In Vivo Calvarial Bone Cell Responses to Dietary Perturbations and the Implications for Mineral Homeostasis (42877)

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Abstract. **Calvariae from small animals have been an important source for *in vitro* studies of bone. However, few *in vivo* studies have been undertaken on quantitative cell changes in calvariae. In the present study of mineral perturbations, rats were first deprived of calcium. After 18 days endosteal osteoclasts and nuclei/osteoclast in the parietal bone had increased 120% ($P < 0.001$) and 26% ($P < 0.001$), respectively, the marrow space had increased 141% ($P < 0.001$), and the bone area experienced a 49% decrease ($P < 0.001$). This thinning and weakening of the calvaria was accompanied by a compensatory increase in the number of endosteal osteoblasts (297%, $P < 0.001$). These rats were then replenished with calcium, and after 14 days the number of endosteal osteoclasts had decreased to 86% ($P < 0.001$) below the control and the endosteal surface was almost completely covered by osteoblasts (866% above the control, $P < 0.001$). Bone area was increased by 51% ($P < 0.01$). Similarly, in calcium-deficient rats in the tibial diaphysis at the fibular junction, the number of endosteal osteoclasts and the medullary space increased 1606% ($P < 0.001$) and 63% ($P < 0.001$), respectively, which were accompanied by a 32% decrease ($P < 0.001$) in cortical bone area. After calcium replenishment, most endosteal osteoclasts in the tibial diaphysis disappeared from the endosteal surface and were replaced by osteoblasts (increased 487%, $P < 0.001$). These results indicate that changes in bone cell activity in response to calcium deficiency are similar in calvariae and long bones, and that mobilization of calcium from the calvaria during calcium deficiency occurs at the expense of the protective action of the calvaria. Therefore, long bones as well as membranous bones are apparently important for the maintenance of mineral homeostasis.** [P.S.E.B.M. 1989, Vol 190]

Membranous bones such as calvariae differ from long bones in embryonic development, postnatal bone structure, and response to stress resulting from mechanical force. Moreover, previous *in vivo* studies have indicated differential changes in osteoclast activity in the two types of bone in calcium-deficient animals—increased in long bones but unchanged in alveolar (membranous) bone (1, 2). Similarly, the rate of medullary cavity occlusion in response to estrogen treatment differed in the long bone and skull, with the latter showing a slower occlusion rate (3). Since the calvaria and its associated bone cells have been widely used for *in vitro* studies (4–12), we undertook to examine whether and how calvarial bone cells respond to hormonal influence or dietary perturbations *in vivo*.

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The skeleton performs two major functions—mineral homeostasis and mechanical (or protective) support. Although total withdrawal of dietary calcium seldom occurs in humans without other dietary deprivation, an extreme calcium deficiency, which can be elicited in experimental animals by dietary manipulation, could be detrimental. In examining the relative importance of the skeletal functions, the calvaria is a good subject, because it protects a vital organ, the brain. This study was undertaken to determine (i) if calvarial osteoclasts and osteoblasts respond to mineral perturbation *in vivo* and (ii) if mobilization of mineral occurs at the risk of impaired protective function.

Materials and Methods

Treatment of Animals. Male Sprague-Dawley rats (Sprague-Dawley Co., Madison, WI) initially weighing about 102 g (9 or 10/group) were individually housed in suspended cages and fed a calcium-deficient diet (0% calcium, 0.6% phosphorus) (1) for 18 days to evoke a mineral stress. They were then changed to a control diet (1.2% calcium, 0.6% phosphorus) for 14 days for

calcium replenishment. Control rats were maintained on the control diet throughout the experiment. At autopsy, blood samples were taken by cardiac puncture, and bone specimens including calvariae and tibiae were removed, freed of adhering soft tissues, and immediately fixed in ice-cold buffered formaldehyde (3.7%, pH 7) (13).

Serum Chemistry. Serum calcium was measured by atomic absorption spectroscopy (14) using a Pye-Unicam atomic absorption spectrophotometer (Pye-Unicam Ltd., Cambridge, England), and serum phosphorus was measured according to the method of Fiske and Subbarow (15) using a Technicon Autoanalyzer (1).

