Effect of Calcium Citrate-Malate on Skeletal Development in Young, Growing Rats[1]

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ABSTRACT **It has been previously demonstrated that calcium from calcium citrate-malate (CCM), a mixture of calcium, citric acid and malic acid, is better-absorbed than calcium from calcium carbonate ($CaCO_3$) in humans and in rats. It was of interest to determine if this differential in absorption would result in differences in bone development under chronic feeding conditions. The present study was designed to compare CCM with $CaCO_3$ for effects on bone development in weanling female C/D rats fed either CCM or $CaCO_3$ at 0.3 or 0.6% dietary Ca for 4 or 12 wk. There was a nonsignificant trend for rats fed CCM to weigh more and have larger bones than rats fed $CaCO_3$. Histologic evaluation of cortical and trabecular bone revealed normal bone formation in all rats. Trabecular bone was significantly affected by calcium level and source. The 0.3% Ca diets (either source) resulted in reduced trabecular bone volumes in tibias. After 4 wk, rats fed CCM had 23–25% more trabecular bone than rats fed $CaCO_3$. By 12 wk, the difference was even greater; rats fed CCM had 44–47% more trabecular bone than rats fed $CaCO_3$. Dietary calcium source did not affect cortical bone. It is concluded that because of its positive effects on bone, CCM is a more bioavailable calcium source than $CaCO_3$. *J. Nutr.* 120:876–881, 1990.**

INDEXING KEY WORDS:

• *calcium* • *bone* • *rats*

Recent publicity regarding a possible connection between low dietary calcium intake and disease has resulted in a large increase in the consumption of calcium supplements. For several reasons, health professionals have recommended calcium supplements to select population groups. These reasons include the recognition that certain at-risk groups (i.e., teen-agers and postmenopausal women) consume less than the Recommended Dietary Allowance for calcium (1, 2), the belief that achieving peak bone mass will protect against later loss (3) and the possibility of lowering blood pressure with calcium (4). There are more than 30 brands of calcium supplements sold today, and these vary in dose level and calcium source. Because there is a general belief that most calcium supplements are similar with respect to bioavailability (5), consumers have no reason to choose one supplement over another.

There is scant information on bioavailability of calcium compounds. What information is available has been derived from either absorption of radiolabeled calcium or from calculated apparent absorption (intake minus excretion). For example, based on absorption, calcium carbonate, calcium citrate and calcium lactate-gluconate have similar bioavailabilities (6–8). Bone meal appears to be an inferior calcium source (6). Greger et al. (9) compared nine commercially available calcium sources and nonfat dry milk for calcium bioavailability in rats fed for 20–27 d. With growth, apparent calcium absorption and bone calcium levels as criteria, calcium from these sources was well-utilized, with no striking differences noted among sources.

Although calcium supplements have been compared based on absorbed calcium, calcium that is absorbed is not necessarily deposited in the skeleton. Absorbed calcium that is retained in bone can be measured by using the whole-body retention technique (10). However, a more direct method by which to assess the effects of a compound on bone is to evaluate bone histology. By using histomorphometry, one can quantify bone volumes, as in trabecular bone volume, or bone area, as in cortical bone area. These techniques require special equipment and training, are time consuming and are labor-intensive. Nevertheless, histomorphometry is a sensitive method to study the effects of calcium supplements on bone over time.

In this experiment, two calcium sources were compared for their effects on bone development. It was previously shown that calcium is better-absorbed from

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calcium citrate-malate[2] (CCM) than from calcium carbonate in humans (11, 12). Therefore, it was of interest to determine whether long-term feeding of CCM affected skeletal development. Bone development was measured by using standard histomorphometric techniques in young, growing rats fed CCM or calcium carbonate for either 4 or 12 wk.

MATERIALS AND METHODS

Animals. Fifty-six weanling female C/D rats (Charles River Breeding Laboratories, Portage, MI) weighing ~50 g were housed individually in hanging stainless steel, mesh-bottomed cages and given access to distilled, deionized water via an automatic watering system. The animal room was maintained at 20–23°C and 50–60% relative humidity with a 12-h light/12-h dark cycle.

