Effects of Copper, Iron, and Ascorbic Acid on Manganese Availability to Rats (43383)

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Abstract. **Four experiments were done to characterize the interactions of copper, iron, and ascorbic acid with manganese in rats. All experiments were factorially arranged. Dietary Mn concentrations were <1 μg/g (Mn0) and 50 μg/g (Mn+). Dietary Cu was <1 mg/g (Cu0) and 5 μg/g (Cu+); dietary Fe was 10 μg/g (Fe10) and 140 μg/g (Fe140). Ascorbic acid (Asc) was not added to the diet or added at a concentration of 10 g/kg diet. Experiment 1 had two variables, Mn and Cu; in Experiment 2, the variables were Mn and Asc. In Experiment 3, the variables were Mn, Cu, and Asc; in Experiment 4, they were Mn, Cu, and Fe. Definite interactions between Mn and Cu were observed, but they tended to be less pronounced than interactions between Mn and Fe. Cu depressed absorption of ^{54}Mn and accelerated its turnover. In addition, adequate Cu (Cu+), compared with Cu0, depressed liver, plasma, and whole blood Mn of rats. Absorption of ^{67}Cu was higher in animals fed Mn0 diets than in those fed Mn+. Ascorbic acid depressed Mn superoxide dismutase activity and increased Cu superoxide dismutase activity in the heart. The addition of ascorbic acid to the diet did not affect Mn concentration in the liver or blood. Absorption of ^{54}Mn was depressed in rats fed Fe140 compared with those fed Fe10. Interactions among Fe, Cu, and Mn resulted in a tendency for Mn superoxide dismutase activity to be lower in rats fed Fe140 than in rats fed Fe10. Within the physiologic range of dietary concentrations, Mn and Cu have opposite effects on many factors that tend to balance one another. The effects of ascorbic acid on Mn metabolism are much less pronounced than effects of dietary Cu, which in turn affects Mn metabolism less than does Fe.** [P.S.E.B.M. 1992, Vol 199]

Multiple interactions among trace elements such as iron, zinc, and copper that affect their bioavailability are well known. Such interactions tend to occur when the chemical forms or electronic structures of the various ions are similar (1). Although manganese, as a transition element, might also be expected to be subject to such interactions, the effects of other trace elements, with the exception of iron, on manganese metabolism are not well characterized. Furthermore, factors such as ascorbic acid, which enhances iron availability and depresses copper availability, could also be expected to have an effect on manganese metabolism.

Although the interactions between copper and manganese are not well characterized, limited data indicate that some interactions exist. The addition of copper to rat diets tended to decrease tissue manganese concentrations (2–5), while manganese deficiency in kids resulted in an increase in femur copper (6). On the other hand, chronic injection of very high amounts of manganese caused increases in the tissue copper of rats, including femur copper (7). Both manganese and copper are known to affect serum cholesterol (8, 9) and both are involved in the metabolism of bone (4). Effects of dietary copper on activity of manganese superoxide dismutase (10, 11) and plasma uptake of manganese (12) have been noted. However, these interactions have mostly been noted incidentally to other work, and they have not been systematically studied. Interactions between iron and manganese (13–16) and iron and copper (17–20) are well known, and their existence raised the question of whether the effects of copper on manganese might be exerted indirectly via effects on iron. The contrasting effects of ascorbic acid on iron (21) and copper (22–26) metabolism make it difficult to predict what the effect of ascorbic acid on manganese metabolism might be.

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We report here the results of a series of experiments done to characterize the interactions of copper, iron, and ascorbic acid with manganese in rats.

Methods

Animal Care. Weanling Long Evans rats were purchased from Harlan Sprague Dawley, Indianapolis, IN. They were housed in individual stainless steel cages in a temperature-controlled room with a 12:12-hr light:dark cycle. Food consumption was monitored on a 5-day schedule and animal weights were recorded weekly. Food and demineralized water were provided *ad libitum*.

Diets. Diets were based on the AIN-76 formulation (27, 28), but were made with a modified AIN-76 mineral mix that contained no added Mn, Cu, or Fe. Adjustments in the amounts of these minerals were made by adding specific mineral mixes containing the mineral of interest triturated in sucrose (Mn) or starch (Cu and Fe). The contents of the special mineral mixes were as follows: Mn, 4.82 mg Mn/g as $MnCO_3$; Cu, 0.5 mg Cu/g as $CuSO_4$; $5H_2O$; and Fe, 2 mg Fe/g as ferric citrate. The additions of minerals or ascorbic acid to the diets were made at the expense of sucrose.

Experiment 1—Interaction Between Mn and Cu. This was a 2 × 2 factorially arranged study with two concentrations of dietary Mn, <1.5 mg Mn/kg (Mn0) diet and >50 mg Mn/kg (Mn+) diet, and two concentrations of dietary Cu, 0.6 mg Cu/kg diet (Cu0) and 6.0 mg Cu/kg diet (Cu+). There were eight rats per group with mean weights of 53.6 ± 0.4 g.

After being fed their respective diets for 2 weeks, the rats were fasted overnight and then fed test meals composed of 3 g of their respective diet plus 3 μCi of ^{54}Mn (DuPont NEN Research Products, Boston, MA). Experimental diets were returned 8 hr after the meal and the rats were allowed to eat *ad libitum* for the remainder of the study.