Bone Histomorphometry. *Tissue processing.* After 24 hr of fixation, calvariae and tibiae were trimmed, then fixation was continued for another 24 hr. The bones were then demineralized in buffered formic acid (5% formic acid:20% sodium citrate, 1:1, pH 4.2) (16), washed and partially dehydrated in 70% ethanol, and infiltrated and embedded in glycol methacrylate using a JB-4 embedding kit (Polysciences) (1). All procedures were carried out at 5°C. The embedded bones were sectioned at 5 μm with a Leitz sliding microtome. Calvarial sections were made 1 mm from and parallel to the median suture line, while the tibiae were cut at the fibular junction and perpendicular to the axis of the bone. The sections were stained for tartrate-resistant acid phosphatase activity by a simultaneous coupling azo dye method (17–19). Incubation was carried out with naphthol ASTR phosphate (Sigma) as a substrate and hexazotized pararosaniline (pararosaniline HCL; Gurr, Santa Monica, CA) as a coupler (20) in 0.1 *M* acetate buffer (pH 5.0) at 37°C for 1 hr in the presence of 10 m*M* sodium tartrate (2). After demonstration of acid phosphatase activity, sections were counterstained with 0.03% methyl green-0.01% thionin in 0.02 *M* citrate buffer at pH 5.8 (21) for cell morphology.

Histomorphometry. Osteoclasts were identified by tartrate-resistant acid phosphatase activity, multinucleation, and cell appearance (e.g., size and foamy cytoplasm), osteoblasts by central clear (negative) Golgi area, eccentric nucleus, and the strong basophilic cytoplasmic stain (2). Bone cells/section were enumerated at the endosteum (including the cortical space continuous with the endosteum) and in the marrow spaces under ×400 magnification. Bone area (mineralized and unmineralized) was measured with a digitizing tablet (Summagraphics, Fairfield, CT) interfaced with a CompuPro System 8/16 computer (Godbout Co., Oakland Airport, CA) (2). Increase in bone area during the period of calcium replenishment was calculated by subtracting the mean basal value from each final value in the control or the test group.

Statistical Analysis. All data were expressed as mean ± standard error of the mean. Student's *t* test was used to evaluate the significant difference in means between the control and the test group.

Results

Body Weight and Serum Chemistry. Body weight was not significantly affected by calcium deficiency or replenishment (Table I). However, serum calcium was significantly decreased (5%) and serum phosphorus increased (10%) by the calcium-deficient diet (Table I). These changes were corrected when animals were switched to a control diet.

Bone Changes in the Calvaria. Gross changes in the calvaria associated with calcium deficiency are shown in Figure 1. The number of endosteal osteoclasts in the parietal bone of the calvaria was significantly increased (120%) by calcium deficiency (Fig. 2), which was attended by a significant increase in the marrow space (141%, Figs. 1, 3A and B, and 4). A highly significant correlation between these two parameters (Fig. 5) suggests that the numerical increase of endosteal osteoclasts accounted for the expanded marrow space

Table I. Body Weight and Serum Parameters during Calcium Deficiency and Replenishment

Group	Body weight (g)	Serum calcium (mg/dl)	Serum phosphorus (mg/dl)
Calcium deficiency			
Control	202 ± 3[a]	10.44 ± 0.16	5.80 ± 0.18
Calcium deficient	204 ± 3	9.94 ± 0.07	6.40 ± 0.19
% change	+1	−5	+10
P	NS[b]	<0.02	<0.01
Calcium replenishment			
Control	258 ± 5	10.08 ± 0.09	5.75 ± 0.16
Calcium replenished	250 ± 3	10.17 ± 0.07	5.80 ± 0.18
% change	−3	+1	+1
P	NS	NS	NS

[a] Mean ± SE, $n = 9$ or 10/group.
[b] Not statistically significant at the 0.05 probability level.

