Copper-65 Absorption by Men Fed Intrinsically and Extrinsically Labeled Whole Wheat Bread

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Six men were fed a diet composed of conventional foods with all bread as whole wheat bread. Intrinsically labeled ^{65}Cu bread (containing 6.5 ppm Cu and 48 atom % ^{65}Cu) was substituted for unlabeled bread for 3 days, and stools were collected for 24 days. Extrinsically labeled bread was then substituted for 3 days and another 24-day stool collection made. ^{65}Cu excretion was measured by mass spectrometry. Mean Cu intake was 1.10 mg of Cu/day. Average Cu balance was −0.06 ± 0.08 mg/day. Average absorption of the intrinsic copper was 72.2 ± 9.3% and of extrinsic Cu 64.2 ± 5.8%. The ratio of extrinsic to intrinsic absorption was 0.906 ± 0.164. Absorption of intrinsic and extrinsic tracers did not differ significantly ($p > 0.05$) by a paired t-test, and the ratio (E/I) was not significantly different from 1. Use of extrinsic Cu tracers to assess Cu absorption is supported by these results.

Although the dietary essentiality of copper is well established, little is known about its absorption by humans or the factors affecting copper's availability for absorption from foods. Ascorbic acid is known to interfere with copper absorption in animals (Van Campen and Gross, 1968; Milne and Omaye, 1980). Women absorbed more copper from formula diets based on animal protein than from diets based on plant protein (Turnlund et al., 1983), but there was no difference in ^{67}Cu absorption by rats from plant or animal foods with an extrinsic ^{67}Cu tracer (Johnson et al., 1985). While Davis et al. (1962) found that isolated soybean protein interfered with Cu utilization in rats, Lo et al. (1984) reported that Cu was available equally to rats from isolated soy protein and copper carbonate. Dietary fiber did not impair Cu absorption in rats (Scheibel and Mehta, 1985), and neither phytate nor cellulose affected copper absorption by men (Turnlund et al., 1985).

Research in copper absorption has been limited because of the short half-lives of the two radioisotopes: 12 h for ^{64}Cu and 62 h for ^{67}Cu. The development of methods for the analysis of stable mineral isotopes has made possible many studies with human subjects previously impossible or unethical. With stable isotopes it is possible to prepare foods that have been isotopically labeled during the growth of the plant or animal from which the food is prepared.

The validity of using extrinsic tracers to assess mineral absorption is a fundamental methodological question that must be answered for each mineral. It is now an accepted practice to use extrinsic tracers to measure the absorption of inorganic or non-heme iron (Consaul and Lee, 1983; Hallberg, 1981). Considerable research has also been done on the validity of extrinsic labels for zinc (Evans and Johnson, 1977; Neathery et al., 1975; Janghorbani et al., 1983; Flanagan et al., 1985; Meyer et al., 1983); absorption of extrinsic zinc generally seems to be correlated with the absorption of intrinsic zinc. Absorption of extrinsic cadmium (Siewicki and Balthrop, 1983; Welch and House, 1980) and magnesium (Schwartz et al., 1980, 1981) tracers seems to be the same as intrinsic tracers, but the absorption of extrinsic calcium or selenium labels is much different from the absorption of intrinsic calcium (Wien and Schwartz, 1983) or selenium (Welch and House, 1980). There was no difference in the absorption by rats of intrinsic or extrinsic ^{67}Cu from chicken liver (Johnson et al., 1988). We also found no difference in the absorption by rats of intrinsic and extrinsic ^{65}Cu from wheat (Johnson and Lykken, 1983). This study was designed to compare the absorption by men of ^{65}Cu added intrinsically or extrinsically to whole wheat bread.

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METHODS

Subjects. Healthy adult male volunteers were admitted to the study after being informed of its purpose and any associated risks. The project was approved by the Institutional Review Board of the University of North Dakota and the Human Studies Committee, USDA—ARS. Informed consent and experimental procedures were consistent with the Declaration of Helsinki. All subjects were chaperoned when they left the metabolic unit to prevent ingestion of unauthorized foods or loss of excreta samples.

Intrinsically Labeled Bread. Hard red spring wheat was grown in a conventional manner outdoors at the North Dakota State University Experiment Station, Langdon Branch. At anthesis, on a sunny day (to promote rapid translocation of nutrients), ^{65}Cu was injected into stems between the first and second nodes below the head. Approximately 0.3 mg of ^{65}Cu in the form of $CuCl_2$ was injected into each stem. Details of the stem injection procedure are given elsewhere (Starks and Johnson, 1985a). At harvest wheat averaging 74.1 ± 4.9 atom % ^{65}Cu and 9.16 ± 1.57 ppm total Cu was obtained. (The natural abundance of ^{65}Cu is 30.91 atom %.) Although this is a higher Cu concentration than the 3.5–5 ppm usually reported for wheat (Erdman and Moul, 1982; Zook et al., 1970), it is similar to the Cu concentration (8.1 ppm) of untreated, control wheat we have grown previously (Starks and Johnson, 1986).

