total protein content of each pellet was determined by the method of Lowry (20), using recrystalized BSA (Fluka, Basel, Switzerland) as a standard. All measurements were normalized to a per mg cell protein basis.

In the ^{64}Cu efflux experiments, the cells were prelabeled with 24 µmol Cu/L for 2 or 16 h at 37 °C. The radioactive medium was then aspirated, and the cells were washed once with Ham's F-10 medium, supplemented with 12% FCS. Total ^{64}Cu efflux in 5 mL of Ham's F-10 medium without FCS at 37 °C for 2 h was measured and analyzed by ultrafiltration for secreted ^{64}Cu-carrying proteins. The cells were washed and processed as described here. Total copper in cells and incubation media were determined in duplicate by atomic absorption spectroscopy (AAS).

Estimation of kinetic parameters. Kinetic parameters K_m and V_{max} were calculated using Lineweaver-Burk analysis of ^{64}Cu uptake after 2 h as a function of the copper concentration. The time dependency study showed that ^{64}Cu uptake was linear over at least the first two hours (chi-square test, X^2 = 0.41 and 0.52 for copper-deficient and copper-sufficient cells respectively). Because no initial rates of uptake (1-min incubations)

were measured, kinetic parameters determined are only estimates based on the 2-h period and are therefore called apparent V_{max} and K_m.

Whole-body counting. Radioactive copper [^{64}Cu]Cu-acetate (specific activity 320 TBq/kg Cu; 0.5 µmol Cu per kg body weight) in a total volume of 0.25 mL of acetate buffer was injected intraperitoneally. Whole-body radioactivity was measured only for up to about 5 days after injection, because for its short physical half-life (12.8 h). The measured radioactivity at 2 h after injection was designated as 100%. Whole-body counting of animals was performed with a specially designed whole-body counter for rats (21). The efficiency of this counter for ^{64}Cu was 14%. Tissue distribution of ^{64}Cu in muscle (flexor digitorum longus), bone (tibia) and plasma was calculated using the following assumptions: muscle, 39% of body weight; bone, 14% of body weight; and plasma, 5.6% of body weight (22).

Histopathology. A complete necropsy and microscopic examination (including heart, kidney, liver, lung, pancreas, thymus, lymphoid, testis) was performed for all animals in this study. Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3 µm and stained using hematoxyline-phloxine-saffron.

Ultrafiltration method. Samples (1 mL) of the efflux medium were ultrafiltrated by centrifugation as described by Bloxam et al (23), using YMT-6 membranes, with a 10-kDa cut-off, in the Amicon's Micropartition system MPS-1 (Publication No.460; Amicon, Danvers, MA), for qualitative comparison, i.e., protein-bound ^{64}Cu and non protein-bound ^{64}Cu.

Other analyses. Ceruloplasmin [EC 1.16.3.1] in plasma was measured by its 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 Sunderman and Notomo (24).

Statistical analyses. Comparisons between the two dietary groups, except for the histological scores, were made by Student's t-test. For this test, a 95% or a 99% level of confidence was considered as significantly different. Histological data were compared by Fischer's two-tailed exact test (25). Chi-square analyses of the data was used for estimation of the linearity of the time-dependency study of ^{64}Cu uptake (26).

RESULTS

In vivo characterization of copper-deficient rats. No histopathological lesions were observed, except for mild cardiac fibrosis observed in about 40% of the copper-deficient rats (Table 1). The overall severity of these abnormalities was considered to be minor.

Body weight, total liver weight and copper concentrations in several tissues are presented in Table 1. Food intake (data not shown) and mean body weights were not affected significantly by the copper level of the diet. However, hematocrit was 20% lower (P< 0.01) in the copper-deficient rats, and copper tissue concentrations were all significantly (P< 0.01) lower because of the copper deficiency. The most severe decrease was found in plasma copper, which was accompanied by a lower ceruloplasmin concentration.

Whole-body loss of ^{64}Cu differed significantly between copper-deficient rats and copper-sufficient rats. In copper-deficient rats whole-body ^{64}Cu was considerably greater as indicated by its biological half-life (Table 1).

Table 2 indicates that the higher ^{64}Cu retention in the copper-deficient rats as compared to copper-sufficient rats is reflected predominately in liver, bone and muscle. These are tissues which have been shown to account for > 60% of the total copper in rats (22).

Table 1. *Characterization of rats fed a copper-sufficient or a copper-deficient diet for 10 wk*[1].

Characteristic	Copper-sufficient	Copper-deficient
Total body weight, g	342 ± 13	335 ± 13
Liver wet weight, g	8.6 ± 0.7	7.9 ± 0.3*
Hematocrit, %	0.48 ± 0.03	0.40 ± 0.02**
Ceruloplasmin, g/L	0.63 ± 0.11	0.10 ± 0.02**
Copper concentrations, μg Cu/g wet weight		
Plasma	1.15 ± 0.13	0.24 ± 0.03**
Liver	4.53 ± 0.21	2.26 ± 0.28**
Muscle	1.19 ± 0.07	0.70 ± 0.10**
Bone	0.41 ± 0.04	0.21 ± 0.04**
Heart	4.39 ± 0.24	2.10 ± 0.40**
Cardiac fibrosis, % of animals	0	42***
Biological half-life of whole-body ^{64}Cu, d	6 ± 1	24 ± 3**

1 Values are means ± SD, n = 5.

* Value differs significantly (P< 0.05, Student's t-test) from copper-sufficient group.

** Value differs significantly (P< 0.01, Student's t-test).

*** Value differs using the 2-tailed exact Fischer test (P < 0.05).

Table 2. *Tissue distribution of ^{64}Cu 24 h after an intraperitoneal injection of ^{64}Cu in rats fed a copper-sufficient or copper-deficient diet for 10 weeks*[1] .

Tissue	Copper-sufficient	Copper-deficient
	% of dose per total tissue	
Plasma	10.9 ± 3.2	8.5 ± 1.9
Liver	15.8 ± 2.1	26.4 ± 3.1*
Muscle	6.2 ± 1.0	8.6 ± 0.9*
Bone	10.0 ± 1.5	16.2 ± 1.9*
Carcass	27.1 ± 5.1	27.8 ± 4.2
Sum of tissues	70.0 ± 6.6	87.5 ± 5.9*

[1] Values are means ± SD, n = 5.

* Value differs significantly (P< 0.05, Student's t-test) from copper-sufficient group.

Characterization of isolated liver parenchymal cells. Isolated parenchymal cells were obtained with a high purity (> 99 ± 1%, n = 12) and good viability (85–95%) from both copper-sufficient and copper-deficient rats. No significant differences were observed in cellular adherence and protein content of the isolated parenchymal cells: for cells from copper-sufficient rats, 2.1 ± 0.1 mg protein per 10^6 cells (n= 6) and for cells isolated from copper-deficient rats, 2.0 ± 0.2 mg protein per 10^6 cells (n = 6). The copper contents of the liver parenchymal cells, as measured by AAS, differed significantly (P< 0.05), 0.05 ± 0.02 μg Cu/mg cell protein for cells from copper-deficient rats (n= 3) and 0.09 ± 0.02 μg Cu/mg protein for cells from copper-sufficient rats (n= 3).

Time dependence of ^{64}Cu uptake. When liver parenchymal cells from copper-deficient or copper-sufficient rats were incubated with a ^{64}Cu solution (24 μmol Cu/L), a linear increase in the net ^{64}Cu accumulation was found at least for 2 h (chi-square test, X^2= 0.52 and 0.41 for cells from copper-sufficient and copper-deficient rats respectively) and a further increase was seen between 4 and 16 h (Fig. 1). From the ^{64}Cu data in Table 3, steady state levels of 0.95 ± 0.41 μg Cu/mg protein for cells from copper-deficient rats and 0.42 ± 0.17 μg Cu/mg protein for cells from copper-sufficient rats can be computed. At all time points, the ^{64}Cu uptake values were higher for parenchymal cells from copper-deficient rats as compared to cells from copper-sufficient rats. To ascertain that the measured increase of ^{64}Cu activity reflects cellular copper accumulation, total copper content was determined by AAS at 2, 4 and 16 h after incubation. The net accumulation of copper during the incubation greatly exceeded the endogenous copper content at 0 h, for cells from copper-sufficient rats and particularly for cells from copper-deficient rats (Table 3). The uptake of copper as

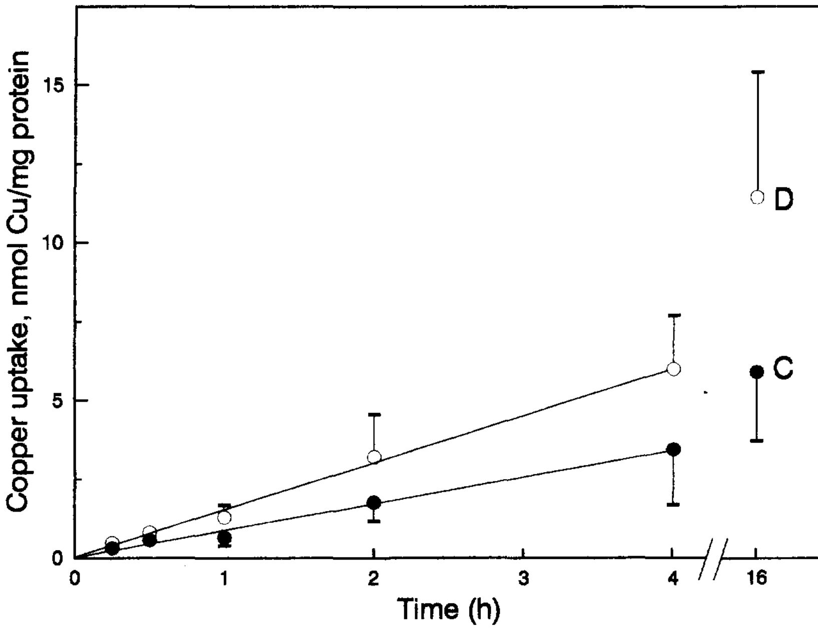

Figure 1. *Time dependence of ^{64}Cu uptake by liver parenchymal cells isolated from copper-sufficient (C) and copper-deficient (D) rats. Cells were incubated in Ham's F-10 medium, supplemented with 12% FCS and contained 24 µmol Cu/L at 37 °C. Each point represents the means ± SD of six separate liver parenchymal cell preparations.*

determined by ^{64}Cu was comparable to the net uptake of copper as determined by AAS. Table 3 also shows calculated specific activities in the cells at the indicated time points in comparison with the administered specific activities of ^{64}Cu in the medium. In contrast to its effect on ^{64}Cu uptake, copper deficiency exerted no effect on ^{65}Zn uptake (data not shown).

Kinetics of ^{64}Cu uptake. The ^{64}Cu accumulation over 2-h periods was used to determine its apparent kinetic parameters. Uptake of ^{64}Cu was measured during 2-h incubations in parenchymal cells from copper-deficient and copper-sufficient rats over the concentration range, 1.5–48 µmol Cu/L. Lineweaver-Burk analysis of the uptake data yielded the kinetic parameters V_{max} and K_m (Fig. 2), the values of which are presented in Table 4. The results indicate a significantly higher V_{max} for cells from copper-deficient rats compared to cells from copper-sufficient rats, but no difference in K_m. Using these kinetic parameters, we can calculate the rate of ^{64}Cu uptake at 24 µmol Cu/L, that is, 2.5 ± 0.3 nmol.mg protein^{-1}.h^{-1} for cells from the copper-deficient rats, which is in agreement with the actual measured value, 3.1 ± 0.4 nmol.mg protein^{-1}.h^{-1} (Table 3).

Table 3. *Comparison of copper uptake by liver parenchymal cells in primary cultures as determined by ^{64}Cu measurements and atomic absorption spectroscopy*[1].

	Time			
Parameter	0 h	2 h	4 h	16 h
^{64}Cu uptake, µg Cu/mg protein				
Cu-deficient (6)	0	0.20 ± 0.02*	0.38 ± 0.12*	0.81 ± 0.21*
Cu-sufficient (6)	0	0.12 ± 0.02	0.21 ± 0.02	0.38 ± 0.14
Total copper, µg Cu/mg protein				
Cu-deficient (3)	0.05 ± 0.02*	0.22 ± 0.05	0.47 ± 0.16	0.82 ± 0.28
Cu-sufficient (3)	0.09 ± 0.02	0.18 ± 0.05	0.28 ± 0.11	0.58 ± 0.18
^{64}Cu specific activity influx (in medium), TBq/kg Cu (6)	320	320	320	320
^{64}Cu specific activity (in cells), TBq/kg Cu				
Cu-deficient (6)	0	247 ± 73	286 ± 109	300 ± 110
Cu-sufficient (6)	0	160 ± 89	217 ± 125	270 ± 99

[1] Values are means ± SD (number in parenthesis).

* Value differs significantly (P < 0.05, Student's t-test) from copper-sufficient cells.

Table 4. *Apparent kinetic parameters, V_{max} and K_m for liver parenchymal cells from copper-deficient and copper-sufficient rats*[1].

Cells	V_{max}	K_m
	pmol Cu/ (mg protein.min)	µmol Cu/L
Copper-deficient	45 ± 4*	27 ± 2
Copper-sufficient	30 ± 3	29 ± 3

[1] Values are means ± SD, n = 6.