Whole body ^{54}Mn retention was determined within 2–3 hr after the meal by using a custom-built, small animal, whole body counter equipped with an ND62 multichannel analyzer (Nuclear Data Instrumentation, Schaumberg, IL). Whole body ^{54}Mn retention was measured every day for a week and then at 2-day intervals for an additonal 2 weeks. The multichannel analyzer was calibrated with ^{137}Cs. Radioactivity of ^{54}Mn was measured between 694 and 974 keV, which includes the γ-peak of ^{54}Mn (834 keV). The measured radioactivity was corrected for background and decay.

Absorption of ^{54}Mn was calculated by extrapolating the linear portion of a plot of ln (percentage of retention) versus time from days 11 to 22 after ^{54}Mn administration (29). Excretion rates were expressed as the biological half-life (BH), where BH = $-ln2$/slope; the slope in this equation is the slope of the linear portion of the semilogarithmic plot of retention versus time. Apparent absorption was not corrected for endogenous excretion by using a similar ^{54}Mn retention curve for injected ^{54}Mn because the slopes of retention curves for orally administered and injected ^{54}Mn are not the same (30). Regression equations were calculated for individual rats, and statistical analyses of data were done on the absorption and BH values calculated from these equations.

After consuming the experimental diets for a total of 5 weeks, rats were fasted overnight and sacrificed by cardiac exsanguination following anesthesia with sodium pentobarbital. Femurs, liver, and blood were collected for trace metal analyses. Part of the liver was frozen at −80°C for the determination of arginase activity by the method of Colombo and Konarska (31). Hearts were analyzed to determine activity of Mn and Cu superoxide dismutase by the method of Marklund and Marklund (32) as modified by Paynter (33). Whole blood was analyzed for Mn; Mn, Cu, and Fe were determined in plasma or serum by atomic absorption spectrophotometry. Serum cholesterol concentrations were measured with a Sigma Diagnostics kit (Procedure 351; Sigma Chemical Co., St. Louis, MO). Hemoglobin and hematocrit were measured with a Coulter Counter (Coulter Electronics, Hialeah, FL). Diets and tissues were lyophilized and wet-ashed with concentrated nitric acid and 30% hydrogen peroxide (34). Whole blood was digested in vented Teflon tubes with concentrated nitric acid (CFS Chemicals, Columbus, OH). The samples were refluxed for 48 hr; hydrogen peroxide (30%) was added to complete the digestion. The samples were heated for 4 hr, cooled, and analyzed for Mn with the Zeeman Graphite Furnace atomic absorption spectrophotometer (Perkin-Elmer 3030). Manganese in bovine liver (National Institute of Standards and Technology SRM 1577a) was also analyzed. The certified value is 9.9 ± 0.8 μg/g; we found 9.6 ± 0.1 μg/g.

All data were subjected to analysis of variance. Differences among the means were tested by Scheffé contrasts (35).

Experiment 2—Interaction Between Mn and Ascorbic Acid. This experiment was similar to the Mn × Cu experiment, except that the dietary variables were two concentrations of dietary Mn, <2 mg Mn/kg diet (Mn0) and >50 mg Mn/kg diet (Mn+), and zero (Asc0) and 10,000 mg added ascorbate/kg diet (Asc+). Rats (8/group) had mean weights of 53.4 ± 0.1 g at the beginning of the experiment.

Experiment 3—Interactions among Mn, Cu, and Ascorbic Acid. A 2 × 2 × 2 factorially arranged experiment was done with Mn, Cu, and ascorbate as the dietary variables. Dietary concentrations of 1 (Mn0) and 50 mg Mn/kg diet (Mn+), <0.5 (Cu0) and 5 mg Cu/kg diet (Cu+), and zero (Asc0) and 10,000 mg ascorbate/kg (Asc+) were used for the study. Experimental conditions were similar to those used in the

other experiments. Both ^{54}Mn (3 μCi) and ^{67}Cu (2μCi) were fed in the test meal (4 g of diet), and absorption and BH of both ^{54}Mn and ^{67}Cu were determined. Rats weighed 74.6 ± 0.4 g at the beginning of the experiment.

Experiment 4—Interactions among Mn, Cu, and Fe. A 2 × 2 × 2 factorially arranged experiment was done with Mn, Cu, and Fe as the dietary variables. Dietary concentrations of 1 (Mn0) and 50 mg Mn/kg diet (Mn+), <0.5 (Cu0) and 5 mg Cu/kg diet (Cu+), and 10 (Fe10) and 140 mg Fe/kg diet (Fe140) were used. Other experimental conditions were similar to those described above, except that alkaline phosphatase activity in serum was also measured in this experiment with the Cobas FARA analyzer (Roche Diagnostics Systems, Nutley, NJ). Rats weighed 58.7 ± 0.4 g at the beginning of the experiment

Results

Experiment 1—Interaction Between Mn and Cu. Although diet did not significantly affect food intake in this experiment, rats fed Cu+ diets were slightly and significantly heavier than rats fed Cu0 diets (Table I). Copper affected several of the indices of manganese metabolism, but the effects of manganese on copper metabolism were not as pronounced as the effects of copper on manganese.

Both plasma and whole blood manganese concentrations were affected by dietary copper, as well as manganese, so that animals fed Cu0 diets had greater concentrations of manganese in blood than did animals fed Cu+ (Table I). There was an interaction between copper and manganese such that the effect of copper on plasma and whole blood manganese was greater in rats fed Mn+ than in those fed Mn0 diets. The liver manganese concentration was also higher in rats fed the Cu0 diets than in those fed the Cu+ diet ($P < 0.05$); dietary manganese did not affect liver copper concentration. Femur manganese and copper concentrations were affected by an interaction between dietary manganese and copper, so that the highest concentrations of manganese and copper were found in femurs of rats fed diets adequate in both elements (Mn+Cu+).

