THE TEMPORAL RESPONSE OF BONE TO UNLOADING

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ABSTRACT

A model of weightlessness in which rats are suspended by their tails at a 40° angle to unload the hindlimbs while maintaining normal weight bearing on the forelimbs has been used to simulate certain conditions of space flight. When we used this model in growing rats, we found that growth in bone mass ceased by 1 week in the hindlimbs and lumbar vertebrae, whereas growth in bone mass in the forelimbs and cervical vertebrae remained unaffected. Within two weeks, however, the accretion of bone mass in the hindlimbs and lumbar vertebrae returned to normal despite continued skeletal unloading.

Since bone mass in the growing rat is primarily determined by bone formation (bone resorption is modest), we investigated the effects of selective skeletal unloading on bone formation during 2 weeks of suspension using radioisotope incorporation (with ^{45}Ca and [^{3}H] proline) and histomorphometry (with tetracycline labeling). The studies using radioisotope incorporation showed that bone formation was inhibited by the 5th day of skeletal unloading. By the 10th to 12th day, bone formation had returned toward normal. In comparison with cortical bone, cancellous bone (lumbar vertebrae and proximal tibiae) incorporated more ^{45}Ca and [^{3}H] proline (indicating greater metabolic activity) and had a greater absolute response to skeletal unloading. The results of these studies were confirmed by histomorphometric measurements of bone formation using triple tetracycline labeling.

We conclude that this model of simulated weightlessness results in an initial inhibition of bone formation in the unloaded bones. This temporary cessation of bone formation is followed by a cessation in the accretion of bone mass, which then resumes at a normal rate by 14 days, despite continued skeletal unloading. We believe that this cycle of inhibition and resumption of bone formation has profound implications for understanding bone dynamics during space flight, immobilization, or bed rest and offers an opportunity to study the hormonal and mechanical factors that regulate bone formation.

INTRODUCTION

The prolonged absence of skeletal loading, whether caused by immobilization (1-5), bed rest (6), or exposure to simulated (8,7) or actual (9-11) weightlessness, consistently results in osteopenia in both the adult and growing animal. The cellular and molecular basis for these changes remains unclear. In the adult, the unloaded skeleton may be protected from progressive, uninhibited bone loss via mechanisms that couple bone resorption to bone formation, but in the modeling skeleton, where formation predominates, bone resorption appears to play less of a role.

Long-term exposure to weightlessness provides an excellent model for investigating the effects of mechanical unloading on skeletal tissue. Data from three Skylab astronauts indicate that space flight is associated with an increase in urinary calcium excretion of 150 mg per day (11) and a continued loss of total body calcium of 4-8 g per month (12). In two of these astronauts the density of the calcaneus had decreased after 84 days of flight (11). However, these investigations did not reveal whether the osteopenia induced in healthy adults by complete unloading is caused by a reduction in osteoblast activity, a stimulation of osteoclast activity, or a combination of the two (13).

Studies in adult patients immobilized in a 1 g environment indicate that osteopenia results from both an inhibition of bone formation and a stimulation of bone resorption. This was shown by Bronner et al. (14) in patients with scoliosis who were immobilized by a plaster jacket, as well as by Heaney (1) in patients who were immobilized by spinal cord injuries. Both studies used radiocalcium kinetics and calcium balance techniques. Minaire et al. (4), using histomorphometric analysis of bone biopsies, found an increase in bone resorption surfaces (although active bone resorption appeared to be reduced

according to biochemical assessments) and reduced bone formation in patients immobilized with spinal cord injuries. In the study by Bronner et al., the inhibition of bone formation appeared to be quantitatively more important than the stimulation of bone resorption in the negative calcium balance that occurs after immobilization. Of interest is that in 2 of the 4 subjects in this study bone formation resumed despite continued immobilization.

The osteopenia that develops in growing animals as a result of weightlessness (10,15,16) or immobilization (2) seems to be due primarily to a reduced rate of bone formation. Klein et al. (2) suggested that a decreased rate of bone formation was primarily responsible for the smaller net gain in bone mass of unilaterally denervated limbs of growing rats, although bone resorption initially increased 10-20% above controls in the immobilized limbs at the same time that bone formation was reduced. As in patients with spinal cord injuries, part of the initial response of bone to denervation may be caused by the stress of the procedure or condition.