* Value differs significantly (P < 0.05, Student's t-test) from copper-sufficient cells.

^{64}Cu efflux studies. The efflux of ^{64}Cu over a 2-h period was measured after 2- and 16-h prelabeling periods respectively. The amounts of copper and the ^{64}Cu specific activities present in the cells at the start of the efflux periods are presented in Table 3.

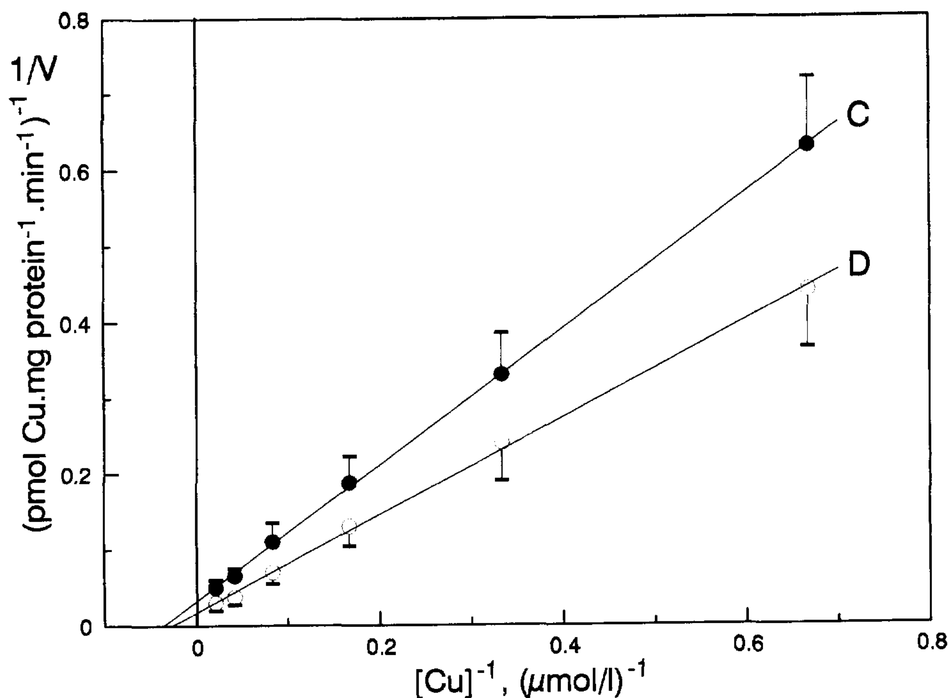

Figure 2. *Kinetics of ^{64}Cu uptake rates by liver parenchymal cells isolated from copper-sufficient (C) and copper-deficient (D) rats were measured at 37 °C in Ham's F-10 medium, supplemented with 12% FCS for 2 h with the indicated copper concentrations. Each point represents the means ± SD of six separate liver parenchymal cell preparations.*

Quantitative copper release from cells from copper-deficient and copper-sufficient rats were: 24 ± 9 and 39 ± 6 ng Cu/mg protein for 2-h prelabeling periods, and 145 ± 74 and 116 ± 15 ng Cu/mg protein for 16-h prelabeling periods, respectively. These data indicate that quantitative effluxes vary with the duration of prelabeling periods for both cells from copper-deficient and copper-sufficient rats; furthermore, cells from copper-deficient rats may release more copper than cells from copper-sufficient rats (cf. 16-h prelabeling period). However, results given in Table 3 show that cells from copper-deficient rats may accumulate more copper than cells from copper-sufficient rats. Considering these copper levels in the cells, efflux rates may be best described by the rate constants, which in turn are reflected by relative efflux values (fraction retained of the uptake level after the prelabeling period), as shown in Figure 3. Independent of duration of prelabeling periods, copper efflux from cells from copper-deficient rats was about 50% of the efflux from cells from copper-sufficient rats.

After the 2-h efflux period, the medium was analyzed for total ^{64}Cu, and an ultrafiltration technique also was applied to determine the fraction of protein-associated ^{64}Cu secreted. A qualitative comparison was made using the ultrafiltration technique (protein-bound vs. nonprotein bound within the 10-kDa boundaries). No difference was observed after the 2-h

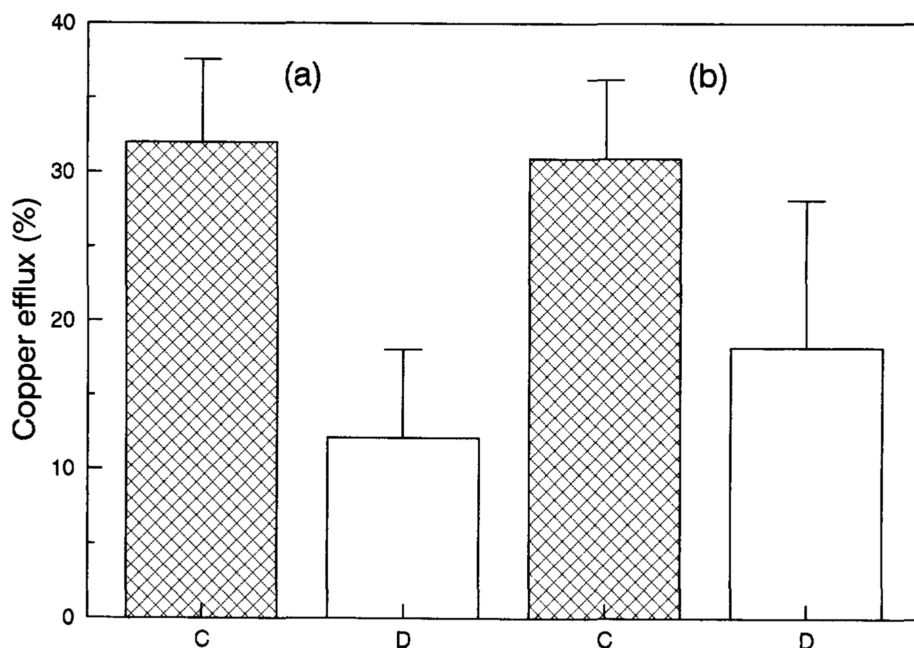

Figure 3. *Efflux of ^{64}Cu from rat liver parenchymal cells prelabeled with ^{64}Cu. Copper-sufficient (C) and copper-deficient (D) cells were prelabeled with ^{64}Cu for 2 h (A) or 16 h (B) in a medium containing 24 µmol Cu/L at 37 °C. Total radioactivity was measured after a 2-h efflux period and expressed as the percentage of cellular radioacitivity after prelabeling. Each bar is the means ± SD of six separate liver parenchymal cell preparations.*

prelabeling period in the percentage of ultrafiltrable radioactivity, i.e, 23 ± 2% for cells from copper-deficient rats and 25 ± 3% for cells from copper-sufficient rats. However, cells from copper-deficient rats had secreted, in absolute amounts, less radioactivity than cells from copper-sufficient rats. After a longer prelabeling period (16 h), a significantly lower percentage ($P < 0.05$) of ultrafiltrable radioactivity was measured for cells from copper-deficient rats, i.e., 13 ± 2% compared to 21 ± 2% for cells from copper-sufficient rats. Because cells from copper-deficient rats secreted only slightly more radioactivity, the only detected effect after both prelabeling periods was that cells from copper-deficient rats secreted more protein-bound ^{64}Cu than cells from copper-sufficient rats.

DISCUSSION

In vivo characterization of copper deficiency. Rats fed the copper-deficient diet developed the classical signs of copper deficiency (Table 1). In copper-deficient rats, whole-body ^{64}Cu retention is increased as indicated by its biological half-life (Table 1); ^{64}Cu tissue distribution (Table 2) is altered, i.e., by an elevated hepatic ^{64}Cu level. Finally,

histopathological examination indicated that no severe pathological lesions occurred.

The whole-body turnover of ^{64}Cu characterized by the calculated biological half-life is significantly slower in the copper-deficient rats than in copper-sufficient animals. This result supports our previous report (9) that whole body retention of ^{64}Cu, after intraperitoneal injection of ^{64}Cu, is a good method to assess copper status in vivo. In the present study, the biological half-life of about 6 d obtained for the copper-sufficient rats is between the values of 4 d for young and 9 d for adult copper-sufficient rats reported by Linder and Roboz (27).

Copper deficiency in vitro. Liver parenchymal cells were isolated from rats maintained on a copper-deficient diet for 10 wk. This was sufficient to deplete their liver copper levels by 50% (Table 1). Copper concentrations in short-term cultured parenchymal cells from copper-deficient rats were also about 50% of the value measured in cells from copper-sufficient rats. Copper concentrations obtained both from in vivo and in vitro measurements were on the same order of magnitude.

The kinetics of ^{64}Cu uptake by freshly isolated parenchymal cells from copper-deficient rats differ from the kinetics of ^{64}Cu uptake by cells from copper-sufficient rats. In contrast, no changes were observed for ^{65}Zn uptake between cells from copper-deficient and copper-sufficient rats, supporting the idea of metal specificity. This agrees with the results of another study in this laboratory (Van den Berg, G.J., unpublished results) in which no difference in total liver zinc levels between copper-sufficient and copper-deficient rats were found, respectively 26.7 ± 2.3 and 26.6 ± 3.7 µg Zn/g wet weight (n= 12). Moreover, uptake of ^{65}Zn by hepatocytes from the brindled mouse model of Menkes disease is also normal (13), and consistent with normal hepatic zinc levels.

The higher apparent V_{max} of copper uptake for cells from copper-deficient rats would explain the higher level of accumulated copper observed in cells from copper-deficient rats as compared to cells from copper-sufficient rats after a short-term incubation with a given amount of ^{64}Cu. No significant difference in the apparent K_m value between cells from copper-deficient and copper-sufficient rats was found, and the values were in accordance with reported values for rat and mouse hepatocytes (13,28). The absolute estimate values of V_{max} obtained in this study are lower than previously reported values (13,28). Assuming a linear ^{64}Cu uptake for 2 h (Fig. 2), the initial copper uptake rate for cells from copper-sufficient rats could be calculated as 1.0 ± 0.7 nmol Cu.mg protein^{-1}.min^{-1}. When we compare this value with the calculated 1.8 ± 0.4 nmol Cu.mg protein^{-1}.min^{-1} using the reported K_m and V_{max} for hepatocytes from copper-sufficient rats (28), the values are of the same order. However, experimental conditions differ considerably; the effective extracellular concentration of free Cu(II) in this study is much lower due to the presence of albumin and histidine, which are the most important copper ligands in vivo under physiological

conditions. Because of the modulating role of these ligands (29), quantitatively comparable amounts of copper can be taken up as compared with free Cu(II) (13,28), but only after a longer incubation time. Although our data show that intracellular copper affects copper uptake, McArdle et al. (30) did not observe this effect when using mouse hepatocytes directly copper depleted by diamsar (a copper chelator) overnight. No clear explanation for this difference can be given, but copper carriers may have changed their properties or redistributed on the cell membrane during copper deficiency.

Copper-deficient rats retained more radiolabeled copper after a single intraperitoneal dose, than did copper-sufficient rats, particularly in the liver; this agrees with reported results (31). There are at least three different compartments in the liver, related to storage, ceruloplasmin synthesis and bile excretion (32). All three compartments are affected in copper deficiency in vivo: 1) Liver copper stores decrease. 2) The synthesis of apoceruloplasmin remains normal in copper-deficient rats, but most of it is secreted as inactive ceruloplasmin (33,34). Restoration of plasma ceruloplasmin oxidase activity by copper administration only occurs after a lag period of a few hours (34). 3) Biliary secretion of copper decreases in copper deficiency (35). The in vitro experiments demonstrate that the copper content of isolated liver parenchymal cells is similar to the copper content of the whole liver, and that copper deficiency results in a twofold reduction in both cellular liver copper and total liver copper. In addition, parenchymal cells from copper-deficient rats accumulate relatively more radiolabeled copper in vitro after administration of ^{64}Cu. The elevated amount of protein-bound ^{64}Cu secreted by cells from copper-deficient rats may reflect this situation, assuming that more ^{64}Cu is available and used for the synthesis of metalloproteins. The higher relative ^{64}Cu efflux from cells from copper-sufficient rats (Fig. 3) may result from differences in chemical copper speciation between cells from copper-deficient and copper-sufficient rats or may result from actively operating release mechanism(s) in hepatocytes responding to intracellular copper concentrations. The changes in relative ^{64}Cu efflux values depend on the duration of the prelabeling periods (Fig. 3). This indicates that control mechanism(s) or differences in chemical speciation depend on intracellular concentrations in cells isolated from copper-deficient rats as well as from copper-sufficient rats.

In conclusion, effects of copper deficiency persist in freshly isolated liver parenchymal cells from copper-deficient rats. Thus, short-term cultures of these cells offer a prospect for the study of the metabolic adaptation of the liver to copper deficiency at the cellular level.

