Calcium Metabolism in Manganese-Deficient and Zinc-Deficient Rats* (33672)

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Dietary deficiencies of manganese and of zinc are known to have profound effects on the skeleton (1). In manganese-deficient chicks, perosis is a prominent deformity. The same deficiency produces in rats disproportionate growth of the skeleton, curvatures of radius and ulna, scoliosis and kyphosis, bony rarefaction, chondrodystrophy, and abnormal development of the proximal tibial epiphysis (1–4). In addition, congenital defects in ossification of the otic capsule (5) and in otolith calcification (6–8) are major effects of manganese deficiency during pregnancy.

The skeletal abnormalities described in manganese-deficient animals could result from abnormal calcium metabolism or from faulty matrix formation. Evidence of various types pointed to abnormalities of cartilage or bone matrix formation in manganese-deficient animals as leading to defects of ossification. On the other hand, findings of lowered ash content and bone rarefaction suggest derangements of calcium metabolism in this deficiency (1, 8, 9).

In zinc-deficient chicks, a perosis occurs similar to that resulting from manganese deficiency. In other animals with a dietary deficiency of this element gross deformities of bones and joints have been reported, as well as radiologic and histologic rarefaction (1). Congenital skeletal disorders are a prominent effect of maternal zinc deficiency (10). In addition, certain histochemical and autoradiographic evidence suggests that zinc plays a specific (although unknown) role in bone formation (1). The present study was therefore undertaken to investigate the metabolism of calcium in manganese-deficient and in zinc-deficient rats.

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Methods. All animals used were Sprague-Dawley rats. For the experiments with manganese deficiency, female rats weighing 80–90 g were purchased from a commercial source and were given a ration containing either 2 ppm added manganese (manganese-deficient diet) or 44 ppm added manganese (manganese-supplemented control diet). The ration had the following composition (percent): casein,[2] 30; salts,[3] 6; corn oil, 8; glucose (Cerelose), 56. Crystalline vitamins were given separately.[4] The calcium content of the diet was 0.8%; the phosphorus content was 0.4%.

The rats were fed these diets until they reached a body weight of 180 g, when they were mated with stock-fed males. The females received a single diet (either manganese-deficient or manganese-supplemented) throughout gestation and lactation; the young were given the same diet after weaning. At 6, 9, or 12 weeks of age, calcium metabolism was studied in the female offspring.

For the experiments with zinc deficiency,

[2] Purified high-nitrogen casein from Nutritional Biochemicals Co.

[3] The salt mix had the following composition (g): $CaCO_3$, 600; $CaHPO_4$, 120; K_2HPO_4, 650; NaCl, 336; $MgSO_4 \cdot 7H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 50; KI, 1.6; $CuSO_4 \cdot 5H_2O$, 0.6; $ZnCO_3$, 0.5; $MnSO_4 \cdot H_2O$, either 0.205 or 4.6, to provide either 2 or 44 ppm, respectively, to the diet.

[4] A mixture of crystalline vitamins in glucose, to which was added cod liver oil and α-tocopheryl acetate, was given 3 times each week in small glass dishes in amounts to provide the following intake (μg/day); Ca-pantothenate, 500; *p*-aminobenzoic acid and riboflavin, each 100; thiamine·HCl, pyridoxine, and nicotinic acid, each 300; menadione, 250; folic acid, 6; biotin, 2.5; vitamin B_{12}, 0.3 and choline chloride, 10 mg; inositol, 5 mg; α-tocopherol and ascorbic acid, each 1 mg vitamin A, 150, and vitamin D, 15, IU each.

weanling female rats were purchased from a commercial source and were given the stock ration[5] for two days. They were then transferred to a complete (control) purified diet of the following composition, in percent: soybean protein,[6] 30.0; sucrose, 57.3; corn oil, 8.0; salt mix,[7] 4.0; DL-methionine, 0.7. Crystalline vitamins were given separately.[4] The calcium content of the diet was 0.5%; the phosphorus content was 0.3%.

The animals were kept in individual stainless steel cages, and extreme care was taken to eliminate sources of zinc contamination from the environment (11). After 7 days of becoming accustomed to the purified diet, some of the rats were given a zinc-deficient diet which was the same ration as the control diet except that zinc was omitted from the salt mix. This ration contained 0 ppm of zinc as determined by X-ray fluorescence analysis, with an error in the method of ± 2 ppm. The control diet contained 60 ppm of added zinc.[8] These conditions of diet and environmental control have produced extreme zinc deficiency in rats (10, 11).