The activity of Mn superoxide dismutase (SOD) was affected only by dietary manganese; the activity of CuSOD was affected by dietary copper and by an interaction between copper and manganese, such that rats fed Mn+Cu+ diets had the highest CuSOD activity in the heart. Plasma iron concentration was affected by dietary copper and by an interaction between manganese and copper, such that rats fed Mn+Cu+ diets had the highest plasma iron concentrations and those fed Mn+Cu0 had the lowest plasma iron concentrations. Hemoglobin and hematocrit were higher in rats fed Cu+ ($P < 0.0001$) than in rats fed Cu0 diets; there was no effect of dietary manganese on these two indices. Cholesterol was significantly increased ($P < 0.001$) in

Table I. Effects of Dietary Manganese and Copper on Manganese Metabolism

	Final wt (g)	Femur		Heart Mn	Heart Cu	Liver			Plasma			Whole blood	Hb	Hct	Chol	Arginase[b]	Mn	Mn biological half-life
		Mn (μg/g)	Cu (μg/g)	SOD[a] (units/mg prot)	SOD[a] (units/mg prot)	Mn (μg/g)	Cu (μg/g)	Fe (μg/g)	Mn (ng/ml)	Cu (μg/ml)	Fe (μg/ml)	Mn (ng/ml)	(g/dl)	(%)	(mg/dl)	(units/g wet wt)	(% absorp)	(days)
Diets																		
Mn0,Cu0	211	5.51[d,e]	5.23[d]	2.79	1.31[d]	3.06	1.50	555	3.30[d]	0.14	1.44[d]	5.2[d]	9.0	26.8	125.8	133.0	6.5	33.0
Mn0,Cu+	235	4.88[e]	5.63[c,d]	2.37	2.04[c]	1.31	16.52	382	3.10[d]	1.26	1.89[c,d]	5.7[d,e]	14.0	41.4	96.6	222.0	4.6	26.4
Mn+,Cu0	214	5.97[c,d]	4.44[e]	4.20	1.29[d]	9.69	2.11	457	6.90[c]	0.11	1.08[d]	17.3[c]	10.3	30.4	130.1	164.0	1.8	9.1
Mn+,Cu+	238	6.49[c]	6.23[c]	5.57	2.29[c]	8.66	15.94	275	5.50[c]	1.22	2.71[c]	11.0[c,d]	14.0	40.5	98.6	240.0	1.5	9.0
Analysis of variance (*P*-values)																		
Mn	NS	0.0001	NS	0.0014	NS	0.0001	NS	0.0092	0.0001	NS	NS	0.0001	NS	NS	NS	NS	0.0001	0.0001
Cu	0.0038	NS	0.0001	NS	0.0001	0.0443	0.0001	0.0001	0.0164	0.0001	0.0001	0.0053	0.0001	0.0001	0.0001	0.0001	0.0004	0.0001
Cu*Mn	NS	0.0086	0.0001	NS	0.0507	NS	NS	NS	0.0506	NS	0.0095	0.0268	NS	NS	NS	NS	0.0107	0.0001

[a] One unit of activity equals the amount of enzyme that reduces the auto-oxidation of pyrogallol (0.2 m*M*) by 50%.
[b] One unit of activity hydrolyzes 1 μmol of L-arginine to 1 μmol of ornithine per minute.
[c,d,e] Values in the same column not sharing a common letter are significantly different, $P < 0.05$.

Cu0 animals, and arginase activity was significantly decreased ($P < 0.0001$) in Cu0 animals.

The absorption and BH of ^{54}Mn were affected by both dietary manganese and copper and by an interaction between manganese and copper, such that both ^{54}Mn absorption and ^{54}Mn BH were greatest in animals fed diets deficient in both manganese and copper (Mn0Cu0). Absorption and BH of ^{54}Mn were least in animals fed diets adequate in both elements (Mn+Cu+).

Experiment 2—Interaction Between Mn and Ascorbic Acid. Added dietary ascorbate affected a few indices of manganese metabolism, but effects were generally small. Food intake and final weight were not affected by the dietary treatments (data not shown). The addition of ascorbate to the diet significantly ($P = 0.05$) decreased the activity of MnSOD, but not the activity of CuSOD in heart (Table II). Liver copper and iron were unaffected by the dietary treatment. Liver manganese was affected by dietary manganese, but unaffected by dietary ascorbate. However, plasma manganese was significantly lower ($P < 0.005$) when ascorbate was added to the diet. Plasma copper was unaffected by the dietary treatments; plasma iron responded to changes in dietary manganese only. Serum cholesterol was not affected by the dietary treatments. Whole blood manganese did not change with dietary ascorbate. The activity of arginase, a Mn-dependent enzyme, was significantly lower ($P < 0.05$) when ascorbate was added to the diet. The absorption and BH of ^{54}Mn did not change when ascorbate was added to the diet.

Experiment 3—Interactions among Mn, Cu, and Ascorbic Acid. All three dietary variables and interactions between copper and ascorbate and manganese and ascorbate affected the weight of animals in this experiment. Rats fed Mn+Cu+ diets were the heaviest (Table III), and rats fed diets deficient in both elements (Mn0Cu0) tended to be the lightest. The addition of ascorbic acid to the diet significantly depressed the weight of animals fed Mn+Cu0. Animals fed Cu0Asc+ diets were noticeably gray at the end of the experiment; this indicated that they were copper deficient.