The wheat was cleaned, ground, and made into bread at the Cereal Science Department of North Dakota State University, Fargo, ND. An accident during milling resulted in the labeled wheat being mixed with unlabeled control wheat. Thus, the enrichment of labeled bread loaves was less than that of the original crop, averaging 48.1 ± 3.8 atom % ^{65}Cu. Copper content of the labeled bread was 6.50 ± 0.94 ppm. Intrinsically labeled bread dough was divided into weighed loaves and stored frozen until just before feeding, when it was thawed, proofed, and baked.

Extrinsically Labeled Bread. Extrinsically labeled bread was prepared from the same variety of wheat and with the same recipe as the intrinsically labeled bread. A solution of $^{65}CuCl_2$ was added to the liquid ingredients of the bread dough. Enrichment of the extrinsically labeled bread averaged 48.4 ± 2.6 atom % ^{65}Cu, and total copper concentration was 7.41 ± 0.64 ppm.

Plan of Study. Subjects consumed the basal whole wheat diet (Table I) for 14 days after admission and

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Table I. Typical Menu

breakfast	lunch
grapefruit juice	grape juice
scrambled eggs	oven-baked breast of chicken
whole wheat bread	mashed potatoes
margarine	whole wheat bread
jelly	butter
skim milk	peach crisp
dinner	snack
orange juice	pineapple juice
spaghetti with meat sauce	cheese
whole wheat bread	pears
margarine	sugar cookie

throughout the study except for two 3-day intervals when ^{65}Cu-labeled bread was fed. On days 15–17 they ate ^{65}Cu intrinsically labeled bread in place of unlabeled bread. On days 39–41, ^{65}Cu extrinsically labeled bread was substituted in the diet. Bread consumption both from the basal diet and during the intervals of feeding labeled bread was about eight slices/day for each volunteer. The ^{65}Cu content of each loaf, the total Cu content, and the weight of each loaf fed were known, so that the ^{65}Cu and total Cu intake from bread could be calculated. Subjects consumed 2.60 ± 0.21 mg of ^{65}Cu from intrinsically labeled bread over the 3-day interval; total Cu intake from bread was 1.70 ± 0.06 mg/day. They consumed 2.85 ± 0.28 mg of ^{65}Cu from extrinsically labeled bread during days 39–41; total Cu intake from bread was 1.92 ± 0.18 mg/day.

It was necessary to feed the labeled bread over a 3-day interval in order to provide an intake of ^{65}Cu large enough to produce detectable levels of ^{65}Cu in feces after part of the dose had been absorbed. The minimum detectable level of ^{65}Cu enrichment was 31.0 atom %. This corresponds to about 0.01 mg of ^{65}Cu above natural abundance in a 3-day composite stool sample.

Copper intake during both intervals when labeled bread was fed was higher than the average intake over the entire study (Table II) due to the higher Cu content of the isotopically labeled bread. All stools were collected beginning at day 15 until the end of the study. They were pooled in 3-day composites for analysis. Thus, stools were analyzed for 24 days (eight 3-day composites) in each of the two parts of the study. Urine was also collected beginning on day 15 in order to measure balance.

Except for substitution of labeled bread during two 3-day intervals, the diet was unchanged throughout the entire study. A 3-day menu cycle based on conventional foods was employed. Duplicate diets were prepared during each ^{65}Cu bread interval and for two other 3-day intervals during the study. Copper content of the diet was 1.10 ± 0.18 mg/day. Copper intake varied slightly with the caloric intake of each subject.

Determination of Copper Enrichment and Calculation of Cu Absorption. Enrichment of ^{65}Cu in bread, diets, and fecal samples was determined mass spectrometrically by a previously described method (Johnson, 1982). This involved dry-ashing of fecal samples, separation of minerals in the ash by anion-exchange chromatography, chelation of Cu with *meso*-tetraphenylporphyrin (TPP), further purification by thin-layer chromatography, and mass spectrometric analysis of the Cu–TPP chelate on a Du Pont DP-102 mass spectrometer. Isotopic enrichment of the fecal samples was calculated from the ratio of the m/z 677 to m/z 675 peaks, corresponding to ^{65}Cu and ^{63}Cu, respectively, after correction for contributions of the TPP to those peaks. The coefficient of variation for duplicate analyses was 2%. Copper absorption was calculated by subtracting stable isotope excreted in feces from that administered in the oral dose and dividing by the amount of the oral dose. The uncertainty in fractional absorption was thus ≤0.02 absorption unit (Johnson, 1982, 1984).