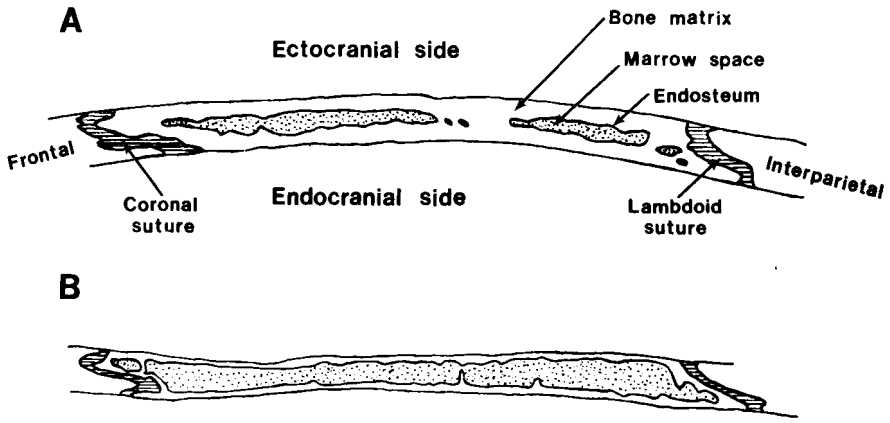

Figure 1. Tracings of 5-μm sagittal sections of rat parietal bone under ×20 magnification from a control rat (A) and a rat kept on a calcium-deficient diet for 18 days (B). Note the expansion of marrow space and thinning of bone matrix in the test rat.

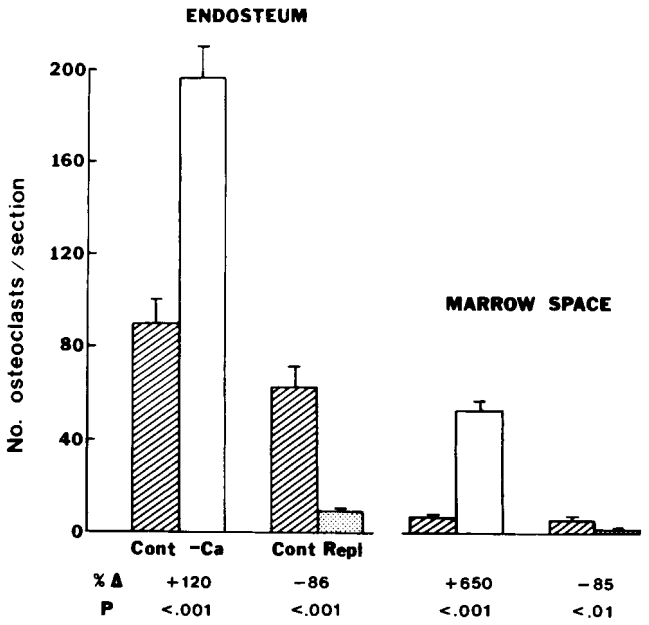

Figure 2. The number of osteoclasts in the parietal bone at the end of 18 days of calcium deficiency and after 14 days of calcium replenishment. Mean and 1 SE above the mean. Group designations for marrow space osteoclasts are the same as shown on the left for endosteal osteoclasts.

observed in these animals. Moreover, bone area was significantly reduced (49%, Fig. 4) despite a marked increase in the number of osteoblasts (297%, Table II), many of which appeared plump, localized in the intracortical spaces continuous with the endosteum (Fig. 3B). A highly significant correlation between endosteal osteoclast and osteoblast number ($r = 0.84$, $P < 0.001$; Fig. 6) suggests that the increases in the two types of bone cells are coupled. As a result of the expansion of marrow elements, which did not become fibrotic, and thinning of the bone matrix, the unmagnified calvaria appeared grossly somewhat reddish and soft, as confirmed by gentle probing.

During calcium replenishment, the number of calvarial endosteal osteoclasts (Fig. 2) and osteoblasts (Table II) in control animals decreased, changes that were apparently related to the animals' increased age. In test animals, however, the decrease in endosteal osteoclast number was much more pronounced (from 90 to 63 in the control and from 197 to 9 per section in the test rats), and the endosteal surface was almost completely covered by osteoblasts, 866% more osteoblasts than the control. These bone cell changes apparently produced a 51% increase in bone area during calcium replenishment (Fig. 7), as further evidenced by the appearance of cement (reversal) lines adjacent to endosteal surfaces (Fig. 3C). In the calvarial marrow space, osteoclast number also significantly increased during calcium deficiency (650%, Fig. 2), but decreased to 85% below the control value after calcium replenishment.