The liver manganese concentration was significantly affected by interactions among all three dietary variables. Liver manganese was highest in rats fed Mn+Cu0Asc+ (10.3 μg/g) and lowest in animals fed Mn0Cu+ diets (1.6 μg/g). Liver copper was significantly affected by both dietary manganese and copper and by an interaction between manganese and ascorbate. Liver copper concentrations were slightly lower in animals fed Mn0 diets than in those fed Mn+ diets.

Plasma manganese and plasma copper were affected by all three dietary variables and by interactions between copper and manganese and between copper and ascorbate. Plasma manganese was highest in animals fed Mn+Cu0Asc+ (11.3 ng/ml), and it was lowest in animals fed Mn0Cu+ (2.5–3.1 ng/ml). Plasma copper was almost undetectable in animals fed Cu0 diets. In animals fed Cu+ diets, plasma copper was higher in rats fed Mn0 and Asc+ diets.

Serum cholesterol was affected by all three dietary variables. As expected, cholesterol was increased in Cu0 animals, but the absence of manganese from the diet somewhat counteracted the effect of copper. The addition of ascorbate to the diet decreased cholesterol.

Absorption of ^{54}Mn was affected by dietary manganese, an interaction between copper and manganese, and a marginal interaction between manganese and ascorbate. When manganese was absent from the diet, the addition of copper decreased ^{54}Mn absorption. The marginal interaction between manganese and ascorbate resulted in decreased ^{54}Mn absorption when ascorbate was added to Mn0 diets and a slight increase in ^{54}Mn absorption when ascorbate was added to Mn+ diets. The BH of ^{54}Mn was affected by all three dietary variables. The biological half-life of ^{54}Mn was decreased by the addition of manganese, copper, or ascorbate to the diet.

Table II. Effects of Dietary Manganese and Ascorbate on Manganese Metabolism

	Femur		Mn SOD[a] (units/mg prot)	Cu SOD[a] (units/mg prot)	Liver Mn (μg/g)	Plasma		Whole blood Mn (ng/ml)	Chol (mg/dl)	Arginase[b] (units/g wet wt)	Mn (% absorp)	Mn Biological half-life (days)
	Mn (μg/g)	Cu (μg/g)				Mn (ng/ml)	Fe (μg/ml)					
Diets												
Mn0,Asc0	5.88[d]	5.70	2.51	1.63	2.51	3.16	2.69	6.5	102.2	246.6	5.3	27.7
Mn0,Asc Hi	6.20[c,d]	5.90	1.93	1.79	3.22	2.42	2.54	4.7	93.8	198.1	4.3	26.0
Mn+,Asc0	6.54[c]	5.78	3.09	1.74	9.33	4.90	1.92	13.5	101.5	271.1	1.5	9.0
Mn+,Asc Hi	6.25[c,d]	5.66	2.85	1.57	9.05	4.00	2.20	13.0	102.6	238.3	1.7	8.6
Analysis of variance (*P*-values)												
Mn	0.0120	NS	0.0010	NS	0.0001	0.0001	0.0260	0.0001	NS	NS	0.0001	0.0001
Asc	NS	NS	0.0517	NS	NS	0.0050	NS	NS	NS	0.0424	NS	NS
Mn*Asc	0.0270	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a] One unit of activity equals the amount of enzyme that reduces the auto-oxidation of pyrogallol (0.2 m*M*) by 50%.
[b] One unit of activity hydrolyzes 1 μmol of L-arginine to 1 μmol of ornithine per minute.
[c,d] Values in the same column not sharing a common letter are significantly different, $P < 0.05$.

Table III. Interactions of Dietary Copper, Manganese, and Ascorbate on Copper and Manganese Metabolism

	Final wt (g)	Heart Mn-SOD[a] (units/mg prot)	Heart Cu-SOD[a] (units/mg prot)	Liver		Plasma		Chol (mg/dl)	Mn (% absorp)	Mn biological half-life (days)	Cu (% absorp)	Cu biological half-life (days)
				Mn (μg/g)	Cu (μg/g)	Mn (ng/ml)	Cu (μg/ml)					
Diets												
Mn0,Cu0,Asc0	267	1.70	1.05[c]	3.08[b]	1.48	3.56	0.03	131.9	5.8	42.7	33.5	2.18
Mn0,Cu0,Asc+	267	0.90	1.80[b,c,d]	2.60[b]	1.72	4.99	0.04	121.0	5.6	41.8	21.1	2.22
Mn0,Cu+,Asc0	328	1.44	1.87[b,d]	1.57[b]	12.24	2.54	1.34	86.9	4.8	40.2	23.9	2.21
Mn0,Cu+,Asc+	330	0.74	2.37[b]	1.60[b]	13.20	3.08	1.53	81.2	2.9	37.7	19.3	2.19
Mn+,Cu0,Asc0	317	2.46	1.22[c,d]	7.60[c]	2.67	7.38	0.03	141.6	2.2	18.1	36.4	1.68
Mn+,Cu0,Asc+	254	1.72	1.54[c,d]	10.27[c]	1.51	11.30	0.03	124.6	3.2	16.1	18.2	1.78
Mn+,Cu+,Asc0	347	2.56	1.86[b,d]	8.01[c]	14.08	4.81	1.06	107.4	3.3	14.8	26.4	1.84
Mn+,Cu+,Asc+	345	1.47	2.64[b]	7.81[c]	13.83	4.99	1.25	90.9	3.4	14.3	29.9	1.74
Analysis of variance (*P*-values)												
Cu	0.0001	NS	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	NS	0.0001	NS	NS
Mn	0.0244	0.0001	NS	0.0001	0.0103	0.0001	0.0039	0.0256	0.0001	0.0001	NS	0.0001
Asc	0.0500	0.0001	0.0001	NS	NS	0.0002	0.0512	0.0110	NS	0.0140	0.0003	NS
Cu*Mn	NS	NS	NS	NS	NS	0.0003	0.0048	NS	0.0056	NS	NS	NS
Cu*Asc	0.0474	NS	NS	0.0315	NS	0.0040	0.0521	NS	NS	NS	0.0007	0.0011
Mn*Asc	0.0355	NS	NS	0.0091	0.0481	NS	NS	NS	NS	NS	NS	NS
Cu*Mn*Asc	NS	NS	0.0260	0.0027	NS	NS	NS	NS	NS	NS	NS	NS