Some of the control rats were fed *ad libitum*. The other control rats had their food intake restricted to the amount of food eaten by the deficient animals. Seventeen days after the zinc-deficient diet was started, the animals were placed in metabolism cages; on day 18, the ^{45}Ca experiment was begun. During the study of calcium metabolism, the restricted-intake controls continued to receive the amount of food eaten by the deficient animals.

Calcium metabolism was studied by the method of Aubert and Milhaud (12, 13), which combines a kinetic study using ^{45}Ca with a short-term classical balance study. After the intravenous injection of a test dose of ^{45}Ca, measurements were made of the disappearance of radioactivity in the blood, the blood calcium level, the amounts of ingested and fecal calcium, and the specific activities of fecal and urinary calcium. The time-course of the plasma specific activity can be described by a two-termed exponential equation, thought to be generated by a two-compartment system (14). From an analysis of this equation, together with the calcium data, one can calculate the following parameters: ingested Ca, V_i; total fecal Ca, F; Ca absorbed during digestion, V_a; urinary Ca, V_u; endogenous fecal Ca, V_f; bone formation rate (Ca into bone), V_{o+}; bone resorption rate (Ca out of bone), V_{o-}; Ca balance, Δ; rapidly exchangeable Ca pool, P; slowly exchangeable Ca in bone, E; rate of slow exchange, V_e; the total amount of Ca lost from body fluids, organs, and soft tissues (i.e., $V_u + V_f + V_{o+}$), V_T, and the absorption coefficient, a (i.e., V_a/V_i).

The animals were housed in stainless steel metabolism cages throughout the experiment. They were given an intravenous injection containing 20–40 μCi of ^{45}Ca as $CaCl_2$. Serial blood samples were taken from the retro-orbital venous sinus at 2, 4, 6, 24, 32, and 48 hr after injection. A 72-hr urine collection was made for measurement of urinary radioactivity. Food intake was measured and fecal excretion was collected during a 48-hr period between 6 and 54 hr after injection. Feces were marked by giving 0.1 ml of 10% carmine solution by gavage at the beginning and the end of the 48-hr period.

Samples of the diet and the feces were ashed in a muffle furnace and brought up to volume with 2 *N* HCl. Urine samples were wet ashed with 1:1 nitric and perchloric acids and brought to volume. Serum samples were analyzed directly. Calcium content of the food and feces was measured by precipitation of the oxalate and titration with $KMnO_4$ (15). Serum calcium was estimated by the differential spectrophotometric method of Radin and Granza based on the change in absorbance of a dye due to the calcium ion (16). Radioactivity was measured at infinite

[5] Commercial rat chow (Wayne Lab Blox) and powdered whole milk, both *ad libitum*.

[6] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cincinnati, Ohio. The soybean protein was treated with the tetrasodium salt of ethylenediaminetetraacetic acid to lower its zinc content.

[7] The salt mix had the following composition (g): $CaCO_3$, 600; $Ca(H_2PO_4)_2 \cdot H_2O$, 220; K_2HPO_4, 650; NaCl, 336; $MgSO_4 \cdot 7H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 50; $MnSO_4 \cdot H_2O$, 4.6; KI, 1.6; $CuSO_4 \cdot 5H_2O$, 0.6.

[8] As $ZnCO_3$.

TABLE I. Calcium Kinetics in Normal and Manganese-Deficient Rats at Various Ages.[a]