Bone Changes in the Tibial Diaphysis. The number of endosteal osteoclasts was markedly increased (1606%, Fig. 8) by calcium deficiency, which resulted in a significant increase in the marrow space (63%, Fig. 9) and a decrease in bone area (32%, Fig. 9). In parallel with our observation in the calvaria, the number of osteoclasts in the marrow space also increased during calcium deficiency (832%, Fig. 8). Although the number of endosteal osteoblasts was increased by the calcium-deficient diet, the change was not statistically significant (Table II), in part due to the lower cell counts in 3 of the 10 specimens examined in which no intracortical spaces and hence no associated osteoblasts were observed.

After calcium replenishment, most of the endosteal osteoclasts present at the end of calcium deficiency almost completely disappeared from the endosteum (Fig. 8) (except in one of the nine specimens examined, which had three osteoclasts remaining per cross section) and were replaced by osteoblasts (487% above the control, Table II). These bone cell changes apparently were responsible for the observed increase in bone area (59%, Fig. 10) seen during calcium replenishment. As in the calvaria, a similar decrease in the number of osteoclasts in the marrow space was observed in the

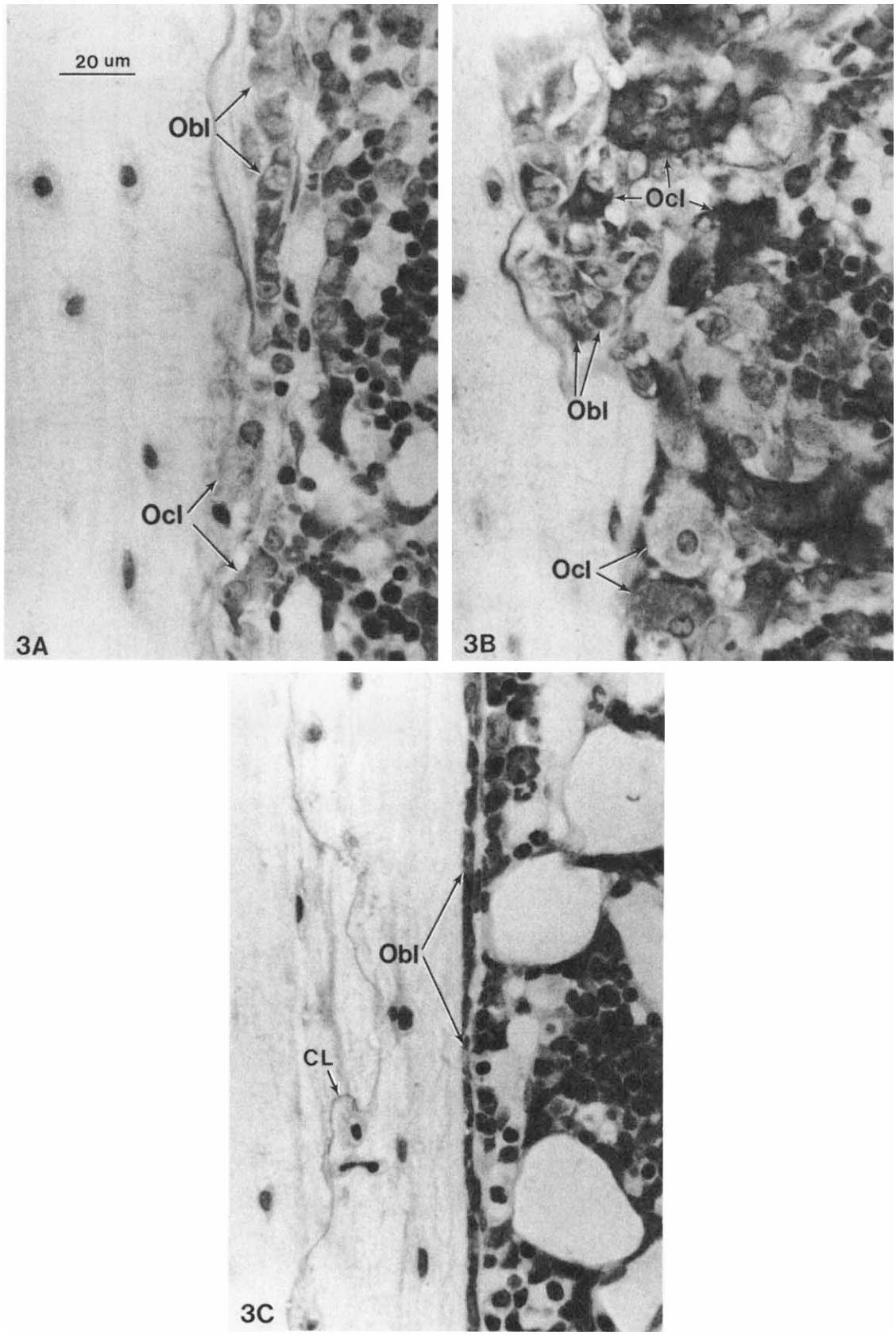

Figure 3. Photomicrographs showing part of the parietal bone sections (5 μm) stained for acid phosphatase activity and counterstained with methyl green and thionin (original magnification X400). CL cement line; Obl, osteoblasts; and Ocl, osteoclasts. (A) from a control rat; (B) from a calcium-deficient rat showing increased numbers of osteoclasts and osteoblasts; and (C) from a rat after calcium replenishment showing the disappearance of endosteal osteoclasts and replacement by osteoblasts.

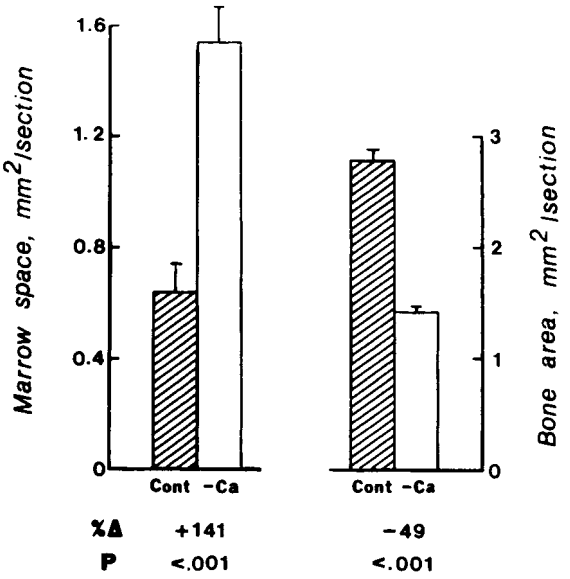

Figure 4. The marrow space and bone area in the parietal bone at the end of calcium deficiency.

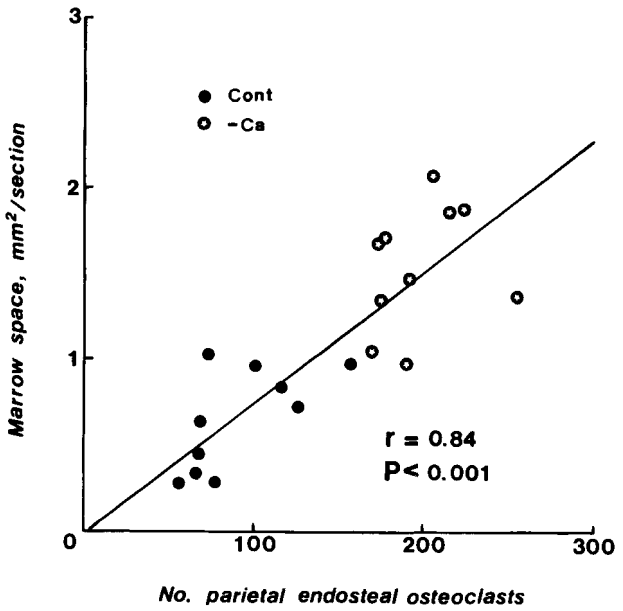

Figure 5. Correlation between the number of endosteal osteoclasts and marrow space in the parietal bone.