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8

EFFECT OF NUTRITIONAL COPPER DEFICIENCY ON COPPER UPTAKE BY PLASMA MEMBRANE VESICLES ISOLATED FROM RAT LIVERS

ABSTRACT

Rat liver plasma membrane vesicles were prepared from rats fed on a copper-adequate (5 mg Cu/kg) and a copper-deficient (0.4 mg/kg) purified diet and used to study copper uptake from copper-dihistidine ($^{64}CuHis_2$) complexes. Cu uptake was temperature dependent and saturable. At 4 °C, vesicles from copper-deficient rats had a higher V_{max} than controls (1.53 $\pm$ 0.20 nmol Cu/(mg vesicle protein.min) versus 1.08 $\pm$ 0.10 nmol/(mg.min). $p < 0.05$, Student's t-test). K_m values were not significantly different. There was no evidence for co-operativity in the binding process. At 25 °C and 37 °C, V_{max} values for copper-deficient rats were also significantly higher than controls (4.66 $\pm$ 0.31 nmol/(mg.min) versus 3.20 $\pm$ 0.25 nmol/(mg.min). $p < 0.05$, 25 °C, and 8.62 $\pm$ 1.03 versus 6.20 $\pm$ 0.49, $p < 0.05$, 37 °C). At these temperatures, n values indicated co-operativity for uptake ($n = 1.70 \pm 0.06$ and 1.69 $\pm$ 0.01 for copper-deficient and control rats respectively). $K_{0.5}$ values were not significantly different (5.72 $\pm$ 0.03 and 5.76 $\pm$ 0.17 µmol/L for copper-deficient and control rats at 25 °C, 5.68 $\pm$ 1.30 and 5.72 $\pm$ 0.12 µmol/L at 37 °C). The effect was specific for copper, since zinc uptake by the vesicles showed no difference between control and copper-deficient rats. The data presented show that copper deficiency in the whole rat results in an increase in the uptake of copper mediated by the liver plasma membrane, probably by increasing the number of transporters in the cell membrane.

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INTRODUCTION

The liver is central to the metabolism of copper. Following uptake across the gut, most of the copper is taken up by the liver, where it is stored, excreted or released into the serum as the glycoprotein ceruloplasmin. The mechanism whereby copper is taken up into the liver has been the subject of considerable study (for a recent review, see 7).

Using hepatocytes in suspension culture, Ettinger's group (5,13) showed that uptake was probably from the copper-histidine, an observation confirmed by McArdle et al. (9) although the latter pointed out that, physiologically, the ternary complex of copper-albumin-histidine was probably more important (10). Hepatic copper uptake is a carrier mediated process not dependent on metabolic energy (5,9,18). Apparent Michaelis constant (K_m) values vary between 4 and 20 μM and the maximum velocity (V_{max}) shows similar variation.

Nutritionally copper deficiency in vivo induces decreased copper concentrations, e.g., in the liver (16,17, see 4 for a review). In a previous study (16) we investigated the effect of dietary copper deficiency on the kinetics of copper transport and accumulation, using cultured hepatocytes. The data showed that the maximum rate of copper uptake was increased without a change in the apparent affinity of the transport process. While this result made teleological sense, other data (Bingham & McArdle, unpublished data) suggested that rapid depletion of hepatocyte copper stores, using chelators, made no difference to the transport kinetic parameters.

Interpretation of the data in terms of a simple change in the properties of the copper transporter are complicated by the fact that, in the cultured hepatocytes, other factors may also be important. For example, there could be contributions from intracellular copper pools, from cellular metabolism and from copper protein synthesis.

Rat liver membrane vesicles constitute a relatively simple experimental system which have recently been demonstrated to be valuable in trace element studies (Bingham & McArdle, unpublished results). In this paper, we have examined the properties of vesicles isolated from the livers of normal and copper-deficient rats and demonstrate that copper deficiency results in an increase in the maximum rate of copper transport across the hepatocyte plasma membrane.

MATERIALS AND METHODS

Animals, Housing and Diets. Weanling male Wistar rats (Hsd/Cpb:WU., Harlan, Zeist, The Netherlands) were housed individually in polycarbonate, wire-bottomed cages (37.5*22.5*15.0 cm). The cages were placed in a room with controlled temperature (20–22 °C), relative humidity (40–65%) and light cycle (light, 06.00–18.00 h). The rats were divided into two groups so that body weight distributions were similar. The rats initial average weight was 64 ± 2 g (mean ± SEM). Each group was assigned randomly to one of

the two experimental diets, a copper-adequate and a copper-deficient diet. The purified diets were formulated according to the nutrient requirements of rats (11) containing the following constant components (g/kg diet): casein, 151; corn oil, 25; coconut fat, 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 (with and without copper), 10; vitamin premix, 12. The purified diets, which were in powdered form, were stored at 4 °C until feeding. Diets and demineralized water were freely available. Feed consumption and body weight were recorded weekly. The experiment lasted 60–70 d.

The two diets were analyzed for their copper content. The copper concentration (mg Cu/kg diet) were: 0.4 $\pm$ 0.1 for the copper-deficient and 5.1 $\pm$ 0.2 for the copper-adequate diet (means $\pm$ SEM, n = 3).

The protocol for this work and facilities were approved by the animal experiments committee of the Erasmus University of Rotterdam.

Chemical analyses. Feed samples (1 g) were ashed at 500 °C for 17 h and dissolved in 6 mol/L HCl. Liver samples were freeze-dried and ashed as described for feed samples. Plasma samples were freeze-dried, and then digested with 14 mol/L HNO_3 at 80 °C until a clear solution was obtained. All copper analyses were determined with flame atomic absorption spectrophotometry (Varian AA-475; Varian Techtron, Springvale, Australia).

Characterization of copper status of the rats. On d 42 all animals of each treatment group were injected intraperitoneally with 0.25 mL of acetate buffer (0.05 mol/L, pH 5.4) of 0.15 mmol/L [^{64}Cu] Cu-acetate (5 MBq/mmol; Interfaculty Reactor Institute, Delft). Radioactivity in individual rats was counted in a specially designed whole-body counter for rats (2) during 4 d every 8 h after dosing.

Blood was obtained from the portal vein canula, just before perfusing the liver, into heparinized test tubes, centrifuged and plasma stored at –20 °C until analysis. Liver samples were obtained just after perfusion of the liver and stored at –20 °C. In addition, a sample of the liver homogenate was taken for copper determination and stored at –20 °C.

Vesicles isolation. Rat liver membrane vesicles were prepared by the method of van Amelsvoort et al. (15), as modified by Rosenthal et al. (12). Rats were anesthetized by Nembutal (Sanofi Sante Animale SA, Paris, France), and the liver was perfused, after severing the inferior vena cava, with approximately 25 mL of isotonic sucrose buffer A (0.25 mol/L sucrose, 0.2 mmol/L $CaCl_2$, 10 mmol/L HEPES-KOH, pH 7.5) heated to 37 °C, through the portal vein with a 23G needle to remove blood. All subsequent procedures were carried out at 4 °C.

The livers were excised, weighed and a sample was taken for copper analysis. The rest of the livers were dissected into smaller fragments while in sucrose buffer A (3 volumes buffer to 1 volume liver), which was discarded and changed repeatedly. The liver was homogenized in sucrose buffer A, in a loose-fitting Dounce homogenizer (0.14 diameter clearance; Braun-Melsungen A.G., Melsungen, Germany) with 12 strokes. After taking samples for copper analysis and for comparison of enzyme activities, the homogenate was centrifuged at 1000×g for 10 min and the supernatant was decanted and spun at 20000×g for 30 min, using a Beckman Spinco L50 ultracentrifuge.

The resulting pellet was resuspended in sucrose buffer A and layered on top of a discontinuous gradient prepared from 460 and 210 g/L sucrose and spun at 50000×g for 2.5 h, using a SW 41 Ti rotor. Membranes isolated at the 210/460 interface of the sucrose gradient were collected and diluted with isotonic sucrose buffer B (0.25 mol/L sucrose, 0.2 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 10 mmol/L HEPES-KOH, pH 7.5) and sedimented at 80000×g for 40 min, using a type 70.1 Ti rotor. This produced a cloudy buff-coloured layer overlying a reddish-brown pellet; only the cloudy layer was collected and resuspended in isotonic buffer B.

The isolated membranes were vesiculated by repeated passage through a 19G needle. Aliquots of vesicles and homogenate were stored at –70 °C.

Assay of enzymes and protein. The activities of cytochrome c reductase (EC 1.9.3.1) (6) and 5^1-nucleotidase (EC 3.1.3.5) (1) were measured in both homogenate and final membrane fractions. The relative enrichment of 5^1-nucleotidase, a plasma-membrane marker enzyme, in the plasma membrane fraction (10–12 fold) was comparable with those reported by others (6), and cytochrome oxidase could not be detected in the final vesicles. There were no differences between the control and copper-deficient rats.

Protein concentrations were measured by the Bio-Rad protein assay (3), using bovine serum albumin as standard. The yield of isolated membranes was 0.7–1.2 mg membrane protein/g wet weight of liver; not different for the two treatment groups.

Experimental methods. ^{64}Cu was prepared by neutron bombardment and was used within 12h. Specific activities were between 150 and 250 MBq/mg Cu.

The incubation medium was as follows: 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$ and 10 mmol/L HEPES, pH 7.4. [^{64}Cu] Cu-acetate was mixed with histidine (pH 7.0) at a ratio of at least 1:10 before added to the incubation medium. The incubation medium, containing [^{64}Cu] $CuHis_2$ (2 µmol/L, 18 MBq/L), and with appropriate additions, was incubated at 25 °C before starting the reaction by adding the vesicles (final concentration 250 µg/mL). At appropriate times (1 min, unless otherwise stated), aliquots

(80 μL) were filtered through 0.45-μm Millipore filters (pre-washed with 9 g/L NaCl containing 500 μmol/L $CuCl_2$ (8) and washed three times with 2 mL of ice-cold incubation medium with 10 mmol/L EDTA. The filters were removed from the filtration apparatus (Hoefer Scientific Instruments, San Fransisco, USA), and radioactivity measured in a Packard 5000 γ-counter (Canberra Packard, Brussels, Belgium). Control experiments (filtering 80-μL aliquots without vesicles) demonstrated that background binding to filters was negligible (< 5% of radioactivity when vesicles were present), but appropriate blanks (incubation medium with appropriate additions and sucrose) were always included.

Uptake measurements were made by incubating the vesicles with a constant amount of radiolabeled [^{64}Cu] $CuHis_2$ and increasing amounts of unlabeled $CuHis_2$ (0 to 15 μmol/L). Estimates of apparent K_m or $K_{0.5}$ and V_{max} were made on the untransformed data of each preparation using an iterative nonlinear least squares method (Ultrafit, Biosoft, Cambridge, UK). Correlation coefficients always exceeded 0.95 and data did not fit equations that included nonspecific binding parameters.

Ceruloplasmin [EC 1.16.3.1] in plasma was measured by its 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 Sunderman & Nomoto (14).

Zinc uptake measurements. Zinc uptake was measured using essentially the same method. Vesicles were incubated in incubation medium containing ^{65}Zn added as [^{65}Zn] $ZnCl_2$. In those experiments measuring uptake at different concentrations, unlabelled $ZnCl_2$ was added to the medium. Samples were taken after one minute and filtered through Millipore filters as described above. Washing and pre-washing was also carried out as described, except that the medium contained 0.5 mM $ZnCl_2$. Binding to the filters was also negligible in these experiments (< 5% total binding).

Statistical analyses. Results are expressed as means ± standard deviation. Correlations were measured using least squares regression, and considered significant when $p<0.05$. Data were fitted to equations using an iterative curve fitting program (Ultrafit, Biosoft, Cambridge, UK). Differences between group means were evaluated with Student's t-test when the data were normally distributed. The level of significance was preset at $p<0.05$.