[a] One unit of activity equals the amount of enzyme that reduces the auto-oxidation of pyrogallol (0.2 m*M*) by 50%.

[b,c,d] Where a three-way interaction was noted, values in the same column not sharing a common letter are significantly different, $P < 0.05$.

Addition of ascorbate to the diet decreased ^{67}Cu absorption significantly, except in animals fed both Mn+Cu+ diets. The BH of ^{67}Cu was significantly shorter in Mn+ animals than in Mn0 animals. The BH of ^{67}Cu was also affected by an interaction between dietary copper and ascorbate.

Experiment 4—Interactions among Mn, Cu, and Fe. Food intake was affected by interactions among all three dietary variables in this experiment so that final weight of animals was also significantly affected by diet composition (Table IV).

Liver and femur mineral concentrations were generally affected by all three dietary variables and by interactions among them (Table V). Liver and femur manganese concentrations were significantly affected by a three-way interaction among Fe, Cu, and Mn, so that femur manganese was most often decreased by the high iron (Fe140) diet, and the lowest femur manganese among Mn+ animals was in those fed diets adequate in copper and high in iron (Cu+Fe140); however, liver manganese concentration was highest in the Mn+Cu0Fe140 animals and lowest in Mn0Cu+Fe140 animals. The femur copper concentration was also affected by a three-way interaction among dietary variables, so that copper was highest in Mn0 animals and lowest in Mn+Cu+Fe140 animals; liver copper was not affected by dietary manganese. The liver and femur iron concentrations were affected by all three dietary variables. Manganese-deficient animals had higher liver and femur iron than did Mn+ animals, except that Mn0Cu+Fe10 animals had low liver iron concentrations and Mn+Cu0Fe140 animals had high liver iron concentrations. Dietary copper and iron were synergistic, so that femur iron was much higher in animals fed Cu+Fe140 diets than for any other combination of copper and iron intake. Liver iron concentrations were higher in copper-deficient animals than in animals fed adequate copper.

The activity of heart MnSOD (Table IV) was affected not only by manganese, but also by two-way interactions among all of the dietary variables. Activity of the enzyme was greatest when animals were fed Mn+Cu0Fe140 diets. Activity of MnSOD was lowest in animals fed Mn0Cu0Fe140 diets. The activity of CuSOD was affected not only by copper, but also by an interaction between manganese and iron. The activity of CuSOD was greatest in the Mn+Cu+Fe10 diet group; it was lowest in the Mn+Cu0Fe140 and Mn0Cu0Fe10 diet groups.

Serum manganese was affected by an interaction between dietary copper and manganese (Table V). In Mn0 animals, copper deficiency decreased serum manganese; in Mn+ animals, copper deficiency resulted in a marked increase in serum manganese. Whole blood manganese was also affected by an interaction between copper and manganese, but the effect in copper was most pronounced in Mn+ animals. Serum copper was nearly undetectable in Cu0 animals. It was affected by an interaction between manganese and copper, so that serum copper in Cu+ animals was higher with the Mn0 than the Mn+ treatment. Serum iron was affected by a three-way interaction among all three dietary variables.

Arginase activity was affected by both dietary manganese and copper (Table IV). Both removal of copper from the diet (Cu0) and addition of manganese (Mn+) to the diet decreased arginase activity. Alkaline phosphatase activity was affected by dietary copper and iron and by an interaction between iron and manganese, so that activity was highest in Mn+Cu+Fe0 animals and lowest (by almost half) in Mn+Cu0Fe140 animals. Serum cholesterol was affected by a three-way interaction among the dietary variables, so that cholesterol was lowest in Mn0Cu+Fe140 animals and highest in Mn+Cu0Fe10 animals. There was a significant three-way interaction effect on ceruloplasmin, but it was overshadowed by the magnitude of the effect of dietary copper; as expected, ceruloplasmin was extremely low in Cu0 animals.

Hemoglobin and hematocrit were affected by interactions among all three dietary variables (Table IV). In animals that were fed diets adequate in both manganese and copper (Mn+Cu+), the amount of dietary iron had only a small effect on hemoglobin and hematocrit. In contrast, in animals fed diets deficient in both copper and manganese (Mn0Cu0), the amount of dietary iron had a pronounced effect on hemoglobin and hematocrit. The lowest values for hemoglobin and hematocrit were found in animals fed Mn+Cu0Fe140.

Manganese absorption (Table IV) was much lower in Mn+ animals than in Mn0 animals; however, ^{54}Mn absorption was equally low in Mn0Cu+Fe140 animals. Absorption of ^{54}Mn was greatest in Mn0Cu+Fe10 animals. The BH of ^{54}Mn was much shorter in Mn+ animals than in Mn0 animals. It was about 25% shorter in Mn0Cu+Fe10 animals than in other groups of Mn0 animals.