We have developed an animal model that uses rats to study the effects of skeletal unloading without surgical manipulation, and thus minimizes the effects of stress due to surgery. This model was developed to simulate some of the changes that occur during space flight. The hindlimbs are selectively unloaded, but not immobilized, by elevating the hindquarters of the rat while leaving the forelimbs weighted. Histomorphometric measurements of the unloaded bones from such animals are comparable to those obtained from rats following space flight (7,16). We (8) recently reported that the unloaded bones (tibiae, L1 vertebrae) cease to gain bone mass (unlike the same bones in pair-fed controls) within two weeks of unloading, whereas the normally loaded bones (humeri, C1 vertebrae, mandibles) are unaffected.

In this report we first investigated whether the inhibition of growth in the unloaded bones in growing rats would continue beyond 2 weeks. Our results showed that after the first 2 weeks of suspension, the accretion of bone mass resumed at a normal rate. We then evaluated more directly the effect of skeletal unloading on bone formation in growing rats using measurements of radioisotope incorporation and histomorphometric analysis. These studies demonstrated that bone formation, which initially is inhibited by skeletal unloading, recovers despite continued unloading.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, PA) were randomly assigned to groups according to weight, with five or six rats per group. For the shorter experiments, a 2.5-inch wide strip of orthopedic tape was wrapped around the base of the tail of experimental rats, and a clip was attached to the orthopedic tape with filament tape (8). For suspended rats in the four-week experiment, the method of Sweeney et al. was used (17). Briefly, the tail was cleaned, dried, and sprayed with benzoyl peroxide (American Hospital Supply, San Francisco, CA), and a strip of orthopedic traction tape (Fastrac, Van Nuys, CA) with a clip in the middle was applied to opposite sides of the tail. The traction tape was secured with elastic bandage and tape. In both cases experimental rats were attached via the clip to a pulley system attached to the top of a plexiglass cage. The system permitted the rats free movement about the cage by pulling with their forelimbs while leaving their hindlimbs non-weightbearing. Except where otherwise noted, animals were fed standard lab chow (Wayne Lab Blox F-6, James Grain Co., San Jose, CA) containing 1.40% calcium and 0.97% phosphorus.

For the shorter periods of suspension, three consecutive experiments were performed. Experiment A contained rats suspended for 2 or 5 days plus a control group. Experiment B contained rats suspended for 7 or 10 days plus a control group. Experiment C contained rats suspended for 12 or 15 days plus a control group. In each experiment the mean food intake of the suspended rats was used to determine the food intake of the nonsuspended rats. All rats were maintained throughout the experimental period on a 12-hour light/12-hour dark cycle. Growth was monitored by maintaining a daily record of weight.

After different periods of suspension animals were guillotined, and selected bones were removed for analysis (see below).

Determination of Bone Mass During Long-Term Suspension

Rats were randomized into groups of five or six for 1, 2, 3 and 4 weeks of suspension, with a pair-fed control group of five or six rats for each period. Animals were 6.5 weeks old on the date of suspension. Six animals killed on the first day of suspension served as base-line controls. All experimental groups were suspended on the same day. Groups were killed sequentially, week by week, together with a group of controls maintained in adjacent cages. Following decapitation, tibiae, humeri, and L1 and C1 vertebrae were removed and cleaned of adhering tissue. Whole bones were extracted 24 h in ethyl alcohol and then 48 h in diethyl ether in a Soxhlet apparatus. Following extraction, the bones were placed in pretared crucibles, dried in a vacuum oven at 105°C for 24 h, and weighed to obtain a fat-free bone weight. The tibiae and humeri were divided into metaphyseal and diaphyseal fragments, weighed again, hydrolyzed in HCl, and evaluated for total calcium as described below.

Radioisotope Uptake

Groups of five or six rats were suspended for 2, 5, 7, 10, 12 or 15 days. At termination, all animals were 8.5 weeks old. A control group of five or six rats was maintained for every two suspended groups as described in the first part of Methods. Twenty-four hours prior to sacrifice, rats were injected intraperitoneally with 10μ Ci [^{3}H] proline per 100 g body weight and 1μ Ci ^{45}Ca per 100 g body weight in 0.9% saline.

Following decapitation, tibiae, humeri, and L1 and C1 vertebrae were removed and extracted as above. The tibiae and humeri were cut with a dental saw perpendicular to the longitudinal axis into three segments consisting of the shaft (diaphysis) and the two ends (metaphyses). Cuts were made at the following sites.

a. The proximal tibia was cut at the midpoint between the distal end of the tibial crest and the epiphyseal-metaphyseal junction.
b. The distal tibia was cut distal to the tibiofibular junction, where the anastomosis of the tibia and fibula is complete.
c. The proximal humerus was cut at the midpoint between the distal end of the deltoid ridge and the epiphyseal-metaphyseal junction.
d. The distal humerus was cut at the point marking the intersection of the humerus and a line drawn at a 45° angle to the shaft from the tip of the inner condyle.