RESULTS

Copper status of the rats. The rats from which the rat liver membrane vesicles were isolated were maintained on a copper-adequate or copper-deficient diet for 8 weeks. This was sufficient to deplete liver copper levels by 40% (Table 1). Copper deficiency was

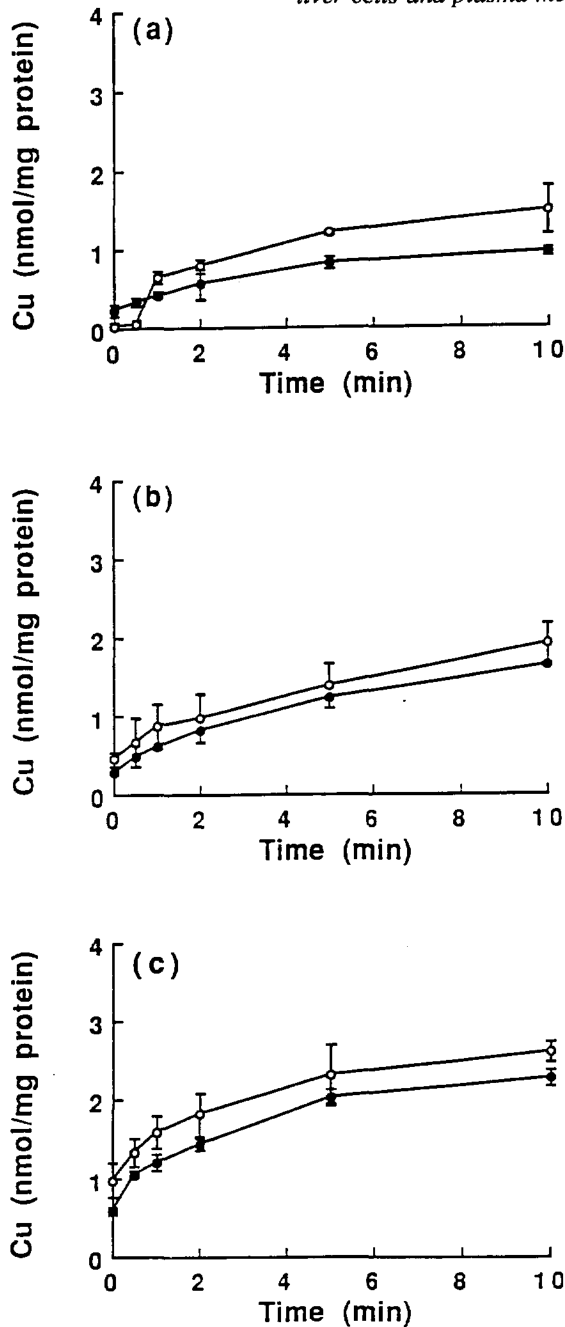

Figure 1. *^{64}Cu uptake by vesicles at (a) 4 ℃, (b) 25 ℃ and (c) 37 ℃ isolated from copper-adequate (●) and copper-deficient (○) rats. Vesicles were incubated as described in Materials and Methods before being separated from the medium by passing through 0.45-μm Millipore filters. The results shown are the mean ± SD of three vesicle preparations and are expressed as nmol Cu/mg vesicle protein.*

Table 1. *Characterization of rats fed a copper-adequate or a copper-deficient diet for 8 wk*[1].

Characteristic	Copper-adequate	Copper-deficient
Final body weight, g	214 ± 6	193 ± 7
Liver wet weight, g	6.4 ± 0.2	6.2 ± 0.3
Ceruloplasmin, g/L	0.505 ± 0.041	0.150 ± 0.040*
Liver, µg Cu/g dry wt	15.4 ± 1.6	9.7 ± 2.3*
Biological half-life of whole-body ^{64}Cu, d	7 ± 1	15 ± 2*

[1] Values are means ± SEM, n = 6.

* Significantly (Student's t-test; $p < 0.01$) different.

further confirmed by significantly lower plasma copper concentrations, depressed ceruloplasmin levels and by a higher ^{64}Cu retention as indicated by its biological half-life (Table 1). These results were in accordance to that previously reported (16).

Copper uptake. Copper uptake by vesicles occurred in approximately two phases. There was an initial rapid rise, followed by an increase approaching equilibrium at about 5 min (Fig. 1). At 4 °C only binding occured while uptake at 37 °C was higher than at 25 °C (Fig. 1).

The initial rates of binding and uptake at different concentrations were determined at 4 °C, 25 °C and 37 °C. At all temperatures, binding and uptake showed saturation (Fig. 2). At 4 °C, there was no evidence for co-operativity in the uptake process; when analysed according to the Hill equation, n was not significantly different from 1. The apparent K_m, or more correctly the $K_{0.5}$ for binding, for the normal and copper-deficient rats were not significantly different (Table 2) at any of the temperatures studied. In contrast, maximum binding was significantly higher in the deficient rat vesicles (Fig. 2).

At 25 °C a similar result was obtained (Fig. 2, Table 2). However, in this case, n values were close to 2, indicating that some form of co-operativety between binding or uptake sites was occuring. Once again, $K_{0.5}$ values were not changed and V_{max} values were higher in the vesicles from copper-deficient rats (Table 2).

At 37 °C V_{max} was further increased, as would be expected for a temperature dependent process, with the rate being higher for the copper-deficient rats (Table 2) and K_m being unaltered. Once again, n values were greater than 1, showing that there was co-operativity in the uptake process ($n = 1.69 \pm 0.01$ for control rats and $n = 1.73 \pm 0.06$ for copper-deficient rats).

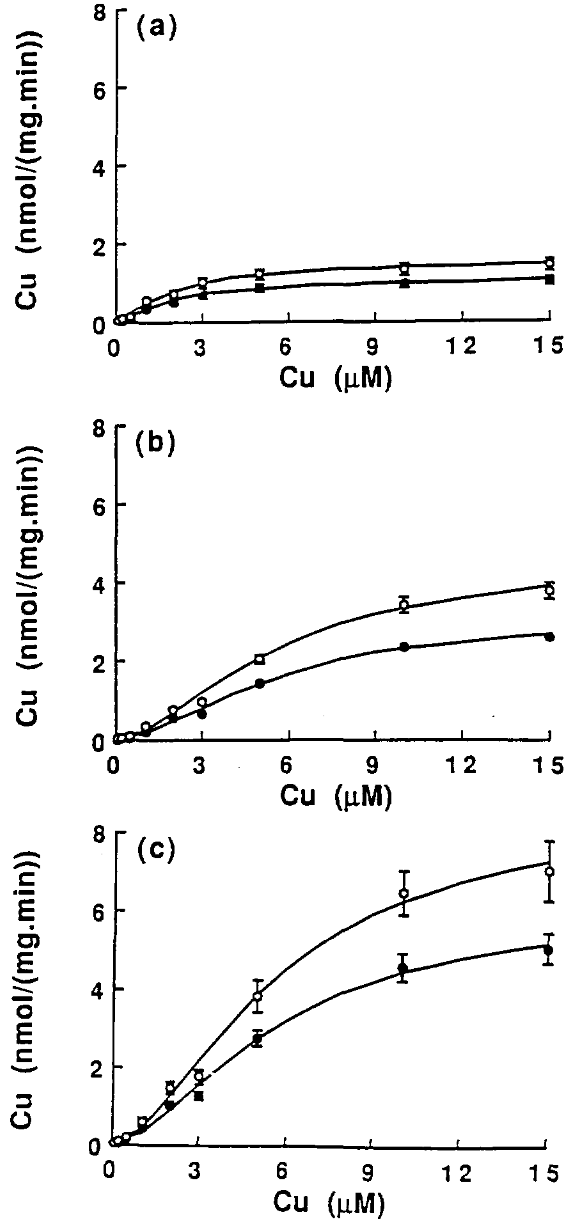

Figure 2. *Copper binding by vesicles at different Cu concentrations. Vesicles isolated from copper-adequate (●) and copper-deficient (○) rats were incubated at (a) 4 °C, (b) 25 °C and (c) 37 °C in increasing concentrations of [^{64}Cu] CuHis$_2$ for 1 min when aliquots were taken and passed through 0.45-μm nitrocellulose filters and washed as described in Materials and Methods. The results are the mean ± SD of three vesicle preparations. Data from each individual experiment were fitted to either the Hill equation or the Michaelis-Menten equation using a nonlinear iterative computer program (see Materials and Methods).*

Table 2. *Kinetic parameters, apparent V_{max}, B_{max} (at 4 °C), and K_m and $K_{0.5}$ (when n> 1) for vesicles isolated from copper-adequate and copper-deficient rats*[1].

Vesicles	V_{max} (B_{max}) nmol Cu/ (mg protein.min)	K_m ($K_{0.5}$) µmol Cu/L	n
4 °C:			
Copper-adequate	1.08 ± 0.10	2.28 ± 0.20	1.30 ± 0.03
Copper-deficient	1.53 ± 0.20*	2.29 ± 0.01	1.29 ± 0.01
25 °C:			
Copper-adequate	3.20 ± 0.25	5.76 ± 0.17	1.70 ± 0.06
Copper-deficient	4.66 ± 0.31*	5.72 ± 0.03	1.69 ± 0.01
37 °C:			
Copper-adequate	6.20 ± 0.49	5.72 ± 0.12	1.69 ± 0.01
Copper-deficient	8.62 ± 1.03*	5.68 ± 1.30	1.73 ± 0.06

[1] Values are means ± SD, n = 3. Data were fitted to the Hill equation using a nonlinear iterative computer program (see Materials and Methods). The B_{max} and apparent K_{max} values relate to data measured at 4 °C, when there is no uptake or co-operativity, while V_{max} and $K_{0.5}$ values are for data measured at 25 and 37 °C, where there is co-operativity for uptake.

* Significantly (Student's t-test; $p<0.05$) different.

Zinc uptake. To test the possibility of a generalised increase in transport, we measured zinc uptake by the vesicles. As may be seen in Figure 3, the parameters for uptake did not appear to be changed, showing that the modifications in the hepatocytes were specific for the copper transport system.

DISCUSSION

The kinetics of copper uptake by membrane vesicles isolated from copper-adequate and copper-deficient rats are quite similar to those found with hepatocytes isolated from normal and copper-deficient rats. These hepatocytes also show no change in K_m and an increase in V_{max} for uptake (16). Several possible explanations were put forward for these observations, but present data indicate that the adaptation does occur at the level of the cell membrane.

Two possibilities exist which explain the mechanism of adaptation; either the cells increase the rate of turnover of the transporter or they increase the numbers of receptors in the membrane. Of these two alternatives, the former seems more likely, since the difference between the two treatments still exist at 4 °C, when no uptake occurs, and modifications in the turnover rate would not be apparent.

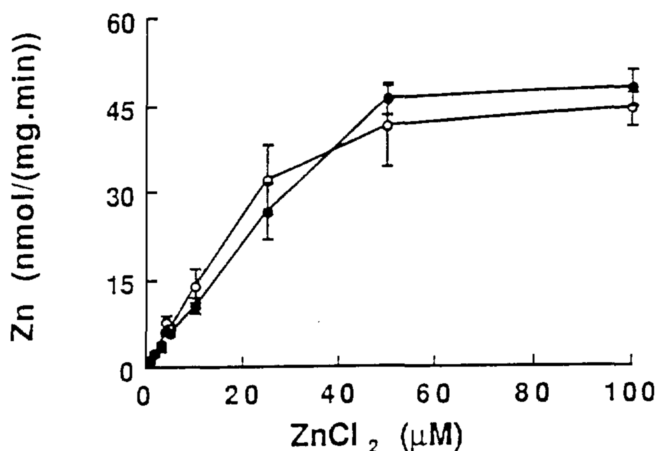

Figure 3. *Zinc uptake by vesicles isolated from copper-adequate (●) and copper-deficient (○) rats. Vesicles were incubated at different concentrations of zinc for 1 minute, after which aliquots were taken and filtered through 0.45-µm Millipore filters as described in Materials and Methods. The results are the mean ± SD of three vesicle preparations.*

The changes in uptake are specific for copper, with zinc uptake being unaltered, showing that the liver has recognition systems which give it the capacity to alter uptake in the presence of altered intracellular copper levels.

It is intriguing that analysing the data using the Hill equation rather than the simple Michaelis-Menten equation gives a better fit for the data and also gives a strong indication for co-operativity. Furthermore, co-operativity only becomes clearly evident at temperatures where uptake, rather than just binding, is taking place. Several groups have shown that the uptake process in the liver is inhibited by sulfhydryl reagents (9,18) and it has been suggested that the transporter is actually a dimer. Our data would support the hypothesis and would further suggest that there are two binding sites. Binding of Cu to one of the sites does not alter the probability of binding to the other, but once one atom is taken up, there is an increased probability of the other being transported. How this may operate in biochemical terms is not clear but one could envisage a mechanism where conformational changes in one molecule forces changes in the other such that the binding site becomes exposed to the interior face of the mebrane.

We have recently shown that uptake of copper is stimulated by a membrane-bound NADH-oxidase (van den Berg and McArdle, Ms submitted) and it is, as yet, unclear how the reduction which takes place prior to uptake may be involved in the co-operativity. Clearly,

much remains to be learned about the mechanism of copper uptake by the liver, but we would suggest that using vesicles isolated from plasma mebrane will help to provide some valuable information about the processes regulating the metabolism of this essential metal.

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9

A PLASMA MEMBRANE NADH OXIDASE IS INVOLVED IN COPPER UPTAKE BY PLASMA MEMBRANE VESICLES ISOLATED FROM RAT LIVER

ABSTRACT

The accumulation of copper (Cu) by hepatocytes is initiated by the binding of Cu in either a $CuHis_2$ complex or as a CuHisAlb ternary complex, followed by transfer of the metal alone across the cell membrane. In this paper, we provide evidence that the transfer involves reduction of cupric (Cu(II)) to cuprous (Cu(I)) and further we show that membrane-bound NADH oxidase can provide the electron required for the reduction. ^{64}Cu uptake by rat liver plasma membrane vesicles is stimulated by the addition of NADH, but not NAD^+. The stimulation increases the V_{max} from 4.75 ± 0.02 to 8.38 ± 0.40 nmol Cu/(mg protein.min) ($p < 0.05$, mean ± SEM, n = 3) without significantly altering the $K_{0.5}$ (1.52 ± 0.17 and 2.10 ± 0.22 µmol/L; with n values of 1.30 ± 0.01 and 1.43 ± 0.10 respectively). Correspondingly, addition of $CuHis_2$ stimulated NADH oxidase activity by a maximum of 7.4 ± 2.1 nmol/(mg protein.min) ($p < 0.01$, mean ± SEM, n = 5) at 5 µmol/L and a NADH concentration of 150 µmol/L. Ascorbic acid also stimulated uptake, presumably by the same mechanism. Our data indicate that there are membrane bound enzymes which can provide an electron for the reduction of copper prior to uptake and suggest a physiological role for the plasma membrane NADH oxidase.