Diets. Diet composition is shown in **Table 1.** Diets contained 0.6% or 0.3% Ca as either $CaCO_3$ or CCM. The phosphorus level of all diets was 0.4% as KH_2PO_4. Twenty kilograms of each diet was made, and four random samples of 2 g each were analyzed for calcium and phosphorus by atomic absorption spectrophotometry. Mineral concentrations were determined based on National Bureau of Standards reference solutions.

Experimental design. On the day they were received, rats were weighed and randomly assigned to one of four experimental groups (n = 14 per group). Each group was fed one of four diets containing calcium (0.3 or 0.6%) as CCM or $CaCO_3$. The calcium levels in this study were chosen to represent marginal and adequate levels for the growing rat, based on recommendations by the National Research Council (14). Body weights and food intake were recorded weekly. Fluorescent bone labels were injected as described below. Rats fed test diets for 4 wk were killed on d 29, and rats fed test diets for 12 wk were killed on d 85.

Bone labeling. All rats were injected subcutaneously on the back with the fluorescent bone label calcein (Sigma Chemical, St. Louis, MO, 20 mg/kg body weight) and tetracycline (Achromycin, Lederle Laboratories, Wayne, NJ, 40 mg/kg body weight). A 1% (wt:v, in 0.9% NaCl) stock solution of calcein, pH 7.2–7.4 was prepared weekly, and a portion was filtered through a 0.45-μm filter before each use. Tetracycline was reconstituted in sterile 0.9% NaCl and stored at 4°C. Animals fed for 28 d were injected with calcein and tetracycline on d 22 and 26, respectively. Animals fed for 84 d were injected with calcein and tetracycline on d 71 and 78, respectively.

Termination. All rats were killed with an overdose of sodium pentobarbital (100 mg/kg body weight) injected intraperitoneally. Right and left hind legs were removed and stripped of skin. Each leg was tagged and placed in a separate container. Left legs were stored in 70% ethanol at 4°C. Right legs were stored at 0°C.

TABLE 1

Composition of diets containing either $CaCO_3$ or calcium citrate-malate (CCM) and being fed to rats at two levels of dietary calcium

	$CaCO_3$		CCM	
Ingredient	0.6% Ca	0.3% Ca	0.6% Ca	0.3% Ca
	g/kg			
Casein[1]	200	200	200	200
Sucrose[2]	288	295	275	290
Cornstarch[3]	300	300	300	300
Corn oil[4]	100	100	100	100
Cellulose[1]	30	30	30	30
Mineral mix[5]	35	35	35	35
Vitamin mix[6]	10	10	10	10
DL-Methionine[1]	3	3	3	3
Choline bitartrate[1]	2	2	2	2
$CaCO_3$[7]	15	7.5	0	0
CCM[8]	0	0	27	13
KH_2PO_4[7]	17	17	17	17

[1]ICN Nutritional Biochemicals, Cleveland, OH.
[2]Diamond Crystal Salt Company, St. Clair, MI.
[3]American Maize Products, Stamford, CT.
[4]Mazola, Best Food, CPC International, Englewood Cliffs, NJ.
[5]Modified from AIN-76 mineral mix for rats (13), Research Diets, New Brunswick, NJ. Made up in sucrose (per kg diet): 1344 mg magnesium oxide, 7700 mg potassium citrate monohydrate, 1820 mg potassium sulfate, 2590 mg sodium chloride, 19.25 mg chromium potassium sulfate, 22.05 mg cupric carbonate, 0.35 mg potassium iodate, 304.5 mg ferric citrate, 2.1 mg sodium fluoride, 122.5 mg manganous carbonate, 0.35 mg sodium selenite and 56 mg zinc carbonate.
[6]AIN-76A (13), Research Diets #V10001, New Brunswick, NJ.
[7]Fisher Scientific, Fair Lawn, NJ.
[8]Calcium citrate-malate in molar ratios 6:2:3.

Bone fat-free dry weight determination. Right hind legs were thawed, and tissue was removed by autoclaving. Femurs and tibias were extracted in a Soxhlet apparatus (Corning Glass Works, Corning, NY) with 2:1 chloroform:methanol for 48 h. Bones were removed from the Soxhlet, dried in an oven at 95°C for 24 h and stored in a dessicator until they were weighed.