Absorption determined by this method approximates true absorption. It differs from true absorption to the extent that absorbed isotopic Cu is excreted during the period that feces are collected for determination of unabsorbed isotope. The absorption and subsequent excretion of isotope during the fecal collection period results in a slight underestimation of true absorption.

Copper content of diet and fecal samples was measured by flame atomic absorption spectrophotometry after wet digestion of aliquots of freeze-dried blended material with nitric and perchloric acids (Analytical Methods Committee, 1960). Urinary Cu was determined directly by graphite furnace atomic absorption spectrophotometry. Analytical accuracy was monitored through periodic analyses of NBS bovine liver standards. The analyzed value was 186 ± 3 μg of Cu/g (n = 12; certified value 193 ± 10 μg of Cu/g). Recovery of Cu from fecal and diet samples was 95.3 ± 1.6% (n = 9).

RESULTS

Balance data are shown in Table II. Copper intake averaged 1.08 ± 0.23 mg/day during period I and 1.13 ± 0.13 mg/day during period II. Average copper balance was −0.06 ± 0.08 mg/day for period I and 0.03 ± 0.10 mg/day for period II. Unabsorbed ^{65}Cu was generally excreted in the first two or three stool collection periods, that is, within 6–9 days of the beginning of the feeding period of the labeled bread. Copper absorption values for the intrinsically and extrinsically labeled bread are shown in Table III. There was no significant difference in the absorption values for the intrinsic and extrinsic tracers by a paired t-test, $p > 0.05$, and the ratio of the extrinsic to intrinsic absorption values, E/I, was not significantly different from unity, $p > 0.05$.

DISCUSSION

This experiment provides the first data on absorption of extrinsic and intrinsic Cu tracers by humans. Future

Table II. Copper Balance[a] (Milligrams of Cu/Day)

	period 1 (intrinsic bread)				period 2 (extrinsic bread)			
subj no.	Cu intake	fecal Cu	urinary Cu	balance	Cu intake	fecal Cu	urinary Cu	balance
2104	1.16	1.12	0.06	−0.02	1.16	1.17	0.05	−0.06
2106	1.16	1.23	0.06	−0.13	1.16	1.22	0.05	−0.11
2107	1.15	1.27	0.05	−0.17	1.15	1.26	0.04	−0.15
2111	0.61	0.60	0.09	−0.08	0.87	0.64	0.10	0.13
2134	1.22	1.16	0.04	0.02	1.24	1.19	0.04	0.01
2135	1.17	1.12	0.05	0.00	1.19	1.11	0.06	0.02
mean	1.08	1.08	0.06	−0.06	1.13	1.07	0.06	0.03
±SD	±0.23	±0.24	±0.02	±0.08	±0.13	±0.25	±0.02	±0.10

[a] All values are the mean for eight 3-day periods: one period during which labeled bread was fed and seven periods during which unlabeled bread was fed.

Table III. Absorption of ^{65}Cu from Intrinsically and Extrinsically Labeled Whole Wheat Bread

subj no.	% Cu absorption		ratio E/I
	intrinsic	extrinsic	
2104	78.6	57.5	0.734
2106	67.1	57.9	0.863
2107	62.3	65.4	1.049
2111	76.6	66.4	0.867
2134	85.1	65.2	0.766
2135	63.2	73.0	1.155
mean ± SD	72.2 ± 9.3	64.2 ± 5.8	0.906 ± 0.164

studies of Cu bioavailability from human diets will be greatly facilitated by the use of an extrinsic ^{65}Cu tracer. Intrinsic labeling of foodstuffs is difficult and sometimes impossible, due to the short half-lives of the radioisotopes and the relatively large amounts of stable Cu that must be used (Starks and Johnson, 1985a,b, 1986).

Strictly speaking, one cannot consider either the intrinsic or extrinsic ^{65}Cu used in this study as a "tracer" because of the relatively large amounts of isotope used. This is necessary because of the high natural abundance of ^{65}Cu (30.91%). We reported previously that although 72–75% of Cu in wheat was associated with protein, stem injection of ^{65}Cu into wheat at levels more than twice those used in this study (22 ppm Cu in wheat) did not change the protein composition of wheat (Starks and Johnson, 1986). In both control and stem-injected wheat, Cu was mainly associated with the glutenin fraction. Thus, although the Cu contents of the wheat and bread used in this study were somewhat higher than usual, the labeling process and the high amounts of tracer should not have had a marked effect on the results. Copper absorption from unlabeled whole wheat bread may be greater than Cu absorption in this study, because percent Cu absorption tends to be inversely related to the dose of Cu (Owen, 1964).