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submitted

INTRODUCTION

The nutritional trace element copper (Cu) is essential to cellular functioning of all living systems, as a cofactor for several intracellular enzymes [1]. Following absorption across the intestine, copper is taken up by the liver and is later released attached to the protein, ceruloplasmin.

The copper that is taken up by the hepatocyte from portal blood is believed to derive from either the Cu-histidine ($CuHis_2$) [2,3], or the Cu-histidine-albumin (CuHisAlb) complex [4] in the plasma. It is possible that the complexes look similar, with the Cu atom coordinating with the imidazole-N and the amino-N of the histidine residues and the remainder of the binding moiety being irrelevant [4]. Before uptake, the Cu is removed from the complex and transferred alone into the cell [2–4].

How the Cu is removed from its ligand is not known. Preliminary data have shown that ascorbic acid can stimulate uptake in cultured rat hepatocytes [5; Bingham and McArdle, unpublished results] and mouse hepatocytes [McArdle, unpublished results]. The process has also been studied in relation to Cu uptake from ceruloplasmin by K562 cells. Percival and Harris [6] have shown that ascorbic acid increases Cu uptake, and that cuprous (Cu(I)) copper chelators block the effect.

We hypothesize, therefore, that reduction of cupric (Cu(II)) copper is a necessary prerequisite for Cu uptake by the hepatocyte. A reductase has been described which could fulfil this function [7]. However, there are no experimental data linking the activity of this enzyme with Cu uptake.

In this paper, we have tested this hypothesis. We have isolated plasma membrane vesicles from rat liver and have incubated them with [^{64}Cu] $CuHis_2$ in the presence of NADH, to determine whether uptake is stimulated. Similarly, we have investigated the effect of $CuHis_2$ on NADH oxidase activity. The results strongly support our theory that the plasma membrane NADH oxidase can provide electrons for the reduction of cupric (Cu(II)) to cuprous (Cu(I)) prior to uptake by the cell.

MATERIALS AND METHODS

Rat liver vesicle preparation. The membrane vesicles were prepared from the liver of adult male Wistar rats using the method of van Amelsvoort and co-workers [8], as modified by Rosenthal et al. [9] and Duthie and McArdle [unpublished data]. Briefly, the animals were anesthetized by Nembutal (Sanofi Sante Animale SA, Paris, France), and the liver was perfused, after severing the inferior vena cava, with approximately 25 ml of isotonic sucrose buffer A (250 mmol/L sucrose, 0.2 mmol/L $CaCl_2$, 10 mmol/L

Hepes–KOH, pH 7.5) heated to 37 °C, through the portal vein with a 23G needle to remove blood. All subsequent steps were carried out at 4 °C.

The liver was removed, weighed, washed and homogenized in 3 volumes of Buffer A. It was centrifuged at 1,000×g for 10 min and the pellet was discarded. The supernatant was then centrifuged at 20,000×g for 30 min. The pellet was resuspended in sucrose Buffer A and layered on top of a sucrose step gradient prepared from 210 and 410 g/L sucrose in 10 mmol/L Hepes-KOH. The membranes were centrifuged at 50,000×g in a SW 41 Ti rotor in a Beckman Spinco L50 centrifuge. Membranes isolated at the 210/460 g/L interface were collected, diluted with isotonic sucrose and sedimented at 80,000×g in a 70.1 Ti rotor. This produced a cloudy, buff coloured layer overlaying a reddish brown pellet. Only the cloudy layer was taken and resuspended in isotonic Buffer B (as buffer A but with 5 mmol/L $MgCl_2$). The membranes were vesiculated by passing through a 19G needle, aliquoted and stored at –70 °C at a concentration of about 5 mg protein/ml. Control experiments have shown that vesicles stored at –20 °C lost transport activity. 5'-nucleotidase activity was increased on average 10 fold in the final pellet, indicating a good degree of purification [10].

Preparation of ^{64}Cu labeled medium. ^{64}Cu was prepared by irradiation of 3 mg copper wire (99.999%, Ventron, Karlsruhe, Germany) for 24 h in a thermal neutron flux of $10^{17}/m^2$ per s in the IRI reactor. The ^{64}Cu solution produced (diluted in 50 mM sodium acetate buffer, pH 5.4) had a specific activity of about 150 MBq/mg.

Unless otherwise stated, the copper was added as 2 μmol/L copper di-histidine ($CuHis_2$) at 5 MBq/L diluted in Balanced Salt Solution (BSS), (136 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 4 mmol/L $NaHCO_3$, 18 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4). ^{64}Cu solution was mixed with histidine (pH 7.0) at a ratio of at least 1:10 before added to the incubation medium.

Experimental procedures. Stimulating agents were added to the medium with vesicles (final concentration 250 (μg/ml) at the concentrations given in the figure legends, and the reaction was started by adding the [^{64}Cu] $CuHis_2$. All uptakes were carried out at 25 °C for 1 min. Aliquots (80 μl) were filtered through 0.45 μm Millipore filters (pre-washed with 9 g/L NaCl containing 0.5 mmol/L $CuCl_2$ and washed three times with 2 mL of ice-cold incubation medium with 10 mmol/L EDTA [11]. The filters were removed from the filtration apparatus (Hoefer Scientific Instruments, San Fransisco, CA), and radioactivity measured in a Packard 5000-gamma counter. Control experiments (filtering 80 μl without vesicles) demonstrated that background binding to filters was neglible, but appropriate blanks (incubation medium with appropriate additions and no vesicles) were always included.

Uptake measurements were made by incubating the vesicles with a constant amount of [^{64}Cu] $CuHis_2$ and increasing amounts of unlabeled $CuHis_2$ (0 to 15 μmol/L), in the

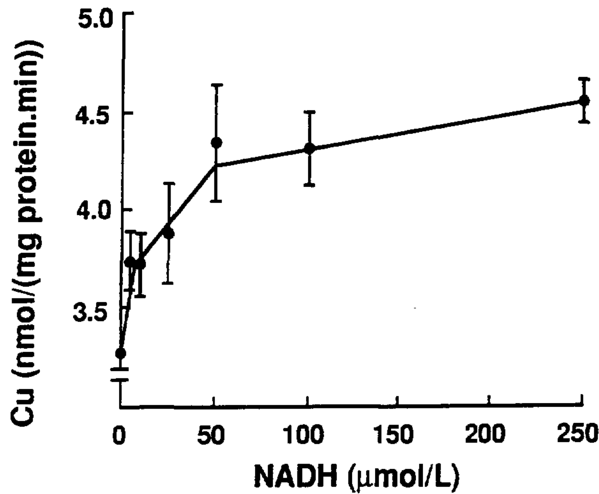

Figure 1. *Effect of NADH on copper uptake by rat liver plasma membrane vesicles at 25 °C for 1 min. Vesicles were incubated with various concentrations of NADH and a [^{64}Cu] CuHis$_2$ concentration of 2 μmol/L. The results are the mean ± SD of five preparations.*

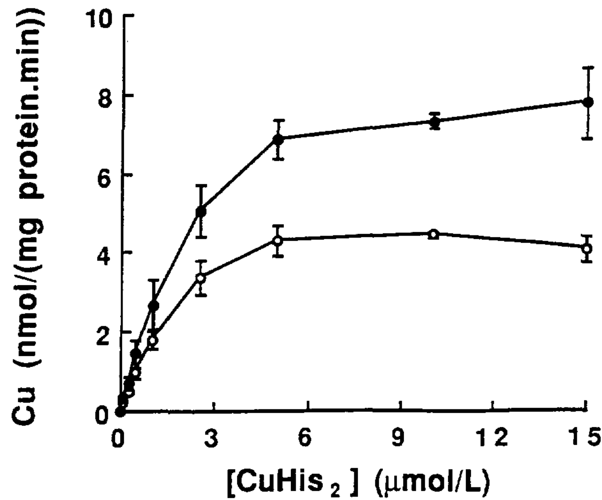

Figure 2. *Uptake of Cu in the absence (○) or presence of 1 mmol/L NADH (●). The uptake rate was measured over 1 min at 25 °C in the presence of increasing concentrations of [^{64}Cu] CuHis$_2$. The results are the mean ± SD of five preparations of vesicles.*

absence or presence of 1 mmol/L NADH (Boehringer, Mannheim, Germany). Estimates of apparent K_m or $K_{0.5}$ and V_{max} were made on the untransformed data of each preparation using an iterative nonlinear least square method (Ultrafit, Biosoft, Cambridge, UK), and considered significantly different when $p<0.05$. Correlation coefficients always exceeded 0.98 and did not fit equations that included nonspecific binding parameters.

NADH oxidase assay. Plasma membrane NADH oxidase was assayed at 25 °C in BSS buffer (pH 7.4), 150 µmol/L NADH (Boehringer, Mannheim, Germany) with 1 mmol/L KCN [12], to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. The assay was started with the addition of 0.1 mg of plasma membrane protein in a final volume of 2.0 ml. The reaction was monitored by the decrease in the absorbance at 340 nm. A blank rate was determined for 5 min. $CuHis_2$ was then added, and the rate measured for a further 10 min. The absorption coefficient used for NADH was 6.21 mM^{-1} cm^{-1}. Protein assays were carried out using the Biorad protein method as described by Bradford [13], using bovine serum albumin as standard.

RESULTS

Addition of NADH stimulated uptake of ^{64}Cu from [^{64}Cu] $CuHis_2$ (Fig. 1). The increase occurred rapidly to a concentration of 50 µM, thereafter increasing more slowly. The stoichiometry of the process (0–50 µmol/L) can be calculated and was found to be 0.91 ± 0.19 ($p< 0.001$, mean $\pm$ SEM, $n= 5$), suggesting that 1 mole of NADH was oxidized per Cu^{2+} transported.

To determine how NADH altered Cu uptake, we incubated the vesicles with increasing $CuHis_2$ concentrations in the presence and absence of 1 mmol/L NADH. The results are shown in Figure 2. NADH stimulated Cu uptake, increasing V_{max} without altering the $K_{0.5}$ (Table 1). As has previously been shown, the n value is greater than 1, indicating co-operativity in the transport process, but n was not altered by the presence of NADH (Table 1).

The effect is specific for NADH, since adding NAD^+ had no effect on uptake (Fig. 3).

The corollary of the effect was assessed by incubating vesicles with NADH in the presence of increasing $CuHis_2$ concentrations. In the absence of added metal, there was a slow oxidation of NADH, as previously reported [7]. The activity was also stimulated by diferric transferrin (data not shown). In the presence of $CuHis_2$, NADH oxidase activity increased, reaching a maximum at about 2 µM $CuHis_2$, with an NADH concentration of 0.15 mM (Fig. 4). As with the analysis of Figure 1, the stoichiometry was determined to be close to 1 (1.1 ± 0.5, $p<0.01$, mean $\pm$ SEM, $n= 5$).

Table 1. *Kinetic parameters, apparent V_{max}, $K_{0.5}$ and n-values, for vesicles incubated in the absence or presence of 1 mmol/L NADH at 25 °C[1].*

NADH mmol/L	V_{max} nmol Cu/ (mg protein.min)	$K_{0.5}$ µmol Cu/L	n
0	4.75 ± 0.02	1.52 ± 0.17	1.30 ± 0.01
1	8.38 ± 0.40*	2.10 ± 0.22	1.43 ± 0.10

1 Values are means ± SEM, n = 5. Data were fitted to the Hill equation using a nonlinear iterative computer program (see Materials and Methods).

* Significantly (Student's t-test; $p<0.05$) different.

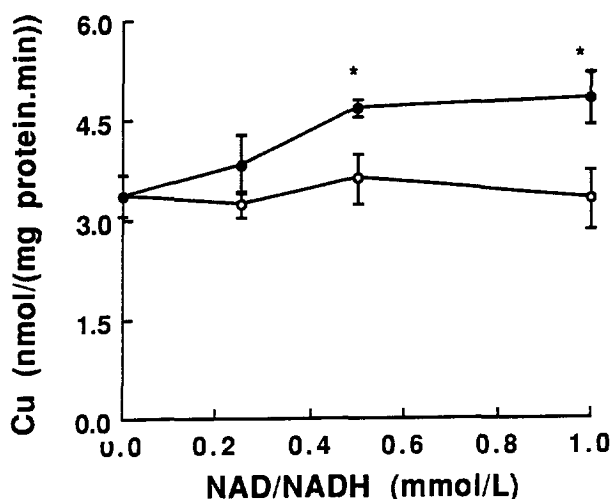

Figure 3. *Effect of NADH or NAD on copper uptake by rat liver plasma membrane vesicles. Vesicles were incubated with 1 mM NADH (●) or NAD^+ (○) and a [^{64}Cu]CuHis$_2$ concentration of 2 µmol/L for 1 min at 25 °C. The results are the mean ± SD of three preparations.*

To determine whether other electron donors could also stimulate uptake, we incubated vesicles in the presence of increasing concentrations of ascorbate and 2 µmol/L [^{64}Cu] CuHis$_2$. As expected, uptake was also increased (Fig. 5) showing that the transport system does not have an absolute requirement for NADH derived electrons.