Bone ash, calcium and phosphorus determinations. Bones previously processed for fat-free dry weight determination were ashed in a muffle furnace at 600°C for 12 h. Ash weights were recorded. Bone ash was dissolved in 0.1 mol/L HCl (containing 0.5% lanthanum as La_3O_2 for calcium determinations), and calcium and phosphorus were measured by atomic absorption spectrophotometry.

[2]Calcium citrate-malate is the term coined to describe the trademarked calcium delivery system resulting from the combination of calcium and organic acids.

TABLE 2

Body weights and food intakes of rats fed calcium citrate-malate (CCM) or $CaCO_3$ for 4 or 12 wk[1]

	$CaCO_3$		CCM		
	0.6% Ca	0.3% Ca	0.6% Ca	0.3% Ca	Pooled SD
	g				
4-Week study					
Weight, d 1	47	48	49	48	4
Weight, d 29	175[a]	178[a]	194[b]	180[ab]	12
Food intake	380	346	370	335	47
12-Week study					
Weight, d 1	47	48	48	48	5
Weight d 85	260	263	278	276	30
Food intake	1149	1183	1135	1203	143

[1]Means for 6–8 rats per group. Means in the same row with unlike superscript letters are significantly different based on two-way analysis of variance and least significant difference test, $p < 0.05$.

TABLE 3

Bone mineral analyses from rats fed calcium citrate-malate (CCM) or $CaCO_3$ for 4 wk[1]

	$CaCO_3$		CCM		
	0.6% Ca	0.3% Ca	0.6% Ca	0.3% Ca	Pooled SD
	mg				
Tibia					
FFDW[2]	178[a]	150[b]	190[a]	148[b]	10
Total Ca	41.9[a]	34.0[b]	43.8[a]	31.8[b]	2.9
Total P	20.9[a]	16.6[b]	21.9[a]	16.7[b]	1.0
Femur					
FFDW	221[a]	178[b]	237[a]	181[b]	15
Total Ca	57.6[a]	43.7[b]	57.6[a]	45.1[b]	2.7
Total P	25.7[a]	19.7[b]	27.4[a]	19.7[b]	1.7

[1]Means for 5–6 rats per group. Means in the same row with unlike superscript letters are significantly different based on two-way analysis of variance and least significant difference test, $p < 0.05$.
[2]Fat-free dry weight.

Bone histomorphometry. Left tibias were dissected from hind legs stored in ethanol. Bones were thoroughly cleaned of soft tissue and were cut on a Lipshaw #25 Safe Section bone saw (Lipshaw Corporation, Detroit, MI) such that the metaphysis and shaft were separated. Bone samples were fixed and dehydrated in ethanol and embedded in methyl methacrylate as described by Schenk et al. (15). Thick sections (~70 μm) were cut on a Leitz 1600 saw microtome (E. Leitz, Inc., Rockleigh, NJ) and were mounted with Eukitts mounting liquid (Calibrated Instruments, Ardsley, NY).

Histological measurements were made by using a Zeiss Zidas image analysis system (Carl Zeiss, Inc., Thornwood, NJ) with a Nikon Optiphot microscope (Nikon, Garden City, NJ) equipped with fluorescence. Measurements of cortical bone were made on unstained tibia shaft cross-sections; cortical bone thickness, tibia shaft area, marrow cavity area and cortical bone area were assessed. Trabecular bone volumes (TBV) were measured on metaphysial sections stained with Von Kossa stain (16). TBV was measured with an eyepiece reticle (100 point grid) at a magnification of 40× (17). Approximately 5 mm^2 were evaluated per section, and at least two sections per rat were counted. Distance between fluorescent labels was measured on unstained sections of shaft and metaphysis. Longitudinal growth rate and cortical bone formation rate were calculated by dividing total distance by time (days) between injections.

Statistics. The 4- and 12-wk feeding studies were analyzed separately. All data were analyzed by two-way analysis of variance (calcium source by calcium level) with a test for interaction. Because no interactions were observed and each factor had two levels, these levels were compared using the least significant difference criterion. The level for significance was 5%.