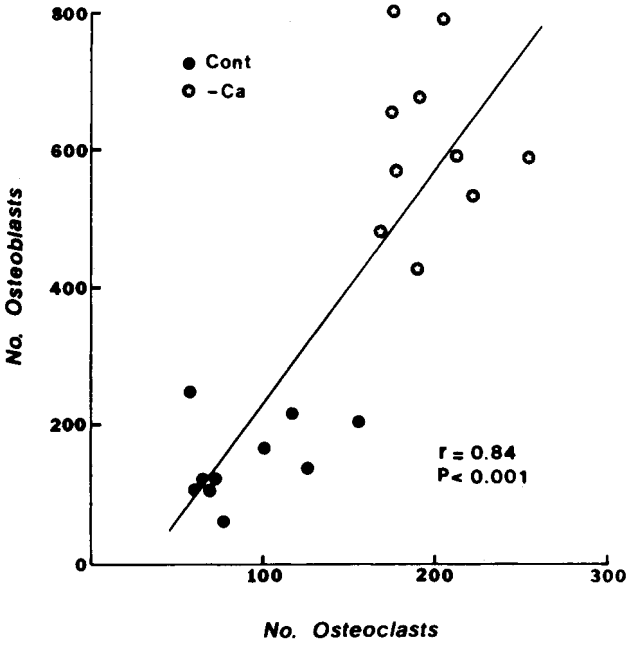

Figure 6. Correlation between the number of endosteal osteoclasts and osteoblasts per section in the parietal bone of the rat calvaria.

Table II. Changes in the Number of Endosteal Osteoblasts in the Calvaria Parietal Bone and Tibia during Calcium Deficiency and Replenishment

Group	No. endosteal osteoblasts/ section	% Change	*P*
Parietal bone			
Control	152 ± 20[a]	—	—
Calcium deficient	603 ± 40	+297	<0.001
Control	58 ± 14	—	—
Calcium replenished	560 ± 76	+866	<0.001
Tibia			
Control	171 ± 10	—	—
Calcium deficient	241 ± 36	+41	NS[b]
Control	52 ± 8	—	—
Calcium replenished	305 ± 9	+487	<0.001

[a] Mean ± SE, n = 9 or 10/group.
[b] Not statistically significant at the 0.05 probability level.

tibial diaphysis during calcium replenishment compared with the value at the end of calcium deficiency—from 17.7 to 0.1 compared with the control from 1.9 to 0.7 per section (Fig. 8).

Number of Nuclei/Osteoclast. The more numerous osteoclasts in calvariae at the endosteum and in the marrow space observed during calcium deficiency were accompanied by significant increases in the number of nuclei/osteoclast (26% and 22%, respectively, Table III), which suggests that calcium deficiency stimulated both proliferation of osteoclast progenitor cells and osteoclast activity. In contrast, no such changes were observed in long bones. During calcium replenishment, most of the osteoclasts seen at the end of calcium deficiency had disappeared from the endosteum and marrow space (Figs. 2 and 8) and thus no quantitative data on the number of osteoclast nuclei were available.

Discussion

Despite the extensive use of calvariae and their associated bone cells for *in vitro* studies of bone metabolism (4–12), few *in vivo* determinations have been made of quantitative cell changes in response to mineral

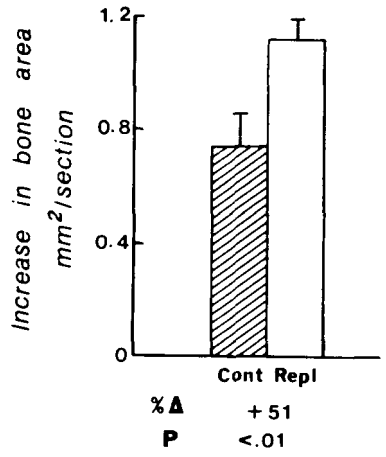

Figure 7. The increase in bone area during calcium replenishment in the parietal bone.