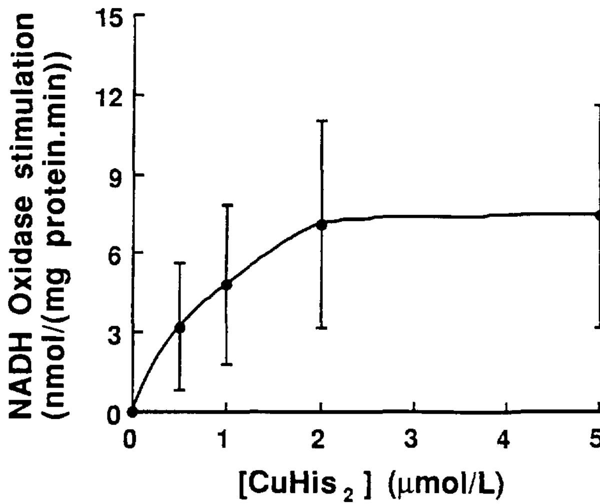

Figure 4. *Oxidation of NADH by rat liver plasma membrane in the presence of $CuHis_2$. In the values presented in the figure, the NADH oxidase activity, prior to the $CuHis_2$ addition was subtracted from the rate of NADH oxidation after $CuHis_2$ addition. All assays contained BSS buffer (pH 7.4), 1 mmol/L KCN and 150 μmol/L NADH and about 0.1 mg plasma membrane protein. The reaction was initiated by the addition of plasma membranes. Absorbance was measured at 340 nm.*

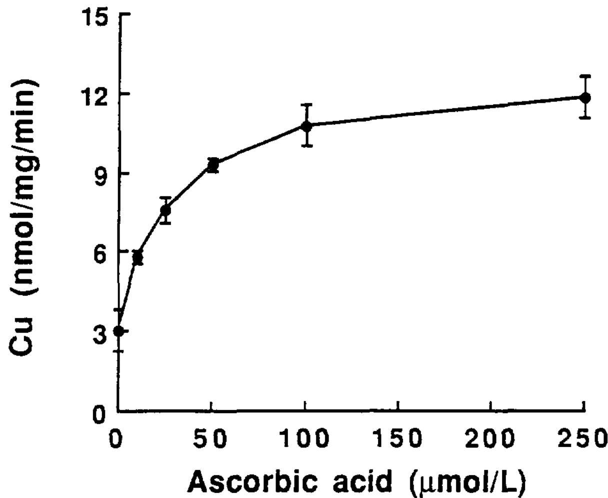

Figure 5. *The effect of ascorbic acid on copper uptake by rat liver plasma membrane vesicles at 25 °C for 1 min. Vesicles were incubated with various concentrations of vitamin C and a [^{64}Cu] $CuHis_2$ concentration of 2 μM. The results are the mean ± SD of four preparations.*

DISCUSSION

Several cell types, including hepatocytes, have been shown to reduce impermeable oxidants such as ferricyanide outside the cell [14] and it was demonstrated that this occurred without the secretion of large quantities of reducing agents. An NADH-oxidizing system in plasma membranes of rodent liver was first identified cytochemically by Morre et al [15] and the finding was supported biochemically by Crane and Low [16].

For many years, the function of this enzyme has remained unclear. Originally, it was thought that it may act to reduce transferrin iron [cf. 17], but nowadays this is not generally accepted. The activity can be modulated by growth factors and hormones, indicating that it may be involved in cell growth regulation [12,14]. We can now suggest that the enzyme is also involved in Cu uptake by the liver.

Copper is delivered to the liver as either $CuHis_2$ or as a CuHisAlb ternary complex [2–4]. Either way, the complex has an extremely high affinity for Cu(II). The way the copper can be removed is to reduce it. How this may be accomplished has previously been unclear.

In K562 cells, Percival and Harris [6] have shown that ceruloplasmin copper can be accumulated through a reduction process, but have suggested that ascorbic acid may play the central role or that electrons can be transported across the cell membrane. Our data show clearly that NADH can provide the electrons for the reduction of Cu(II), as the first step in the Cu uptake process. The stoichiometry of 1:1 fits well with each NADH oxidized providing one electron for each Cu(II). Whether the enzyme involved is NADH oxidase or NADH-ferricyanide reductase, which have been shown to be separate enzymes [12], is not so clear.

How the transport system is associated with the enzyme(s) remains to be investigated. Since ascorbic acid also stimulates uptake, it is unlikely that the enzyme itself is the transporter. Rather, we would suggest that they are in close proximity on the plasma membrane.

Our model is presented in Fig. 6. It incorporates not only data presented in the present paper but uses also information gained earlier by ourselves and others [18,6]. The transporter is a dimer, linked by at least one disulphide bridge. There is co-operativity between the binding sites, although the mechanism at the molecular level is not known. The oxidation of NADH provides the electron required for releasing Cu(II) from its associated ligand and it is moved through the membrane to the cell. Within the cell we have suggested that it is first bound to glutathione as has been demonstrated by Freedman and collaborators [19].

Clearly, the model is preliminary but does explain most of our current knowledge of Cu uptake across the liver, in fact it may well be that this mechanism underlies uptake of copper from all the ligands available to the metal in plasma [6,18].

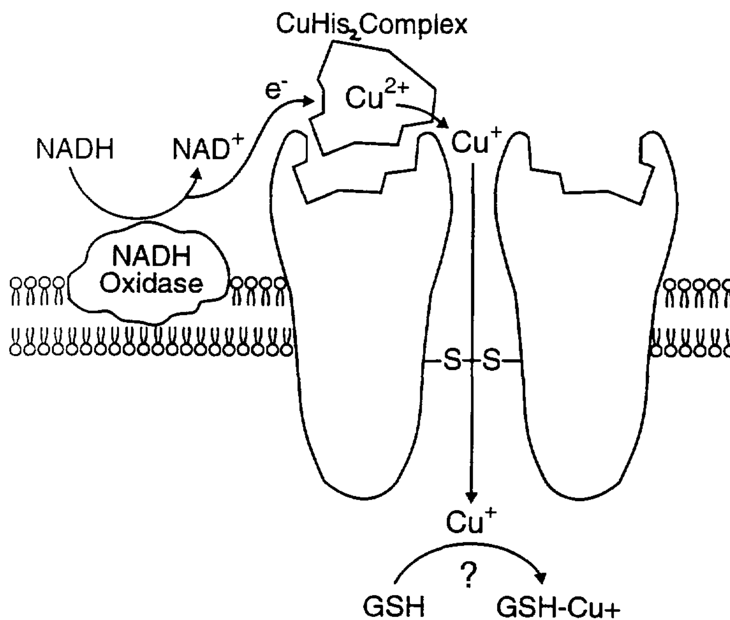

Figure 6. *Scheme of copper uptake from CuHis*$_2$.

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10

COPPER STATUS IN RATS FED DIETS SUPPLEMENTED WITH EITHER VITAMIN E, VITAMIN A OR β-CAROTENE

ABSRACT

Copper status was measured in rats fed copper-adequate purified diets supplemented with either vitamin E (250 IU/kg), vitamin A (40,000 IU/kg), or β-carotene (2 g/kg). It was hypothesized that the extra intake of the antioxidants would spare vitamin C resulting in a decreased copper status as shown previously after supplementation with vitamin C. A significant increase in plasma ascorbate concentration was observed after β-carotene supplementation, but not after supplemental vitamin E or vitamin A. Extra intake of either β-carotene or vitamin A slightly, but significantly, raised plasma copper concentrations. β-Carotene also slightly raised liver copper concentration. Supplemental vitamin E had no effect on plasma and liver copper concentrations. It is concluded that the observed, relatively small effects of supplemental vitamin A and β-carotene on copper status in rats are not mediated by changes in plasma vitamin C concentration.

Biological Trace Element Research 37 (1993) 253-259

IINTRODUCTION

In rats fed diets supplemented with vitamin C (ascorbic acid), indicators of copper status such as plasma ceruloplasmin oxidase activity and copper concentrations in plasma and liver are decreased (1,2). High doses of vitamin C also lower plasma copper concentrations in guinea pigs (3) and humans (4). The impairment of copper status by supplemental vitamin C is associated with increased levels of ascorbate in plasma (1,2,4–7), which may be in part responsible for the change in copper metabolism (2).

Dietary supplementation with vitamin E raises plasma levels of vitamin C in rats (5,8) and guinea pigs (6), suggesting that the antioxidant property of vitamin E spares vitamin C. Other vitamins with antioxidant properties, such as β-carotene and vitamin A, may also affect the circulating level of vitamin C (9). This study with rats was performed to obtain clues whether dietary supplementation with either vitamin E, vitamin A, or β-carotene affects copper status as a result of influencing the concentration of ascorbate in plasma.

MATERIALS AND METHODS

Animals and Diets. Male outbred Wistar rats (Cpb:WU), about 21 d old, were used. The rats were derived from the colony of the Laboratory Animals Center, Wageningen Agricultural University, The Netherlands. All rats went through a pre-experimental period of 10 d during which the purified control diet containing 5 mg Cu/kg (Table 1) and demineralized water were supplied *ad libitum*. The control diet was formulated according to the nutrient requirements of rats (10). The rats were housed in groups of 4–5 animals in stainless steel cages (60×21×19 cm) with wire mesh bases. The cages were placed in a room with controlled temperature (20–22 °C), humidity (55–75%) and lighting (12-h light cycle).

At the end of the preexperimental period (day 0 of the experiment), the rats were divided into 4 groups of 12 animals each on the basis of body weight. One group remained on the control diet. The other groups received the control diet supplemented with either vitamin E (250 IU/kg), vitamin A (40,000 IU/kg) or β-carotene (2 g/kg). The composition of the experimental diets is given in Table 1. The diets and demineralized water were provided *ad libitum*. The powdered diets were stored at 4 °C until feeding.

During the experiment, which lasted 42 d, the rats were housed individually in metabolic cages (Tecniplast Gazzada, Buguggiate, Italy). Food intake was recorded and the rats were weighed weekly. Feces of each rat were collected quantitatively during day 35–40, and stored at –20 °C until analysis. At the end of the experiment the animals were anesthetized with diethyl ether, and blood samples were collected in heparinized tubes by orbital puncture. The anesthetized animals were killed by decapitation. Liver and femur were removed and immediately frozen at –20 °C until analysis.

Table 1. *Composition of the purified, experimental diets.*

	Dietary Supplement			
	None = control	Vitamin A (40,000 IU/kg)	β-Carotene (2 g/kg)	Vitamin E (250 IU/kg)
Components (mg or g/kg diet)				
Glucose, g	329.7	329.7	319.7	329.45
Corn starch, g	329.7	329.7	319.7	329.45
Vitamin A[1], mg	–	80	–	–
β-Carotene[2], g	–	–	20.0	–
Vitamin E[3], mg	–	–	–	500
Constant components[4], g	340.6	340.6	340.6	340.6

1 Rovimix A 500[R], 522,000 IU/g (F. Hoffman-La Roche & Co Ltd, Basel, Switzerland).

2 Beadlets, 100 mg β-carotene/g (F. Hoffman-La Roche & Co Ltd, Basel, Switzerland).

3 D,L-alpha-tocopheryl acetate, 500 IU/g (Boom, Meppel, The Netherlands).

4 The constant components consisted of (g/kg diet): casein, 151; corn oil, 25; coconut fat, 75; cellulose, 30; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; $NaH_2PO_4.2H_2O$, 15.1; $CaCO_3$, 12.4; 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; $CuSO_4.5H_2O$, 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; corn meal, 9679.2. The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacinamide, 20; D,L-calcium panthothenate, 17.8; choline chloride, 2000; pyridoxine, 6; cyanocobalamin, 50; folic acid, 1; biotin, 2; menadione, 0.05; D,L-alpha tocopheryl acetate, 60; retinyl acetate and retinyl palmitate, 8 (4000 IU); cholecalciferol, 2 (1000 IU); corn meal, 9826.15.

Chemical analyses. Blood hemoglobin and hematocrit were determined using a hematology series cell counter (100[TM], Baker Instruments, Windsor, UK). Plasma ceruloplasmin was estimated by measurement of its p-phenylenediamine oxidase activity (11). Feces were freeze-dried, ashed at 500 °C for 18 h and dissolved in 6 mol HCl/L. Liver and femur were freeze-dried and then digested with 65% nitric acid and 30% hydrogen peroxide. Feed samples were pretreated as described for feces. Copper analyses were performed by flame atomic absorption spectroscopy (Varian AA-475, Varian Techtron, Springvale, Australia). Vitamin C (ascorbic acid) in plasma (12) and vitamin E (13), vitamin A and β-carotene (14) were estimated as described.

Statistical analyses. Results are presented as means ± SD. All results within diet groups were found to be normally distributed (Kolmogorov-Smirnov one-sample test).

Student's two-sample t-test was used to evaluate differences between dietary groups. To take into account the increased probability of a type I error because of multiple comparisons, the criterion of statistical significance was pre-set at $p < 0.017$ instead of $p < 0.05$. All statistical analyses were carried out using a SPSS/PC$^+$ software package.