(weeks):	6		8		12	
	Mn+	Mn−	Mn+	Mn−	Mn+	Mn−
No. of rats	4	3	13	13	4	5
Body wt. (g)	119 ± 8	85 ±19	170 ± 6	138 ± 6	224 ±10	193 ±13
Serum Ca (mg/100 ml)	10.9 ± 0.1	10.5 ± 0.1	10.5 ± 0.2	10.4 ± 0.1	11.0 ± 0.2	10.6 ± 0.2
V_i (mg/day)	60.1 ± 2.8	55.5 ± 7.8	77.7 ± 2.6	74.4 ± 3.2	75.0 ± 5.9	71.2 ±10.0
F (mg/day)	28.8 ± 4.6	20.0 ± 3.8	47.6 ± 5.0	45.3 ± 4.8	50.4 ± 9.9	46.1 ± 7.5
V_a (mg/day)	35.2 ± 1.9	38.5 ± 8.2	35.1 ± 3.7	35.7 ± 4.3	31.4 ± 6.2	31.7 ± 6.7
V_u (mg/day)	1.0 ± 0.2	0.9 ± 0.1	0.5 ± 0.1	0.5 ± 0.04	0.4 ± 0.04	0.6 ± 0.2
V_f (mg/day)	3.9 ± 0.7	3.0 ± 0.7	5.0 ± 0.4	6.4 ± 0.8	6.7 ± 1.2	7.0 ± 1.9
Δ (mg)	+30.3 ± 2.8	+34.6 ± 8.1	+29.6 ± 4.0	+28.6 ± 4.7	+24.2 ± 7.1	+24.5 ± 5.6
a (%)	59.2 ± 5.2	68.3 ± 7.8	45.9 ± 5.2	48.1 ± 5.2	42.6 ± 9.6	42.9 ± 6.3
V_{o+} (mg/day)	53.1 ± 3.9	45.8 ± 5.6	65.4 ± 3.1	64.9 ± 3.9	44.5 ± 4.6	38.8 ± 3.1
V_{o-} (mg/day)	22.8 ± 5.9	11.3 ± 4.8	35.8 ± 4.9	36.3 ± 3.6	18.2 ± 5.4	14.4 ± 7.6
P (mg)	25.7 ± 1.3	21.9 ± 3.8	22.6 ± 1.3	21.2 ± 1.5	15.5 ± 1.7	15.6 ± 2.8
V_T (mg/day)	58.0 ± 4.4	49.4 ± 4.7	70.9 ± 3.4	71.9 ± 3.8	51.6 ± 3.7	46.5 ± 3.7
V_e (mg/day)	72.6 ±10.7	65.7 ±11.8	78.4 ± 5.3	82.2 ± 4.1	72.6 ± 3.7	73.9 ± 8.5
E (mg)	62.8 ± 8.5	61.7 ±18.2	47.7 ± 3.4	52.5 ± 4.1	38.4 ± 2.8	41.8 ±10.7

[a] Means ± SE.

thinness on an automatic gas-flow counter.

Results. The various parameters of calcium metabolism in manganese-deficient as compared with normal rats at three ages are summarized in Table I. Body weight was lower in manganese-deficient rats than in controls of the same age. There were, however, no differences in any of the parameters of calcium metabolism including pool size (P), endogenous fecal calcium (V_f), urinary calcium (V_u), or in the rates of calcium entering or leaving bone (V_{o+} and V_{o-}).

The results of the experiments with zinc-deficient rats are summarized in Table II. Both the restricted-intake controls and the zinc-deficient rats showed decreases (as compared with *ad libitum* controls) in calcium balance (Δ), absorbed calcium (V_a), and total amount of calcium lost from body fluids, organs, and soft tissues (V_T), and, of course, ingested calcium (V_i). The reduction in these parameters in zinc-deficient rats thus appears to be at least partially due to the decrease in food intake which accompanies this deficiency state.

The zinc-deficient animals, however, showed significant decreases, as compared with the restricted-intake controls, in the rate of bone anabolism (calcium going into bone, V_{o+}), in bone resorption rate (calcium coming out of bone, V_{o-}), pool size (P), calcium lost from the pool, organs, and soft tissues (V_T), rate of slow exchange (V_e), and slowly exchangeable calcium in bone (E). Urinary calcium (V_u) was slightly increased, but the difference was insignificant ($p>0.10$). The zinc-deficient animals also had higher values for the specific activity of plasma as compared with either of the control groups, although the serum calcium level was the same in all three groups.

Discussion. In the studies of manganese-deficient rats, no disturbances in the metabolism of calcium were detected. This suggests that the skeletal lesions and other defects of calcification observed in manganese-deficient animals are not the result of abnormal calcium metabolism. Substantial evidence has now been accumulated that in manganese-deficient animals there is defective production of the organic matrix (8). The present results lend additional support to the hypothesis that osteogenesis and calcification are impaired in the absence of manganese

TABLE II. Calcium Kinetics in Normal and Zinc-Deficient Rats.[a]