Only the proximal end and shaft of the tibia were analyzed. Both ends of the humerus were combined and analyzed together; the shaft of the humerus was analyzed separately. Bone segments were dried and weighed to obtain a fat-free weight.

Concentrated HCl in the following amounts was then added to the crucibles: tibial metaphysis, 2.0 ml; tibial diaphysis, 1.5 ml; humeral metaphysis, 1.5 ml; humeral diaphysis, 1.0 ml; L1 vertebrae, 1.0 ml; and C1 vertebrae, 1.0 ml. The crucibles were loosely capped and heated at 65oC for 30-40 min. The bone digest was transferred to graduated cylinders, and the crucibles were rinsed with two washes of 0.8 ml of 0.11 M HCl. The bone hydrolysate was brought to a total volume of 6 ml for the tibial metaphysis and 3 ml for all other bone segments using distilled water. The amounts of ^{45}Ca and [^{3}H] proline in the bone digests were determined by liquid scintillation spectroscopy, and calcium content was determined by atomic absorption spectrophotometry.

Tetracycline Labeling

Animals were fed a synthetic diet containing 1.2% calcium and 0.8% phosphorus, which was supplemented with 25 IU vitamin D_3 (per rat) given orally in 0.2 ml Wesson oil three times per week beginning 12 days prior to the first day of the experiment. Rats were injected subcutaneously on day 2 of the experimental period with 10 mg/kg demeclocycline, on day 8 with 20 mg/kg oxytetracycline, and on day 13 with 20 mg/kg tetracycline-HCl. The time between days 2 and 8 was designated as period 1; the time between days 8 and 13, as period 2. Animals were killed on day 14. The tibiae were removed, adherent soft tissue was excised, and the tibiae were then processed as previously described (10,16,18). After several days in acetone, the tibiae were dehydrated in a series of six acetone washes, followed by eight ethyl ether washes. The tibiae were embedded in polyest casting resin (Chemco, San Leandro, CA) and dried at 40oC. The blocks were trimmed to orient the bone and cut into thin sections (50-70μ) at the tibiofibular junction. Sections were

mounted on slides and photographed under UV light. Photographs were enlarged to 70 X and placed on a digitizing tablet. Each fluorescent label was traced with a graphic stylus, and the area between labels was calculated by computer. The amount of bone formed between two labels was calculated using the formula for the area of a polygon, where

$$\text{area} = \sum_{n=1}^{n=max} (x_n - x_{n+1})(y_n + y_{n+1})/2.$$

The bone formation rate was calculated by dividing the area of bone formed by the time interval between labels (in days).

Statistical comparisons were made using Student's t test, with $P < 0.05$ accepted as significant. Data are reported as mean $\pm$ S.E. unless otherwise indicated.

RESULTS

Bone Mass and Calcium Content

During the 4-week experimental period, tibial, humeral and vertebral bone mass in control animals increased in a linear fashion (Fig. 1), with a net mean increase in mass of 93% in the L1 vertebrae, 50% in the C1 vertebrae, 70% in the tibiae and 64% in the humeri. However, no significant net gain in the mass of the unloaded tibiae or L1 vertebrae from suspended rats occurred during the 2nd week of suspension. The subsequent rates of change in mass in the tibiae and L1 vertebrae were comparable to control levels between the 2nd and 4th weeks of suspension. The humeri and C1 vertebrae of suspended animals, which were weighted in this model, gained in mass at the same rate as controls throughout the 4 weeks. No significant differences in the amount of body weight gained were observed between the suspended and control animals.

To determine whether this cessation of bone growth affected both the cortical (diaphyseal) and cancellous (metaphyseal) regions of the tibia, we divided the long bones into these two regions and weighed them again. The results depicted in Figure 2 indicate that both the metaphyseal and diaphyseal regions of the tibia stopped growing between the lst and 2nd week of suspension, whereas the comparable regions of the humerus were unaffected. Quantitatively, the changes in bone mass induced by suspension were greater in the metaphyseal region than in the diaphyseal region; after 4 weeks the mass of the metaphyseal region was reduced 15% in comparison with control values, whereas the mass of the diaphyseal region was reduced 10%.