RESULTS

Table 2 shows that final body weight, growth and feed intake were not significantly influenced by any of the three supplements. Vitamin E and β-carotene concentrations in plasma were significantly increased after dietary supplementation (Table 2). By contrast, plasma vitamin A concentrations were unaffected by supplemental vitamin A. Plasma vitamin C concentrations were significantly elevated after dietary supplementation with β-carotene, but supplemental vitamin E and vitamin A had no noticeable effect.

The selected indicators of copper status were not affected by dietary supplementation of vitamin E (Table 3). Plasma copper concentrations were significantly increased after supplementation with either vitamin A or β-carotene. Liver copper concentration was

Table 2. *Growth performance and plasma vitamin concentrations of rats fed the experimental diets for 42 d*[1].

	Dietary Supplement			
	None = control	Vitamin A (40,000 IU/kg)	β-Carotene (2 g/kg)	Vitamin E (250 IU/kg)
Body weight, g				
Initial, day 0	77 ± 8	77 ± 9	77 ± 8	77 ± 8
Final, day 42	254 ± 23	264 ± 27	256 ± 22	257 ± 24
Growth, g/d	4.1 ± 0.4	4.4 ± 0.5	4.2 ± 0.4	4.2 ± 0.5
Feed intake, g/d	16.7 ± 1.5	17.2 ± 1.4	16.6 ± 1.4	16.8 ± 1.2
Vitamin E, µmol/L	16.0 ± 3.6	17.8 ± 4.2	18.4 ± 2.1	30.6 ± 5.5*
Vitamin A, µmol/L	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.2
β-Carotene, µmol/L	< 0.02	< 0.02	0.99 ± 0.37*	< 0.02
Vitamin C, µmol/L	39.3 ± 8.4	38.3 ± 8.9	47.0 ± 7.1*	37.0 ± 5.0

[1] Results are given as means ± SD for 12 animals per dietary group.

* Significant difference ($P < 0.017$) versus dietary group without supplement.

Table 3. *Copper status of rats fed the experimental diets for 42 d*[1].

	Dietary Supplement			
	None = control	Vitamin A (40,000 IU/kg)	β-Carotene (2 g/kg)	Vitamin E (250 IU/kg)
Ceruloplasmin, g/L	0.37 ± 0.07	0.35 ± 0.07	0.38 ± 0.07	0.39 ± 0.05
Plasma Cu, μg/mL	1.03 ± 0.08	1.15 ± 0.12*	1.19 ± 0.09*	1.07 ± 0.09
Liver Cu, μg/g dry wt	14.86 ± 2.53	15.20 ± 2.17	17.00 ± 2.19*	15.52 ± 1.78
Femur Cu, μg/g dry wt	3.41 ± 0.32	3.53 ± 0.36	3.69 ± 0.28	3.51 ± 0.31

[1] Results are given as means ± SD for 12 animals per dietary group.

* Significant difference (P< 0.017) versus dietary group without supplement.

significantly raised by supplemental β-carotene, but was not altered by vitamin A. None of the three supplements influenced plasma ceruloplasmin concentrations.

Intake of copper by the control rats was 87.5 ± 6.9 μg/d (means ± SD, n= 12) as based on chemical analysis of the diet and feed intake data. Copper intakes by the three experimental groups were almost identical to that by the control rats. Fecal excretion of copper by the control rats was 67.5 ± 11.2 μg/d (means ± SD, n= 12). Copper excretion in feces was not significantly influenced by supplemental vitamin A, β-carotene or vitamin E.

Hemoglobin and hematocrit values in the control rats were 9.4 ± 0.3 mmol/L and 47.8 ± 1.7% (means ± SD, n= 12). Any of the three supplements did not significantly affect hemoglobin and hematocrit values.

DISCUSSION

As would be expected (5,8), dietary supplementation with vitamin E and β-carotene resulted in higher plasma concentrations of vitamin E and β-carotene, respectively. Vitamin A concentration of plasma was not influenced by supplemental vitamin A, owing to tight metabolic control of the plasma concentration of this vitamin (15). Thus, the observed effects of supplemental vitamins on their plasma concentrations indicate that this study can be considered suitable to address its objective.

Dietary supplementation with β-carotene significantly increased plasma vitamin C levels. In a study with humans, β-carotene supplementation also raised plasma vitamin C levels (16). Although there is evidence in the literature (9) that supplemental vitamin A raises plasma vitamin C concentrations, this was not seen in the present study. Dietary supplementation with vitamin E did not affect plasma vitamin C concentrations, which

contradicts previous results in rats (5,8) and guinea pigs (6). The reason for this discrepancy is not evident.

It is clear from this study that copper status was not substantially influenced by the experimental treatments. Supplemental vitamin A and β-carotene had a slight, but significantly elevating effect on plasma copper concentrations. β-Carotene also slightly raised liver copper concentrations in the rats. Supplemental vitamin E did not influence the selected indicators of copper status. This study also indicates that supplemental vitamin A and β-carotene did not affect copper status through altering plasma vitamin C concentrations. The two lines of evidence are as follows. First, vitamin A and β-carotene had similar effects on copper status, but only β-carotene influenced plasma vitamin C concentration. Second, a rise in vitamin C as induced by supplemental vitamin C reduces copper status (1,2), but β-carotene raised plasma vitamin C concentration which was associated with a slightly improved copper status.

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GENERAL DISCUSSION

High intakes of ascorbic acid impair copper status. Earlier studies in various animal species [1-4] and humans [5,6] indicate a negative interaction of dietary ascorbic acid with copper bioavailability. The action of the ascorbic acid has not been defined, although studies in rats [7] suggest that ascorbate reduces the intestinal absorption of copper. More recent studies indicate a facilitating role for the vitamin in post-absorptive copper metabolism, although it is not well understood [8]. Thus, two areas are of physiological importance in the interaction between ascorbic acid and copper, that is during the *pre-absorptive stage*, viz. at the intestinal absorption level, and during the *post-absorptive stage*, viz. at the level of hepatic copper uptake and biliary copper excretion.

Suitability of the rat as model to study ascorbic acid-copper interactions. Chapters 1, 2, 4, 6 and 10 show that feeding of rats on diets enriched with ascorbic acid resulted in marked increases in plasma ascorbate concentrations. Dietary concentrations of ascorbic acid above 1 g/kg diet, equivalent to an average daily intake of 20 mg/rat, does not much further increase plasma ascorbate concentrations. This is comparable with literature data for rats [9,10], guinea pigs [9], mice [11], and humans [12], in which intake of excessive amounts of ascorbic acid resulted in an increase in urinary excretion of ascorbate [9,11,12]. A high variance in plasma ascorbate levels in our studies was noticed, a phenomenon that has also been reported for humans [12]. Because ascorbate status can be modulated by ascorbic acid intake, the rat as used in our studies is considered to be a suitable model to study the effects of dietary ascorbic acid supplementation.

Ascorbic acid-copper interactions at the pre-absorptive stage. Ascorbic acid has been frequently reported to decrease apparent copper absorption in rats [4,7]. The studies described in this thesis are confirmatory on this point [Chapter 2]. Chapters 4 and 6 show that duration of ascorbic acid feeding is an important factor in the interpretation of the balance data. Dietary ascorbic acid lowered apparent absorption after periods of up to 4 weeks, but not after periods longer than 6 weeks. Dietary ascorbic acid depressed tissue copper concentrations after long, but not after short feeding periods. The ascorbate-induced lowering of copper status may have increased the efficiency of copper absorption. Thus, the primary effects of ascorbate itself and the secondary effects of the ascorbate-induced reduced tissue copper concentrations should be distinguished [Chapter 2].

Simultaneous oral administration of ^{64}Cu and ascorbate increased the recovery of ^{64}Cu in the feces within one day [Chapter 1], and Van Campen and Gross [7] have shown that the disappearance of ^{64}Cu from ligated intestinal segments was depressed by the addition of

ascorbate to the lumen. Thus, although both radioisotope studies suggest that ascorbic acid directly inhibits the intestinal absorption of copper, probably the primary effect mentioned above, no insight in the mechanism at the absorption level is provided.

In Chapter 4, it is shown that true copper absorption, as measured with the whole-body method was not depressed by ascorbic acid but there was a lower fecal excretion of endogenous copper. The latter observation was supported by direct measurement of biliary copper excretion in rats with a bile duct cannula. The ascorbate-induced lowering of endogenous copper excretion in rats may be related to the ascorbate-induced copper-deficiency of these animals, and represents a compensatory mechanism to preserve body copper. At this stage a simplified statement can be made, in that the amount of daily loss from the body pool is some function of the size of that pool. Because the daily intake of copper is relatively small to the total copper pool, it will take some time to achieve a new equilibrium. The important conclusion from a physiological point of view may be that negative balances do not persist, provided that the daily intake is above the loss, and that a new equilibrium can be established. The results of the experiments in Chapters 4 and 6 indicate that the feeding of a diet with 10 g ascorbic acid/kg and 5 mg Cu/kg for 6 or 8 weeks will result in a small reduction of copper status without compromising growth. Eventually, a new steady state will be reached in rats fed ascorbic acid which biliary copper excretion is depressed and copper absorption may be somewhat enhanced.

Ascorbic acid can reduce cupric ions to cuprous ions [13], which may lower copper solubility at least in vitro [14,15]. In vivo, dietary ascorbic acid caused a significant decrease in the copper concentration in the liquid phase of the small intestinal lumen [Chapter 6]. In the light of the idea that only soluble copper may cross the intestinal epithelium, it can thus be explained that copper absorption is reduced in rats fed after short-term ascorbic acid feeding. Comparable results were observed when rats were fed fructose instead of glucose [Chapter 5], supporting the view that copper solubility may be an important determinant of copper absorption.

Ascorbic acid-copper interactions at the post-absorptive stage. Data supporting a role for ascorbate in copper transport link the vitamin with the dissociation of copper from ceruloplasmin [8], the most abundant plasma source of copper is derived from the liver. Cells somehow can sequester copper from ceruloplasmin [8] or obtain the metal from other ligands such as albumin, histidine and the ternary complex (AlbCuHist) [16], or even from transcuprein [17] derived from the intestine. Cuprous copper Cu(I) has been considered the form that is most more readily dissociable from ceruloplasmin [18]. Reduction either with ascorbate or with a redox substance weakens the copper-protein bounds, causing the copper to dissociate [19] or exchange more readily with copper in the medium [20]. It was tested whether reduction is a condition for copper to be taken up by the liver.

Ascorbate, administered intravenously together with ^{64}Cu, caused an increase of ^{64}Cu in the liver *in vivo* [Chapter 2]. This effect of ascorbate was observed at a dose of 0.1 mg/rat, causing a maximum increase of plasma ascorbate by about 115 µmol/L. Within this concentration range ascorbate clearly increased copper uptake *in vitro* by isolated hepatocytes [Chapter 2], and by plasma membrane vesicles isolated from the rat liver [Chapter 9], a further proof for requirement of a reductive step in the transport mechanism of Cu at the membrane level. Chapter 9, shows that membrane-bound enzymes can provide an electron for the reduction of copper prior to uptake, suggesting a role for a plasma membrane NADH oxidase. A model for such a mechanism is presented in Chapter 9, which incorporates presented data and information gained by others [8,16]. Although speculative, it may well be that the described mechanism holds for uptake of copper from all the ligands in plasma [16], and also for copper uptake by other cell types.

Ascorbate, administered intravenously together with ^{64}Cu, stimulated ^{64}Cu accumulation in bile in rats with a bile duct cannula [Chapter 2]. The ascorbate-induced stimulation of biliary ^{64}Cu excretion probably reflects the increased hepatic uptake of ^{64}Cu and, thus, may be the result of an increased specific activity of copper in liver pools. It is unlikely that ascorbate promotes biliary excretion of copper mass because the combination of impaired intestinal absorption of copper, after short-term feeding of ascorbic acid, and increased biliary excretion of copper would not allow for a new steady-state of body copper to be reached. Indeed, Chapter 4 illustrates that biliary copper mass is depressed in rats fed on ascorbic acid.

The radioisotope approach assumes that the radioactivity entering the liver of the rat was mixed with the existing hepatic copper mass, resulting in radioisotope dilution. In other words, a homogeneous specific activity is assumed, but it ignores the fact that copper newly taken up and that from existing pools may be metabolized in different ways. It remains unknown whether the ascorbate-mediated extra uptake of copper is excreted via rapid component of biliary copper excretion [21,22].

Ascorbic acid-copper interactions: are they specific for ascorbic acid? A part of the study was performed with other vitamins (vitamin E, vitamin A, or β-carotene) to determine the specific effect of ascorbate on copper metabolism. A possible direct or indirect interference with copper metabolism is not known, but it was hypothesized that the extra intake of the antioxidants would spare vitamin C [23-26]. Copper status could be influenced by manipulating the concentration of ascorbate in plasma, as shown after supplementation with ascorbic acid (cf. Chapters 1, 2, 4 and 6). However, no effect on copper absorption was observed by supplementation of one of the other vitamins, and the observed relatively small effects of supplemental vitamin A and β-carotene on copper status in rats were found not to be mediated by changes in plasma vitamin C concentration [Chapter 10].