RESULTS

In this study, effects of dietary calcium level and source on weight gain were small (**Table 2**) although there was a trend for rats fed CCM to weigh more than rats fed $CaCO_3$. By 4 wk, rats fed 0.6% Ca as CCM were significantly heavier than rats fed $CaCO_3$ at either level. After 12 wk, the trend for better growth with CCM continued; however, there were no significant differences among groups for body weight or food intake at this time.

Tibia and femur fat-free dry weights (FFDW) were lower in rats fed 0.3% Ca than in rats fed 0.6% Ca at 4 wk (**Table 3**) but not at 12 wk (**Table 4**). Likewise, calcium and phosphorus content and concentration (mg/g FFDW, data not shown) of tibia and femur at 4 wk and in femur at 12 wk were lower in rats fed the lower calcium diet. Calcium source did not affect bone FFDW, mineral content or mineral concentration.

Bone histomorphometry revealed that cortical bone was affected by dietary calcium level at 4 wk but not at 12 wk. At 4 wk, cortical bone thickness and area were reduced and marrow cavity area was increased in rats fed 0.3% Ca compared to rats fed 0.6% Ca, regardless of source (**Table 5**). Bone formation rates were not affected by calcium source or level.

TABLE 4

***Bone mineral analyses from rats fed calcium citrate-malate (CCM) or $CaCO_3$ for 12 wk*[1]**

	$CaCO_3$		CCM		
	0.6% Ca	0.3% Ca	0.6% Ca	0.3% Ca	Pooled SD
	mg				
Tibia					
FFDW[2]	329	309	337	327	27
Total Ca	83.4	76.7	79.8	74.0	9.3
Total P	36.7	36.2	39.2	36.4	3.0
Femur					
FFDW	393	378	411	390	34
Total Ca	105.8[a]	100.1[b]	104.0[a]	94.3[b]	8.5
Total P	46.2[a]	43.5[b]	47.8[a]	43.5[b]	4.2

[1]Means for 6–8 rats per group. Means in the same row with unlike superscript letters are significantly different based on two-way analysis of variance and least significant difference test, $p < 0.05$.
[2]Fat-free dry weight.

Trabecular bone was affected by calcium source and level (**Table 6**). After both 4 and 12 wk, rats fed CCM had significantly more trabecular bone than did rats fed $CaCO_3$. In **Figure 1**, the increased amount of trabecular bone in the tibia metaphysis of rats fed CCM can be seen. For both calcium sources, rats fed 0.6% Ca had more trabecular bone than rats fed 0.3% Ca. Longitudinal bone growth rate was significantly increased in rats fed 0.3% Ca as CCM compared to rats fed 0.6% Ca as $CaCO_3$ at 4 wk. At 12 wk, the longitudinal bone growth rate was similar among all groups (Table 6).

TABLE 6

***Trabecular bone volumes (TBV) and longitudinal bone growth (LBG) of tibias from rats fed calcium citrate-malate (CCM) or $CaCO_3$*[1]**

	$CaCO_3$		CCM		
	0.6% Ca	0.3% Ca	0.6% Ca	0.3% Ca	Pooled SD
4-Week study					
TBV, %	28.8[a]	18.7[b]	35.5[c]	23.4[ad]	6.3
LBG, μm/*d*	148.8[a]	159.8[ab]	157.3[ab]	170.6[b]	10.1
12-Week study					
TBV, %	27.5[a]	19.5[b]	40.5[c]	28.0[ad]	9.7
LBG, μm/*d*	34.4	34.7	32.9	34.3	3.0

[1]Means for 6–8 rats per group. Means in the same row with unlike superscript letters are significantly different based on two-way analysis of variance and least significant difference test, $p < 0.05$.