Because bone mass includes bone matrix and mineral and because unloading could affect them differently, we hydrolyzed the bones to determine the calcium content directly. The calcium content of bones from the control animals increased linearly with time: 169% in the L1 vertebrae, 83% in the C1 vertebrae, 100% in the tibial metaphysis, 149% in the tibial diaphysis, 104% in the humeral metaphysis, and 95% in the humeral diaphysis (Fig. 3). These rates of calcium accumulation are actually greater than the rates of bone mass accretion. In a pattern similar to that for bone mass accretion, the calcium content in the tibiae and L1 vertebrae of suspended animals failed to increase significantly between the lst and 2nd week of suspension.

These results suggested that skeletal unloading resulted in an inhibition of bone formation by 1 week, with a resumption in bone formation by 2 weeks. To define this temporal sequence more carefully, we evaluated the effects of 2, 5, 7, 10, 12, and 15 days of suspension on bone formation, which we assessed by both radioisotope incorporation (^{45}Ca and [^{3}H]proline) and histomorphometric analysis. (In preliminary experiments we had determined that both ^{45}Ca and

[^{3}H] proline maximally labeled bone by 24 hours.) ^{45}Ca incorporation was used to assess mineral accretion, and [^{3}H] proline incorporation was used to assess matrix accretion. All animals were killed at the same age (8.5 weeks) to eliminate any influence of age on bone formation in the different groups. Because of the large numbers of animals involved, three successive experiments were performed in identical fashion, except for the duration of suspension. Each experiment contained a control group that was pair-fed to the animals suspended for the longest period in that experiment.

No significant differences in body weight or bone mass (Table 1) were observed among the three control groups, suggesting that the differences in the duration of pair-feeding did not influence the results. In suspended rats the mass (Table 1) and calcium content (Fig. 4) of the tibiae and L1 vertebrae were less than that of controls by 7 days. The humeri and C1 vertebrae were not affected. The decreases in mass and calcium content in the L1 vertebrae and tibiae of suspended animals were more pronounced in cancellous bone (L1 vertebrae, tibial metaphysis) than in cortical bone (tibial diaphysis). By the 10th day of suspension the mass of the L1 vertebrae and the tibial metaphysis had decreased to 75% and 82% of controls, respectively ($P < 0.001$). The rate of decrease appeared to slow or stop between the 10th and 15th day of unloading. By the 15th day, the mass of the L1 vertebrae and the tibial metaphysis were 81% and 78% of controls, respectively ($P < 0.001$), and the calcium content had decreased to 71% and 67% of controls, respectively ($P < 0.001$). This greater loss of calcium in the unloaded L1 vertebrae and tibiae, in comparison with the loss in total mass, resulted in bone that was less mineralized (87% and 86% of controls, respectively; $P < 0.05$) than that in controls.

Radioisotope Uptake

All control groups demonstrated comparable levels of ^{45}Ca and [^{3}H] proline incorporation (Table 2). Incorporation of these radioisotopes was approximately threefold greater in the tibial metaphysis than in the shaft (diaphysis).

^{45}Ca uptake into the diaphysis and metaphysis of the unloaded tibiae and L1 vertebrae in suspended animals reached a nadir between the 5th and 10th day of suspension; the decrease was quantitatively greater in the tibial metaphysis than in the diaphysis (Fig. 5). As a percent of control, the maximal decrements were 29%, 20%, and 24% for the L1 vertebrae, the tibial metaphysis, and the tibial diaphysis, respectively. After 10 days ^{45}Ca incorporation appeared to increase in both the tibiae and L1 vertebrae. This restoration of ^{45}Ca incorporation was especially apparent when the specific activity of calcium in bone was evaluated (Fig. 6). Using this analysis, ^{45}Ca incorporation into both the L1 vertebrae and the tibial metaphysis exceeded control values after 12 days of suspension.

[^{3}H]proline incorporation into unloaded bones showed temporal variations comparable to those associated with ^{45}Ca incorporation. [^{3}H]proline uptake into the L1 vertebrae and tibiae reached a nadir between the 5th and 10th day of suspension, but returned to control levels by the 12th to 15th day (Fig. 7). At its nadir the decrement in [^{3}H] proline incorporation was 22%, 13% and 24% for the L1 vertebrae, tibial metaphysis, and tibial diaphysis, respectively. If [^{3}H]proline incorporation is normalized to bone mass (19) as a more accurate reflection of the rate of matrix formation, [^{3}H]proline incorporation in both the L1 vertebrae and tibial metaphysis of suspended animals exceeded control values by day 15 (Fig. 8).