No. of rats:	+ Zn control *ad libitum* 6	+ Zn control restricted intake 10	Zinc deficient 10
Body wt. (g)	155 ± 6	128 ± 4 [b]	100 ± 4 [c]
Serum Ca (mg/100 ml)	10.2 ± 0.2	10.3 ± 0.2	10.2 ± 0.1
V_i (mg/day)	50.6 ± 5.0	25.8 ± 2.0[b]	27.8 ± 1.7
F (mg/day)	11.6 ± 2.5	9.4 ± 1.0	10.1 ± 1.5
V_a (mg/day)	41.0 ± 2.6	19.1 ± 1.8[b]	20.2 ± 1.6
V_u (mg/day)	0.3 ± 0.04	0.3 ± 0.05	0.5 ± 0.1
V_f (mg/day)	2.1 ± 0.7	2.7 ± 0.3	2.5 ± 0.4
Δ (mg)	+38.6 ± 2.9	+16.1 ± 1.8[b]	+17.2 ± 1.7
a (%)	82.5 ± 3.3	73.4 ± 2.9	73.1 ± 3.9
V_{o+} (mg/day)	63.8 ± 8.8	45.1 ± 4.3	31.2 ± 2.2[c]
V_{o-} (mg/day)	25.1 ± 7.0	29.0 ± 4.2	14.0 ± 2.8[c]
P (mg)	19.7 ± 2.7	18.0 ± 1.3	12.4 ± 1.3[c]
V_T (mg/day)	66.1 ± 8.4	48.1 ± 4.3	34.3 ± 2.3[c]
V_e (mg/day)	79.9 ± 8.3	64.3 ± 4.8	46.8 ± 5.1[c]
E (mg)	61.6 ± 9.8	56.8 ± 6.3	33.7 ± 4.7[c]

[a] Means ± SE.

[b] Significantly different from *ad libitum* controls ($p < 0.01$, Student's t test).

[c] Significantly different from restricted intake controls ($p < 0.05$, Student's t test).

because of faulty matrix formation rather than because of deranged calcium metabolism.

The results of the studies on calcium metabolism in zinc-deficient rats show some similarities to those obtained by Moukhtar *et al.* (17) in hypophysectomized rats. Under the latter conditions, bone anabolism and bone catabolism, pool size, and balance were reduced, while urinary excretion was increased, as was the case in the zinc-deficient animals. The hypophysectomized rats, however, were found to have a reduced absorption coefficient, unlike the zinc-deficient rats.

Comparison of the studies in manganese-deficient and zinc-deficient rats indicate that two decidedly different sets of conditions prevail with respect to calcium metabolism. In both deficiency states, there is significant retardation of growth. In the case of manganese deficiency, however, there is no evidence that calcium metabolism *per se* is affected. In zinc deficiency, on the other hand, specific effects on calcium metabolism were noted. Thus it appears that zinc, but not manganese, is required for normal metabolism of calcium.

Summary. The metabolism of calcium was studied in manganese-deficient and zinc-deficient rats by a method combining a kinetic study using ^{45}Ca with a short-term classical balance study. There were no differences in manganese-deficient rats as compared with controls in any of the parameters of calcium metabolism, including pool size, endogenous fecal calcium, urinary calcium, or in the rates of calcium entering or leaving bone. The zinc-deficient animals, however, showed significant decreases, as compared with both *ad libitum* and restricted-intake controls, in a number of parameters of calcium metabolism. These included the rates of calcium entering and leaving bone, pool size, the rate of slow exchange, and the slowly exchangeable calcium in bone. The results suggest that the skeletal abnormalities observed in manganese-deficient animals are not the result of abnormal calcium metabolism. In zinc deficiency, on the other hand, specific effects on calcium metabolism were noted. Thus it appears that zinc, but not manganese, is required for normal metabolism of calcium.

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Characteristics of the Aminoaciduria Resulting from Cycloleucine Administration in Pair-Fed Rats* (33673)

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Cycloleucine (1-aminocyclopentane carboxylic acid) is known to inhibit transport of amino acids into cells. Initially, interest in the mode of action of this compound was prompted by its carsinostatic effect in human carcinomas (1, 2) and on certain experimental ascites tumors (3). The effect on ascites tumor cells is associated with a decreased rate of incorporation of amino acids into protein, which in turn is the result of competition between cycloleucine and naturally occurring amino acids for transport into cells (4). The competition for transport in this system is greatest for glycine, a monoamino-monocarboxylic acid, least for lysine, a dibasic amino acid and intermediate for valine and leucine (4). Cycloleucine does not appear to interfere with other steps in protein synthesis (5).

Employment of cycloleucine for the treatment of multiple myeloma in humans results in greatly increased urinary excretion of the dibasic amino acids, lysine, ornithine, arginine and the sulfur-containing amino acids, cystine (6). Plasma levels of these amino acids are not elevated suggesting that the aminoaciduria is the result of impairment of renal tubular transport. The specificity of the competitive action of cycloleucine on the active transport of amino acids in the renal tubule appears to differ, therefore, from that occurring in ascites tumor cells, but does resemble the transport defect characteristic of cystinuria, an inborn error of metabolism in humans.

The present study was undertaken to define the effect of cycloleucine on the transport of amino acids in the renal tubule of the rat. Plasma levels and renal clearances of fifteen amino acids were measured after three daily injections of cycloleucine in doses

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