CONCLUSIONS

In the rat ascorbate status can be modulated by ascorbic acid intake and thus appears to be a suitable model to study the effects of ascorbic acid supplementation on copper absorption, transport and biliary excretion. The dose of 1 g ascorbic acid/kg diet produced results comparable to those seen in species other than the rat, including man.

Ascorbate appears to play a role in copper metabolism at two stages. During the *pre-absorptive stage* it reduces the concentration of intestinal, soluble copper, resulting in a decreased copper absorption. Due to secondary effects caused by reduced tissue copper concentrations in animals fed ascorbic acid copper absorption may not be depressed after long-term feeding of ascorbic acid. Thus, primary and secondary effects of ascorbate feeding should be distinguished. During the *post-absorptive stage* ascorbate stimulated copper uptake by the liver. This effect was also seen in parenchymal cells and plasma membrane vesicles isolated from livers of copper-deficient rats. A copper reduction step seems a prerequisite for copper transport across the membrane and the membrane-bound enzymes, NADH oxidase or NADH- ferricyanide reductase, may play an important role.

Although ascorbate stimulates hepatic uptake of copper it was found that in rats fed ascorbic acid biliary excretion of copper was depressed. This effect probably is secondary to the impaired copper status and contributes to achievement of a new steady state.

Most studies on dietary ascorbic acid-copper interactions, including in this thesis, have been conducted with young, growing animals. The growing animal has an increased copper requirement. Feeding diets supplemented with ascorbic acid in combination with a low copper content, therefore will result in a more rapid reduction of plasma and tissue copper levels in young than in adult animals. There are no studies reported to prove this view. In adult monkeys and man small reductions in serum copper and serum ceruloplasmin levels have been observed only after feeding high levels of ascorbic acid for periods up to months [1,6], indicating that only long-term high ascorbic acid intakes may affect on copper status in adults.

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SUMMARY

The effects of feeding supplements of ascorbic acid on dietary copper availability and copper status were studied in rats. Male outbred Wistar (Hsd/Cpb:Wu) rats were fed on purified diets adequate in copper (5 mg Cu/kg) without or with 1 or 10 g ascorbic acid/kg diet. Rats fed on diets with a restricted amount of copper (about 1 mg Cu/kg) were included as positive control and reference groups, to compare and contrast the effects of copper deficiency with those of ascorbate on copper metabolism.

In the rat ascorbate status can be modulated by ascorbic acid intake and thus appears to be a suitable model to study the effects of ascorbic acid supplementation on copper absorption, transport and biliary excretion. The dose of 1 g ascorbic acid/kg diet produced results comparable to those observed in species other than the rat, including man.

Ascorbate appears to play a role in copper metabolism at two stages. During the *pre-absorptive stage* it reduces the concentration of intestinal, soluble copper, resulting in a decreased copper absorption. Due to secondary effects caused by reduced tissue copper concentrations in animals fed ascorbic acid, copper absorption may not be depressed after long-term feeding of ascorbic acid. Thus, primary and secondary effects of ascorbate feeding should be distinguished. During the *post-absorptive stage* ascorbate stimulated copper uptake by the liver. This effect was also seen in parenchymal cells and plasma membrane vesicles isolated from livers of copper-deficient rats. A copper reduction step seems a prerequisite for copper transport across the membrane and the membrane bound enzymes, NADH oxidase or NADH ferricyanide reductase, may play an important role. Although ascorbate stimulates hepatic uptake of copper it was found that in rats fed ascorbic acid biliary excretion of copper was depressed. This effect probably is secondary to the impaired copper status and contributes to achievement of copper homeostasis.

SAMENVATTING

Koper speelt een essentiële rol in biologische en biochemische processen in het lichaam van mens en dier. Het is slechts in kleine hoeveelheden nodig, maar komt ook in kleine hoeveelheden in de voeding voor, vandaar dat koper een essentieel spoorelement genoemd wordt. Een tekort, overschot of een verkeerde koperbalans heeft dan ook negatieve gevolgen voor de gezondheid. In het algemeen is het niet de geconsumeerde hoeveelheid koper die van belang is, maar de mate waarin koper beschikbaar is voor de biologische en biochemische processen in het lichaam.

Onder de omstandigheden die heersen in de maag en in de darm kunnen diverse componenten in de voeding oplosbare of onoplosbare complexen vormen met koper. Deze voedingscomponenten verhogen of verlagen de beschikbaarheid voor opname uit de darm, en hiermee de biologische beschikbaarheid van koper. Een van de vele faktoren die de biologische beschikbaarheid van koper kunnen beïnvloeden is vitamine C (ascorbinezuur is de chemische naam voor vitamine C). In dierexperimenten werd gevonden dat de gevolgen van een kopertekort door de aanwezigheid van extra ascorbinezuur in het voer wordt versterkt. Er zijn bovendien aanwijzingen dat sommige suikers invloed hebben op de biologische beschikbaarheid van koper. Met rattenproeven kon worden aangetoond dat de gevolgen van een te lage inname van koper werden versterkt als het voer een hoog gehalte aan fructose bevat. Het is nog niet duidelijk of dit alleen te maken heeft met een verminderde biologische beschikbaarheid van koper (complexvorming) of dat ook na absorptie interactie van koper met ascorbinezuur of fructose optreedt.

Er bestaan diverse mechanismen in het lichaam van mens en dier om de huishouding van koper te reguleren. Deze mechanismen zorgen voor een voldoende absorptie uit het maagdarmkanaal, transport in de bloedbaan, en gebruik en uitscheiding van koper (zie Figuur 1, p. xiii, General Introduction) voor een vereenvoudigd schema van de koperhuishouding bij de rat). De homeostase wordt gereguleerd op orgaan nivo door een aantal slechts ten dele bekende mechanismen, met name de opname van koper in de darm en in de lever, en de uitscheiding van leverkoper, gebonden aan plasmaeiwitten waaronder ceruloplasmine, en de uitscheiding in de gal. De route via de gal is het voornaamste uitscheidingsmechanisme. Koper verlaat het lichaam via de feces. De homeostase wordt eveneens gereguleerd door mechanismen op cellulair nivo, waarbij de lever gezien zijn centrale rol in de koperhomeostase veel aandacht geniet.

De gerapporteerde negatieve invloed van ascorbinezuur op de koperhuishouding vormde onder andere aanleiding om de rol van ascorbinezuur, met name een verhoogde inname via de voeding, nader te onderzoeken. Het in dit proefschrift beschreven onderzoek is uitgevoerd

met ratten. Met behulp van in vivo methoden, balansonderzoek en absorptiemetingen met radioisotopen (^{64}Cu), werd de beschikbaarheid van koper uit de voeding vastgesteld. Om een beter inzicht te verkrijgen in de mechanismen op orgaan en cellulair nivo zijn in vitro methoden gebruikt. Enerzijds kunnen hiermee in vivo omstandigheden nagebootst worden, anderzijds hebben in vitro methoden een belangrijk voordeel dat de proefomstandigheden goed beheersd en gestandaardiseerd kunnen worden. Ten gevolge van de biologische variatie zijn bij in vivo studies vaak grote aantallen proefdieren nodig om statistische significantie te verkrijgen.

De algemene inleiding beschrijft de achtergronden en de experimentele opzet van het onderzoek in dit proefschrift.

In de hoofdstukken 1 en 2 wordt ingegaan met name op het effect van een hoge inname van ascorbinezuur op de absorptie van koper en de koperstatus. Inderdaad blijkt de koperabsorptie verlaagd te zijn, en een door ascorbinezuur geïnduceerde verlaging van de koperstatus werd vastgesteld. Deze resultaten stemmen overeen met literatuurgegevens. Een verklaring is daarmee nog niet gegeven. Koper, dat meestal in de tweewaardige vorm aanwezig is, kan door reducerende stoffen als ascorbinezuur gereduceerd worden. De absorptie van dit éénwaardige koper wordt geacht veel slechter te verlopen dan van tweewaardig koper. In hoofdstuk 6 wordt een verminderde oplosbaarheid van koper in de dunne darm beschreven bij een verhoogde hoeveelheid ascorbinezuur in de voeding, waardoor de verlaging van de koperabsorptie verklaard zou kunnen worden. Ook is een verminderde oplosbaarheid van koper in de dunne darm waargenomen onder invloed van fructose in de voeding (hoofdstuk 5). Deze verminderde oplosbaarheid kan het gevolg zijn van het reducerend vermogen van fructose of zijn complexerende eigenschappen, waardoor de koperabsorptie verlaagd wordt. Met voornoemde waarnemingen lijkt aannemelijk gemaakt te kunnen worden dat de verlaagde koperabsorptie een afspiegeling is van de verminderde oplosbaarheid van koper in de dunne darm.

In hoofdstuk 4 wordt ingegaan op de invloed van de door ascorbinezuur geïnduceerde verlaging van de koperstatus en de gevolgen op de koperexcretie via de gal. Het blijkt dat de door ascorbinezuur geïnduceerde verlaging van de koperstatus een tijdsafhankelijke vermindering van de koperexcretie via de gal tot gevolg heeft. Met andere woorden, het lichaam probeert zijn koperexcretie te minimaliseren om zo goed mogelijk de koperbalans te handhaven. Ook bestaat een tendens tot een verhoogde koperabsorptie na wat langere tijd. Waarschijnlijk induceert de door ascorbinezuur verlaagde koperstatus een verhoogde efficiëntie van de koperabsorptie. Op dit punt kan dan ook geconcludeerd worden dat een primair effect van ascorbinezuur op absorptienivo, voor een deel gemaskeerd zou kunnen worden door secundaire effecten, namelijk een verlaagde koperstatus. Dit geeft tevens de complexiteit aan bij het uitvoeren van in vivo studies.

In hoofdstukken 7-9 wordt ingegaan op het effect van ascorbinezuur, en een verlaagde koperstatus op de koperopname op cellulair nivo met behulp van in vitro methoden. Deze

aanpak heeft als voordeel dat genoemde secundaire effecten niet interfereren. Ascorbinezuur stimuleert de koperopname door geïsoleerde levercellen en membraanfragmenten van deze levercellen, de zgn. vesicles. Hier wordt dan ook geconcludeerd dat een koper-reductie-stap een voorwaarde is voor koperopname in de cel. Deze in vitro methoden illustreren tevens hoe nuttige informatie verkregen kan worden met betrekking tot het mechanisme van de koperopname door de levercel.

In hoofdstuk 10 wordt ingegaan op de specificiteit van het effect van ascorbinezuur op het kopermetabolisme. Door de mogelijke rol van ascorbinezuur als antioxidant is gespeculeerd dat andere vitaminen met antioxidant werking het plasma-ascorbaat-nivo hoog zouden kunnen houden (zgn "sparen"). Daardoor zou indirekt de koperstatus negatief beïnvloed kunnen worden. Door middel van suppletie van de vitamines E, A en β-caroteen kon echter geen effect op de koperstatus gevonden worden. Dit wijst op een specifiek effect van ascorbinezuur op het kopermetabolisme.

In het onderzoek beschreven in dit proefschrift is de interactie tussen ascorbinezuur en de koperhuishouding bij de rat vanuit diverse gezichtspunten bestudeerd, gebruik makend van zowel in vivo als in vitro technieken. In de algemene discussie worden de meest kenmerkende discussiepunten en conclusies van het gehele onderzoek gepresenteerd.

CURRICULUM VITAE

Gerrit J. van den Berg (GJ) was born in 1947 on November 17th, at Tiel. GJ graduated from high school (HBS-B) in 1966 in Tiel. After performance of the National Service GJ started in 1968 his study Biology at the State University of Utrecht, The Netherlands. GJ acquired his M.Sc. in Biology in 1973 (Utrecht University): principal subjects were Biophysical Chemistry and Immunology, and Microbiology as a subsidiary subject.

From July 1st 1973 GJ worked as a scientific researcher at the Interuniversity Reactor Institute, Department of Radiochemistry lateron (September 1988) the Interfaculty Reactor Institute of the Delft University of Technology.
The investigations described in this thesis were carried out during the last 5 years in close cooperation with the Department of Laboratory Animal Science, State University of Utrecht (Prof dr ir AC Beynen) and the Department of Human Nutrition, Agricultural University of Wageningen (Prof dr ir AC Beynen).

During July 1-22th 1991, GJ was a member of Task Group No. 2, IAEA-Chernobyl project and participated in the environmental assessment of the radiological consequences in the USSR from the Chernobyl accident by collecting lichens as biomonitors, and by measuring the amount of radionuclides in these plants as well as heavy metals. This work was performed at the Radiochemistry Department, Delft University of Technology.

During the 5-years period of executing the work described in this thesis GJ was also involved in the diagnosis of Menkes' disease, work performed in collaboration with Dr WJ Kleijer, Department of Clinical Genetics, Erasmus University Rotterdam.

In August 1993 GJ entered the postgraduate course (Utrecht University; Prof dr LFM van Zutphen) as a requirement for the performance of the function of animal welfare officer according to the Dutch 1977 Experiments on Animals Act.

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EPILOGUE

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Gerrit J van den Berg