Discussion

The existence of interactions between iron and manganese metabolism has been known for many years. Iron and manganese are antagonistic toward one another, so that low iron status enhances Mn absorption and predisposes to Mn toxicity; high dietary iron or iron status decreases Mn absorption (13–16). An interaction between copper and manganese has been noticed in a few instances (2–5, 10–12), but never systematically examined. The results of our studies indicate that manganese and copper do interact negatively with one another, but the interaction is not as strong as that between manganese and iron.

Some definite signs of an interaction between copper and manganese were noted in the first experiment.

Table IV. Interactions of Dietary Copper, Iron, and Manganese on Manganese Metabolism

	Food intake (g/d)	Final weight (g)	Heart MnSOD[a] (units/ mg prot)	Heart CuSOD[a] (units/ mg prot)	Hb (g/dl)	Hct (%)	Chol (mg/dl)	Alk. Phos[b] (units/ liter)	Arginase[c] (units/mg protein)	Cerulo (mg/dl)	Mn (% absorp)	Mn biological half-life (days)
Diets												
Mn0,Cu0,Fe10	13.7	220[f]	1.71	1.29	6.3[f,g]	20.0[f]	136[d]	207	4.33	1.9[d]	6.7[e,g]	41.8[f]
Mn0,Cu0,Fe140	15.0	236[d,f]	1.22	1.48	9.8[e]	29.7[e]	129[d,f]	228	4.27	2.0[d]	3.6[d,g]	42.5[f]
Mn0,Cu+,Fe10	18.3	299[d]	2.97	2.27	14.3[d]	41.4[d]	103[d,f]	328	4.72	38.9[e]	11.0[f]	30.6[e]
Mn0,Cu+,Fe140	16.8	278[d,f]	1.41	2.43	13.8[d]	39.9[d]	79[e,f]	294	5.16	30.8[e]	1.9[d]	40.7[f]
Mn+,Cu0,Fe10	16.3	263[d,f]	3.73	1.34	10.1[e]	30.4[e]	149[d]	290	2.59	2.2[d]	1.5[d]	9.7[d]
Mn+,Cu0,Fe140	13.4	214[d,f]	4.68	1.27	4.8[g]	15.2[f]	129[d,f]	173	3.14	2.3[d]	1.7[d]	9.8[d]
Mn+,Cu+,Fe10	17.8	299[d]	3.93	3.14	13.5[d]	39.3[d]	104[d,f]	337	3.78	27.4[e]	1.2[d]	8.5[d]
Mn+,Cu+,Fe140	17.4	287[d]	3.69	2.29	14.3[d]	41.3[d]	109[d,f]	282	4.30	36.1[e]	1.3[d]	10.3[d]
Analysis of variance (*P*-values)												
Cu	0.0001	0.0001	NS	0.0001	0.0001	0.0001	0.0001	0.0001	0.0002	0.0001	NS	0.0008
Mn	NS	NS	0.0001	NS	NS	NS	0.0310	NS	0.0001	NS	0.0001	0.0001
Fe	0.0200	0.0191	NS	NS	NS	NS	0.0289	0.0068	NS	NS	0.0001	0.0014
Cu*Fe	NS	NS	0.0229	NS	NS	NS	NS	NS	NS	NS	0.0002	0.0052
Cu*Mn	NS	NS	0.0236	NS	NS	NS	NS	NS	NS	NS	0.0281	0.0019
Mn*Fe	0.0301	0.0488	0.0060	0.0236	0.0001	0.0001	NS	0.0189	NS	0.0212	0.0001	0.0228
Cu*Mn*Fe	0.0003	0.0082	NS	NS	0.0001	0.0001	0.0462	NS	NS	0.0220	0.0004	0.0490

[a] One unit of activity equals the amount of enzyme that reduces the auto-oxidation of pyrogallol (0.2 m*M*) by 50%.
[b] One unit of activity equals the amount of enzyme that hydrolyzes 1 μmol of 4-nitrophenylphosphate to 1 μmol of 4-nitrophenomide ion per minute.
[c] One unit of activity hydrolyzes 1 μmol of L-arginine to 1 μmol of ornithine per minute.
[d,e,f,g] Where a three-way interaction was noted, values in the same column that do not share a common letter are significantly different, $P < 0.05$.

Table V. Interactions of Dietary Copper, Iron, and Manganese on Tissue Mineral Concentrations