No differences were found in absorption of intrinsic and extrinsic Cu from whole wheat bread fed to six men. The mean ratio of extrinsic to intrinsic absorption did not differ from unity. These data are supportive of the validity of using an extrinsic tracer to measure Cu absorption.

The variance in the E/I ratio is large, from 0.734 to 1.155, indicating a substantial subject to subject variation in the E/I ratio. The apparent between-subject variation in the E/I ratio may be partly due to day to day differences in absorption by the subjects that are unrelated to the differences in the form of the tracer. Since only ^{65}Cu can be used as a tracer, it is impossible to feed the intrinsic and extrinsic tracers in the same meal and thereby eliminate the influence of day to day variation on the intrinsic–extrinsic comparison.

There are limited data comparing the absorption of intrinsic and extrinsic copper by rats. In one experiment, the absorption of intrinsic and extrinsic ^{67}Cu from chicken liver by rats was found to be the same (Johnson et al., 1988). Absorption of intrinsic and extrinsic copper from whole wheat by rats was also found to be the same (Johnson and Lykken, 1983).

Copper balance in these subjects was negative or very close to zero. The inclusion of sweat loss in the balance calculation would give negative balance figures for all subjects. Copper loss in sweat was reported to be 0.34 ± 0.24 mg/day for men consuming 1.1–1.5 mg of Cu/day (Jacob et al., 1981). These data indicate that an average daily copper intake of 1.1–1.2 mg was insufficient for these subjects.

Dietary fiber in this diet was not analyzed. However, subjects consumed eight slices/day of whole wheat bread, made from 100% extraction flour. The high Cu absorption values obtained in this study would imply that relatively large amounts of fiber from whole wheat do not adversely affect Cu absorption. Turnlund et al. (1985) found no effect of cellulose or phytate on Cu absorption from formula diets. Studies with purified fiber sources have shown varying effects on Cu balance (Allen and Solomons, 1984). Addition of 26 g/day of wheat or corn bran to the diet of adult men resulted in a more positive balance than was achieved in the absence of the fiber (Sandstead et al., 1978); however, Cu intake was not held constant between the control and bran feeding periods. Addition of bran tended to increase Cu intake in that study.

In summary, absorption of ^{65}Cu tracers added extrinsically or intrinsically to whole wheat bread was found to be the same in six men. This supports the validity of using extrinsic Cu tracers to measure Cu absorption from foods. Under the conditions of this study, Cu absorption was relatively high, from 57 to 85%. The rather high amount of high fiber bread (eight slices/day) apparently did not adversely affect Cu absorption. An intake of approximately 1.1 mg of Cu/day was insufficient to maintain a nonnegative Cu balance in these subjects.

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Ochratoxin A Found in Commercial Roast Coffee

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Of 68 samples of commercial roast coffee purchased in 1987, 5 were found to contain ochratoxin A at concentrations of 3.2–17.0 μg/kg by our previous HPLC method. This is the first report of the detection of ochratoxin A in commercial roast coffee samples.

Ochratoxin A (OCT-A) is a nephrotoxic fungal metabolite produced by *Aspergillus spp.* and *Penicillium spp.* and has a high acute toxicity in duckling, rats, mice, chick, rainbow trout, etc. (Krogh, 1978). This mycotoxin may be responsible for kidney disease of swine in Denmark (Krogh, 1978) and was shown to cause hepatoma and renal cell tumors in mice (Kanisawa and Suzuki, 1978).

Although several studies have demonstrated the natural occurrence of OCT-A in various agricultural products (Nakazato, 1983) and many strains of OCT-A-producing *Aspergillus ochraceus* have been isolated from green coffee beans (Stack et al., 1983; Tsubouchi et al., 1984), OCT-A has rarely been detected in green coffee beans, and there has been no report of its detection in roast coffee samples. The reasons for this were considered to be that caffeine in coffee beans had inhibited the growth of the fungi and the production of OCT-A (Buchanan and Fletcher, 1978) and that the roasting procedure had destroyed the toxin in the coffee beans (Levi et al., 1974). However, the analytical methods employed would not have been sufficiently sensitive to detect low levels of OCT-A.

Recently, we reported that some strains of *A. ochraceus* isolated from green coffee beans grew well and produced high levels of OCT-A in YES medium containing 0.1–1.0%

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