DISCUSSION

The experiments reported here provide evidence that CCM builds more trabecular bone than calcium carbonate ($CaCO_3$) in young, growing animals. If one defines bioavailability as the absorption and utilization of a

TABLE 5

***Cortical bone histomorphometry of tibia from rats fed calcium citrate-malate (CCM) or $CaCO_3$ for 4 or 12 wk*[1]**

	$CaCO_3$		CCM		
	0.6% Ca	0.3% Ca	0.6% Ca	0.3% Ca	Pooled SD
4-Week study					
Thickness, *mm*	0.500[a]	0.439[b]	0.512[a]	0.432[b]	0.021
Cortical area, mm^2	2.416[a]	2.176[b]	2.561[a]	2.185[b]	0.119
Shaft area, mm^2	3.279	3.192	3.462	3.258	0.203
Marrow cavity area, mm^2	0.863[a]	1.016[b]	0.901[a]	1.073[b]	0.127
Formation rate, μm/*d*	7.54	7.47	7.73	7.44	1.30
12-Week study					
Thickness, *mm*	0.637	0.605	0.636	0.633	0.033
Cortical area, mm^2	3.333	3.249	3.381	3.373	0.246
Shaft area, mm^2	4.134	4.146	4.191	4.213	0.284
Marrow cavity area, mm^2	0.802	0.897	0.810	0.840	0.108
Formation rate, μm/*d*	2.36	2.65	2.28	2.56	0.37

[1]Means for 6–8 rats per group. Means in the same row with unlike superscript letters are significantly different based on two-way analysis of variance and least significant difference test, $p < 0.05$.