	Femur			Liver			Serum			Whole blood
	Mn (μg/g)	Cu (μg/g)	Fe (μg/g)	Mn (μg/g)	Cu (μg/g)	Fe (μg/g)	Mn (ng/ml)	Cu (μg/ml)	Fe (μg/ml)	Mn (ng/ml)
Diets										
Mn0,Cu0,Fe10	0.63[b,d]	2.91	46.4	2.82[d]	2.34	422[c]	2.66	0.03	1.83[b]	4.3
Mn0,Cu0,Fe140	0.44[d]	2.17	53.6	1.55[d]	1.38	471[c]	1.94	0.03	2.73[b,c]	2.4
Mn0,Cu+,Fe10	0.57[d]	2.95	60.5	2.09[d]	12.96	148[b]	3.10	2.00	5.95[a,c]	3.7
Mn0,Cu+,Fe140	0.47[c,d]	2.87	85.7	0.87[d]	10.42	411[c]	2.89	1.73	4.46[a,c]	2.2
Mn+,Cu0,Fe10	1.16[a]	1.25	43.3	8.46[a,c]	2.63	253[b,c]	11.13	0.04	2.34[b,c]	21.1
Mn+,Cu0,Fe140	1.13[a]	1.60	51.2	10.19[a]	1.60	483[c]	10.54	0.03	2.26[b,c]	15.3
Mn+,Cu+,Fe10	1.11[a]	1.96	56.0	8.07[a,c]	13.63	124[b]	5.38	1.61	6.13[a]	11.5
Mn+,Cu+,Fe140	0.81[b]	0.89	73.5	6.80[b,c]	11.45	253[b,c]	8.58	1.59	7.01[a]	8.3
Analysis of variance										
Cu	0.0004	NS	0.0001	0.0001	0.0001	0.0001	0.0065	0.0001	0.0001	0.0001
Mn	0.0001	0.0001	0.0121	0.0001	NS	0.0022	0.0001	0.0215	NS	0.0001
Fe	0.0001	NS	0.0001	0.0438	0.0001	0.0001	NS	NS	NS	0.0012
Cu*Mn	0.0028	NS	NS	0.0199	NS	NS	0.0002	0.0182	NS	0.0001
Cu*Fe	NS	NS	0.0022	0.0041	NS	NS	NS	NS	NS	NS
Mn*Fe	NS	NS	NS	0.0040	NS	NS	NS	NS	NS	NS
Cu*Mn*Fe	0.0015	0.0215	NS	0.0031	NS	0.0042	NS	NS	0.0266	NS

[a,b,c,d] Where a three-way interaction was noted, values in the same column that do not share a common letter are significantly different, $P < 0.05$.

Copper depressed absorption of manganese and accelerated its turnover. This is consistent with the findings of Freeland-Graves and Lin (12), who found that plasma uptake in humans of a 40 mg dose of manganese was decreased by almost half when 2 mg of copper was given at the same time; however, the differences in their study were not statistically significant. In a series of studies with men and women fed between 0.6 and 6.5 mg Cu/day, increasing dietary copper tended to depress manganese balance, but the effect did not reach statistical significance (P. E. Johnson and D. B. Milne, unpublished data). Consistent with its effect on manganese absorption, adequate dietary copper depressed liver, plasma, and whole blood manganese of rats in the first experiment here. Strause *et al.* (4) found that plasma manganese of female rats was slightly, but not significantly, lower in rats fed a diet low in manganese and adequate in copper for 12 months than in rats fed a diet low in both elements. Nielsen and Shuler (5) found that rats fed 6 ppm of dietary copper had lower liver manganese concentrations than rats fed diets with no added copper; the effect was more striking in female rats than in male rats. Similarly, King *et al.* (2) found that copper deficiency increased manganese concentrations in liver and kidney. The same group reported earlier (36) that rats fed a corn and skim milk diet (<1 ppm of Mn, 8 ppm of Fe, and 5 ppm of Cu) had higher kidney and liver manganese concentrations than did rats fed a purified diet (<1 ppm of Mn, 61 ppm of Fe, and 0.1 ppm of Cu); however, the diets also differed in zinc, protein, and fat content. We found exactly the opposite effects when we covaried manganese, iron, and copper in the fourth experiment, which gave results consistent with the first experiment. Evidently, other factors in addition to copper and iron affected the findings in the earlier work of King *et al.* (36), inasmuch as more recent reports (2, 4, 5) are consistent with our observations that, compared with deficient amounts of dietary copper, adequate copper depresses absorption and tissue concentrations of manganese. Despite its effect on tissue manganese concentrations, dietary copper did not affect the activity of heart MnSOD in any of the present experiments, so dietary copper apparently did not affect functional manganese metabolism at the concentrations studied. This is in contrast to the findings of Paynter (37) who found effects of dietary copper on MnSOD activity in both heart and liver as part of a larger study involving effects of selenium, vitamin E, copper, and manganese on lipid peroxidation. Activity of arginase, which is a manganese-containing enzyme that is responsive to dietary manganese, was increased by increasing dietary copper; although activity tended to be higher in rats fed the adequate level of manganese, there was not a significant effect of dietary manganese on arginase activity in the first two experiments here. However, in Experiment 4, low dietary manganese significantly depressed arginase activity. The functional significance of changes in arginase activity is not known (8).

Within the range of dietary concentrations studied, manganese had much less effect on copper metabolism than copper had on manganese metabolism. This is consistent with the findings of Strause *et al.* (4), who found no change in plasma copper of female rats after either 6 or 12 months of a low manganese diet. However, Kirchgessner and Heiske (3) found that manganese deficiency reduced the copper concentration in the tissues of male rats. Female rats are known to be

less susceptible to copper deficiency and the effects of fructose on copper metabolism than males (38, 39); perhaps they are also more resistant to other factors affecting copper metabolism. Although dietary manganese did not affect copper balance in pregnant swine, nor plasma copper in newborn piglets, dams fed high manganese tended to have lower milk copper concentrations than dams fed low manganese (40). Gubler *et al.* (41) reported that administration of manganese to rats increased plasma and brain copper and decreased kidney copper concentrations; however, manganese was given both via the diet (~18 mg/kg) and by intraperitoneal injection of $MnCl_2$, so it is difficult to compare their findings to the present experiments. Likewise, Scheuhammer and Cherian (7) found increases in copper in several organs of rats after injections of manganese.