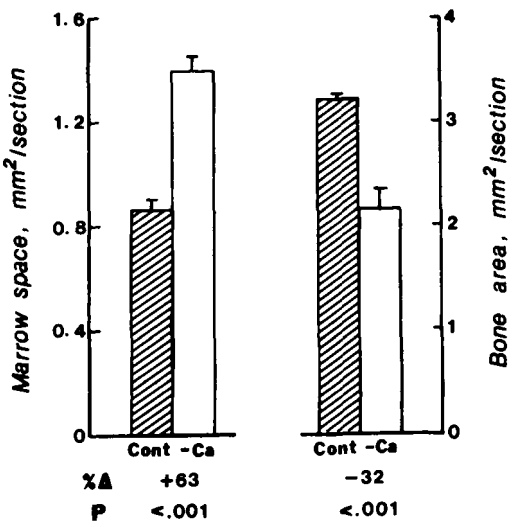

Figure 9. The marrow space and bone area in the tibial diaphysis at the end of calcium deficiency.

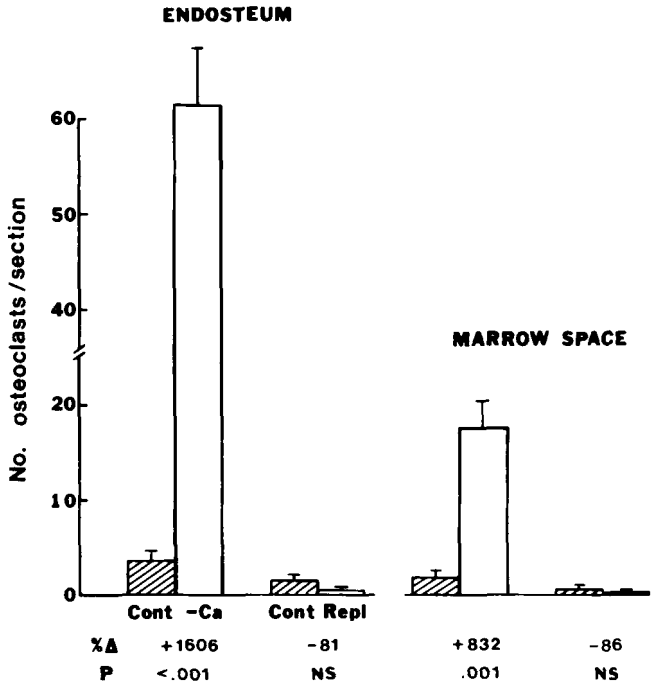

Figure 8. The number of osteoclasts in the rat tibial diaphysis at the fibular junction (cross-section) at the end of calcium deficiency and replenishment. Group designations for marrow space osteoclasts are the same as shown on the left for endosteal osteoclasts.

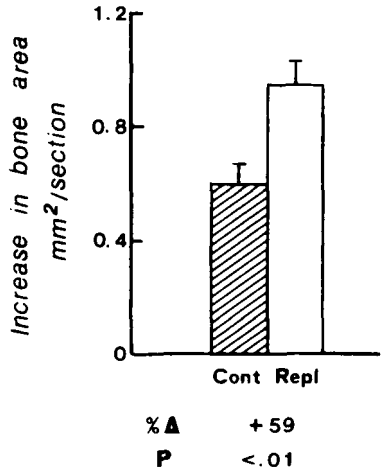

Figure 10. The increase in bone area during calcium replenishment in the tibial diaphysis.

perturbations. Moreover, bone cell activity may differ *in vivo* vis-á-vis *in vitro* because of hormone interactions *in vivo*, e.g., the effect of parathyroid hormone on osteoblast activity is influenced by growth hormone (22). With a previously established calcium-deficiency model in rats (1, 2, 23–25), we demonstrated that the number of endosteal osteoclasts and osteoblasts in the parietal bone both increase during calcium deficiency. Furthermore, a marked decrease in calvarial endosteal osteoclasts, which occurred during calcium replenishment, was attended by a nearly 9-fold increase in calvarial endosteal osteoblasts. These results, along with the previous observation of changes in serum calcium, inorganic phosphate, parathyroid hormone, and 1,25-dihydroxyvitamin D_3 during calcium deficiency and replenishment (1), indicate that calvarial bone cells respond to changes in serum ions and/or calcium-regulating hormones *in vivo*. This suggestion is in accordance with the report of intense erosion of the rat calvaria in response to direct implant of human parathyroid adenoma *in vivo*, albeit no cell changes were evaluated (26).