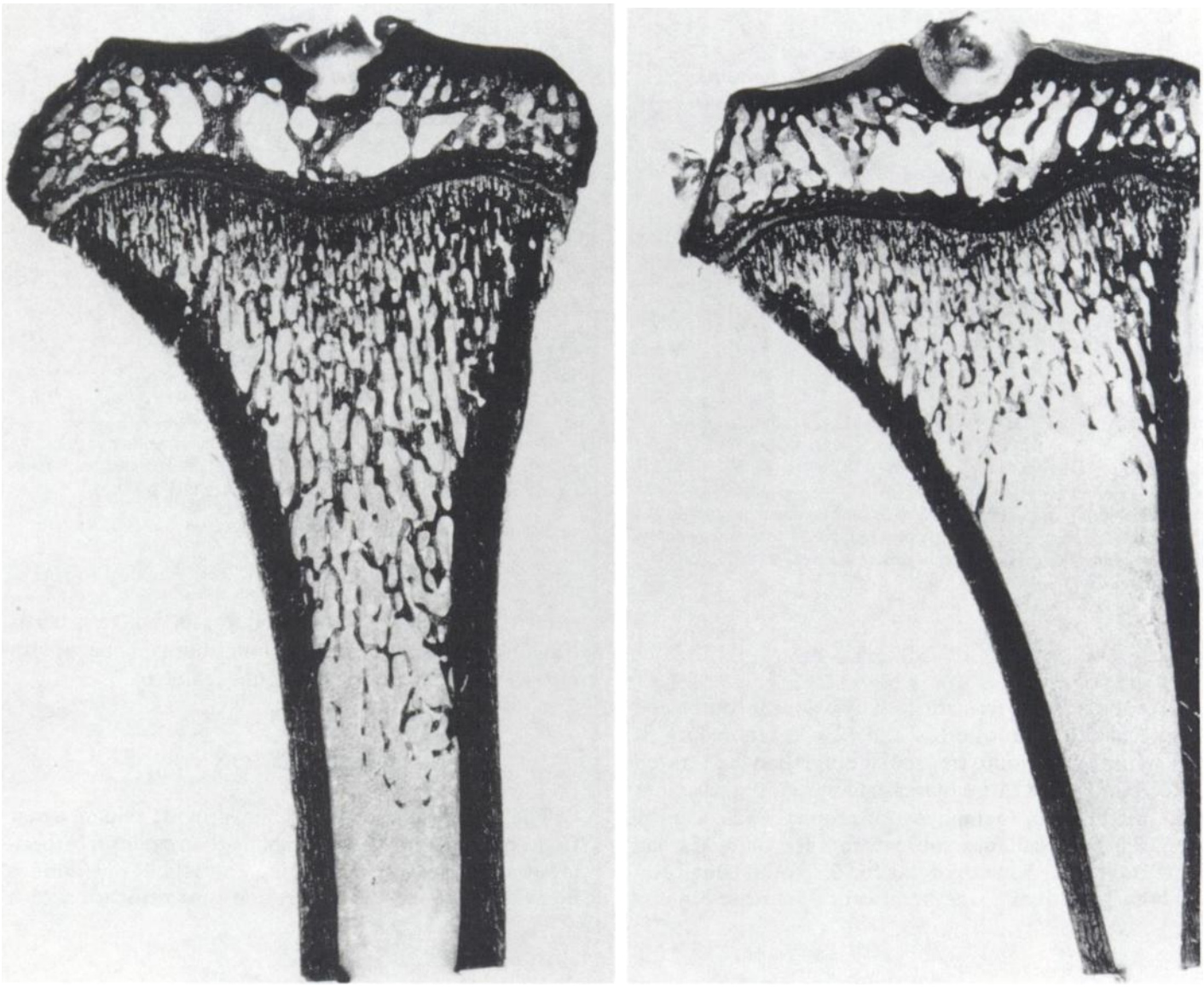

FIGURE 1 Longitudinal sections of tibias from rats fed 0.6% calcium as either calcium citrate-malate (***left***) or calcium carbonate (***right***) for 12 wk. Von Kossa stain.

nutrient, CCM is a more bioavailable calcium source than $CaCO_3$. After 4 wk of feeding, rats fed CCM had 23–25% more trabecular bone than rats fed $CaCO_3$. By 12 wk, the difference between these two calcium sources was even greater. Rats fed CCM had 44–47% more trabecular bone than rats fed $CaCO_3$.

The dietary calcium levels used in this study were carefully chosen. It was hypothesized that differences in bioavailability between CCM and $CaCO_3$ would most likely be observed when the calcium sources were fed at marginal dietary calcium levels. At adequate dietary calcium levels, it was expected that no difference between the two calcium supplements would be observed. The results did not support the initial hypothesis. Instead, an effect of calcium source on the amount of bone was seen, and this effect was independent of calcium level. This suggests that CCM is a more bioavailable calcium source than $CaCO_3$ at both marginal and adequate dietary calcium levels.

The reason for the increased amount of trabecular bone in tibias from rats fed CCM is not clear. The amount of bone present at any given time is determined by the balance between the processes of formation and resorption. If more calcium from CCM is absorbed, more calcium should be available for bone formation. Two lines of evidence argue against CCM stimulating bone formation. First, total bone calcium and phosphorus did not differ with calcium source. However, it is possible that these measures were not sensitive enough to detect small differences in bone calcium levels because only trabecular bone, not cortical bone, was increased in rats fed CCM. Because trabecular bone

represents only about 20% of the total skeleton (18), changes in calcium concentration here would be difficult to detect by total bone mineral analysis. The second piece of evidence arguing against an effect of CCM on bone formation is that cortical bone formation rates and longitudinal bone growth rates were not different among groups at comparable calcium levels.

A second process involved in determining the net amount of bone present is resorption. It has long been postulated that calcium inhibits bone resorption by decreasing the activation of new bone-remodeling units (19). In support of this, calcium supplementation to osteoporotic women resulted in decreased urinary hydroxyproline levels (20). Because more calcium is absorbed from CCM than from $CaCO_3$ in humans (11, 12), CCM could inhibit bone resorption to a greater extent than $CaCO_3$, although this remains to be proved.

By using the sensitive techniques of bone histomorphometry, it was demonstrated that the calcium source CCM is more bioavailable than calcium carbonate. This conclusion is based on the observation that rats fed CCM for either 4 or 12 wk had more trabecular bone in tibias than rats fed $CaCO_3$. The increase in trabecular bone was observed at both marginal (0.3% Ca) and adequate (0.6% Ca) dietary calcium levels.

Future experiments should be designed to elucidate the mechanism for the changes in trabecular bone observed with CCM feeding. These studies should include actual measures of bone resorption, such as hydroxyproline excretion or loss of isotope from a pre-labeled skeleton. Additional studies are needed to determine the effects of CCM on bone when dietary calcium is higher than 0.6%. Also, because the prevention of bone loss is primarily a concern in adults, feeding studies in older rats that are not in the rapid growth phase would be appropriate.

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