Ascorbic acid affects both copper and iron metabolism and might be expected to affect manganese metabolism as well, either indirectly through effects on copper or iron, or directly through chelation or an effect on the oxidation state of manganese. However, when manganese and ascorbic acid were the only dietary variables, the effects of added ascorbic acid on manganese metabolism were minimal. There was a small effect on femur manganese concentrations, but not on manganese concentrations in liver or blood. Interestingly, the addition of ascorbic acid did decrease the activity of MnSOD, although it had no effect on the activity of CuSOD. This probably should be considered a deleterious effect as reductions in MnSOD activity have been shown to result in increased lipid peroxidation and mitochondrial damage in growing rats (8). In humans fed diets with low or adequate copper and ~50 mg or 1.5 g ascorbic acid/day, ascorbic acid supplementation had no effect on manganese balance (P. E. Johnson and D. B. Milne, unpublished data). Kies *et al.* (42) reported that 237 mg ascorbic acid/day compared with 37 mg/day significantly enhanced retention of manganese in humans; however, experimental details were not described. A related paper (43) showed an apparent subset of the data; there were substantial differences in dietary manganese between the ascorbate and nonascorbate groups that may have confounded their results.

In the third experiment in this series, when both dietary copper and ascorbic acid were varied, ascorbic acid depressed MnSOD activity as it had in the second experiment, but, in addition, the activity of CuSOD was increased by the addition of ascorbate to the diet. The increase in CuSOD activity may have been in response to the decrease in MnSOD activity, but because CuSOD is a cytosolic enzyme and MnSOD is mitochondrial, such a compensatory response is unlikely to be sufficient to provide protection against peroxidation reactions in the cell. The activity of CuSOD was maximal when both copper and ascorbic acid were high in the diet. The increase in activity of CuSOD in response to increased dietary ascorbic acid could be a result of ascorbate's role as an initiator of lipid peroxidation (44, 45) through release of iron from cytosolic ferritin. In the third experiment, added ascorbic acid depressed ^{54}Mn BH slightly. A similar trend existed in the second experiment, but differences were too small to be significant. Added ascorbic acid depressed ^{54}Mn absorption when dietary manganese was adequate and increased it when dietary manganese was deficient, but dietary copper did not affect ^{54}Mn absorption. King *et al.* (2) also found that increasing dietary copper from 0.4 ppm to 5 ppm had little direct effect on ^{54}Mn retention, although they felt that dietary copper concentration tended to modify the effect of lactose on ^{54}Mn retention. Copper absorption was also measured in this experiment, and ^{67}Cu absorption was significantly higher when dietary manganese was deficient than when it was adequate, again suggesting a sort of compensatory response in which copper is being absorbed to replace some function of manganese.

Davis *et al.* (46) found decreased MnSOD activity in animals fed high amounts (109 mg/kg diet) of iron, but suggested that an effect of iron on manganese absorption that reduced tissue manganese concentrations and hence MnSOD activity, rather than increased lipid peroxidation, was responsible for the change in SOD activity because tissue iron concentrations were not elevated in manganese-deficient rats. We did not find a main effect of dietary iron on MnSOD activity, but, like Davis *et al.* (46), we found that interactions between iron and copper, and manganese, resulted in a tendency for MnSOD activity to be lower in rats fed the higher amount of iron. However, the effects of iron on manganese absorption in our experiment were far more pronounced than iron effects on MnSOD, which suggests that changes in tissue manganese, although present, are not the whole explanation for changes in MnSOD activity. The depressing effects of high iron and of high ascorbate on MnSOD activity especially in Mn0 animals, were similar and generally accompanied by increases in CuSOD activity. This suggests that these effects may have occurred because iron and ascorbate were affecting manganese metabolism through an effect on copper.

The effects of dietary iron on manganese metabolism were consistent with those reported by other investigators (9–12). The interaction between copper and iron, as reflected in data for hemoglobin, ceruloplasmin, and other indices, was similar to those reported by others. The most striking evidence of an interaction between iron and copper affecting manganese metabolism is seen in the ^{54}Mn absorption data. Absorption of ^{54}Mn is usually quite low when dietary manganese is adequate, and higher when dietary manganese is defi-

cient. In this experiment, the effects of varying copper and iron in manganese-deficient diets were pronounced; when iron was high and copper adequate in the manganese-deficient diet, ^{54}Mn absorption was as low as it was for the adequate manganese groups, but when the iron was reduced to a marginal level, ^{54}Mn absorption increased 5-fold. However, the animals fed manganese-deficient diets with the higher amounts of both copper and iron had a BH of ^{54}Mn similar to that for other manganese-deficient animals. The high ^{54}Mn absorption in rats fed the adequate copper, low iron, manganese-deficient diets was partly counterbalanced by a ^{54}Mn BH that was 25% shorter than for other manganese-deficient groups of animals. Somewhat similar patterns of response were found for MnSOD activity and liver manganese concentration.

Although ascorbic acid might be expected to have an effect on manganese metabolism because of its oxidation-reduction potential or its chelating ability, and because ascorbic acid also has pronounced effects on the metabolism of iron and copper, the effects of ascorbate on manganese metabolism in these experiments were minimal. It is possible that the effects of ascorbic acid on manganese metabolism might be more evident in humans or in an animal such as the guinea pig, which cannot manufacture its own ascorbic acid. However, the limited data available from human studies argue against this.

The concentrations of dietary manganese and copper in these experiments ranged from deficient to adequate but not excessive. Within this range of variation, copper and manganese seem to have opposite effects on many factors that tend to balance each other. When dietary copper is low, manganese absorption and manganese in tissues increase. When dietary manganese is low, copper absorption and copper in tissues increase.

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