Primary hyperparathyroidism in humans (idiopathic, adenoma, or carcinoma), especially in the advanced stages, is associated with a generalized decrease in bond density (27). In this condition skull is characterized by a "ground glass" ("salt and pepper" or "moth-eaten") appearance (28), which is thought to be due to increased bone resorption, although no quantitative bone cell changes have been reported. Our finding of comparable osteoclast changes in calvariae and long

Table III. Changes in the Number of Nuclei/Osteoclast in the Parietal Bone and Tibia during Calcium Deficiency

	No. nuclei/osteoclast per section in parietal bone		No. nuclei/osteoclast per section in tibia	
	Endosteum	Marrow space	Endosteum	Marrow space
Control	1.25 ± 0.04[a]	1.11 ± 0.07	1.46 ± 0.16	1.41 ± 0.20
Calcium deficient	1.57 ± 0.06	1.35 ± 0.09	1.63 ± 0.09	1.18 ± 0.05
% change	+26	+22	+12	−16
P	<0.001	<0.05	NS[b]	NS

[a] Mean ± SE, $n = 9$ or 10/group.
[b] Not statistically significant at the 0.05 probability level.

bones (Figs. 2 and 8) in dietary-induced secondary hyperparathyroidism (1) raises the possibility of similar osteoclast changes in the skull of the hyperparathyroid patients. This suggestion is supported by the fact that the pathologic changes in bones in adult humans are indistinguishable between primary and secondary hyperparathyroidism (27) and by histomorphometric studies of other parts of the skeleton (iliac crest) in patients with hyperparathyroidism, which show a similar increase in osteoclast activity (29, 30).

Hyperparathyroidism is characterized not only by increased bone resorption but increased formation as well (31). This coupling phenomenon, first described by Frost in human adult bone remodeling (32), has been shown consistently in some (29, 33–35) but not all (36–38) studies of hyperparathyroid patients and in animals in response to direct parathyroid hormone treatment (39, 40). However, comparable quantitative cell changes in response to calcium deprivation, especially in membranous bone, remain relatively undefined. In rat long bones, no significant correlation between the number of endosteal osteoclast nuclei and the number of endosteal osteoblasts was found in weanling animals subjected to calcium deficiency. Only when data from calcium-deficient rats and phosphorus-deficient (hypoparathyroid) rats were combined and pooled with control values could a highly significant correlation between these two parameters be obtained (19). In the present study, we demonstrated a significant correlation between the number of endosteal osteoblasts and osteoclasts in calvariae, but not in long bones, in animals deprived of calcium when data were pooled with the control values. Since bone loss was greater in calvariae (49%) than in long bones (32%), which is the reverse of findings reported for estrogen-treated mice (3), the differential coupling response could be related to the severity of bone loss. This possibility is strengthened by the correlation between plasma alkaline phosphatase and urinary total hydroxyproline in patients with primary hyperparathyroidism when bone disease is evident (33). The coupled increase in osteoblast activity may represent an attempt to repair bone lost during continuous parathyroid hormone excess (27, 41). Our results, along with clinical observations (29, 33–35), suggest that in humans and animals, hyperparathyroidism (notably attended by severe bone loss) is associated with increased bone turnover, with resorption exceeding formation.

Increased bone-resorbing activity during calcium deficiency led to decreased bone area, thinning of bone matrix, and resultant weakening of the supportive (protective) action of the calvaria. This finding, which is in line with clinical observations of softening of bones and hence deformities and fractures in patients with hyperparathyroidism (42–44), has interesting implications for the view that one of the important functions of the calvaria is to protect the underlying delicate brain tissues from injury during mechanical impact. Our work, therefore, emphasizes the importance of long bones as well as membranous bones in the maintenance of mineral homeostasis despite the fact that it occurs at the expense of protective/supportive function. This suggestion is further appreciated by the facts that many physiologic processes (e.g., cardiac contraction, coagulation of blood, maintenance of neuromuscular excitability, and cellular enzyme activities) require certain levels of calcium ions in the extracellular fluid. Depletion of such ions without mobilization of calcium from bone in the face of calcium deficiency would result in extreme immediate effects such as tetany, loss of cardiac rhythmicity, and possibly death, the latter of which is often seen in calcium-deficient animals whose ability to resorb bone is deprived by parathryoidectomy (unpublished data).

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