Although initially ^{45}Ca and [^{3}H]proline incorporation into bone were parallel, by the 15th day of suspension [^{3}H] proline incorporation had recovered more than had ^{45}Ca incorporation. At this time the ratio of ^{45}Ca to [^{3}H] proline incorporation was 88%, 91%, and 87% of control values for the L1 vertebrae, tibial metaphysis, and tibial diaphysis, respectively ($P < 0.05$). These results are consistent with the observation, based on the ratio of total bone calcium to fat-free weight, that skeletal unloading eventuates in undermineralized bone.

Tetracycline Labeling

The bone formation rate at the tibiofibular junction in control rats remained essentially identical for periods 1 (days 2-8) and 2 (days 8-13) (Table 3). The bone formation rate in suspended rats decreased to 49% of control levels during period 1, but returned toward control levels during period 2. The difference between these two rates was significant ($P < 0.025$).

DISCUSSION

Elevating the hindlimbs of a rat causes a cephalad fluid shift and a change in musculoskeletal loading in the hindlimbs comparable to that induced by weightlessness (11,20). These rats quickly develop osteopenia in their hindlimbs. Since elevated levels of corticosteroids are associated with a reduction in bone formation (21), it would seem possible that the osteopenia induced in growing animals by suspension (7) and space flight (16) could be the result of sustained hypersecretion of corticosteroids. It is not known whether weightlessness per se stresses rats since inflight analysis of serum corticosteroid levels has not been performed. However, most evidence indicates that osteopenia induced by tail suspension is not the result of excess corticosteroids. First of all, the weights of adrenal glands and serum levels of

corticosteroids of experimental animals remain comparable to control levels at 1, 2, and 3 weeks of tail suspension (10, and Holton EM, et al., unpublished observations). Secondly, if elevated levels of circulating corticosteroids were primarily responsible for the osteopenia induced by this model, changes in all bones, (i.e., loaded and unloaded) would be expected; however, this was not the case. In previous investigations (8) and in the current experiments, osteopenia was localized to unloaded bones, such as the tibia and lumbar vertebrae. Osteopenia was not observed in such bones as the humerus (8), mandible (8,22), and first cervical vertebra (current report), which remain weightbearing. Thus, these data suggest that the osteopenia observed in this model is not caused by a sustained hypersecretion of corticosteroids in response to tail suspension. Whether other models such as immobilization or surgical denervation result in excessive corticosteroid secretion is unknown.

During skeletal modeling, as in the growing rat, bone mass increases when the rate of bone formation exceeds the rate of bone resorption. In this study unloading the hindlimbs of growing rats for one week temporarily suppressed bone growth in the unloaded bones (tibiae, L1 vertebrae). After two weeks this inhibition was reversed, and the rate of growth was comparable to that of controls. The growth pattern of the humeri and C1 vertebrae in suspended animals was indistinguishable from that of controls throughout the 4-week period. Since nonspecific tissue reactions to surgical trauma are avoided in this model, these data suggest that the induction of osteopenia was a locally-mediated phenomenon, initiated by mechanical unloading.

The precise mechanism by which weightbearing influences bone formation and resorption remains poorly defined. Bassett and Becker (23) suggest that skeletal tissue acts as a transducer, converting the mechanical stimulus

of weightbearing into an electrical signal. The electrical signal, in turn, initiates the cascade of biochemical events that results in new bone formation. Findings from studies of growing rats exposed to weightlessness support the hypothesis that mechanical pressure plays a central role in bone formation. Arrest lines at the periosteum and endosteum of long bones in rats subjected to space flight suggest that bone formation may even cease within 12-14 days of weightlessness (10). It is conceivable that bone formation would be reinitiated during longer flights, but this has yet to be determined.

Although no arrest lines were evident in the unloaded bones of rats in the current studies, a 50% reduction in bone formation at the tibiofibular junction of suspended rats in comparison with controls was observed between the 2nd and 8th day of suspension. These data are consistent with previous experiments using this model (7), and with the finding of a 47% reduction in periosteal bone formation in rats exposed to weightlessness for 3 weeks (16). In our current study, the rate of bone formation in the unloaded limbs of rats returned toward control levels between the 8th and 13th day of suspension, suggesting that the growing rat responds to continued skeletal unloading with an initial inhibition of bone formation, followed by its resumption.

Measurements of bone formation using tetracycline as a marker of bone growth reflect primarily mineral apposition. Therefore, a modification of the [^{3}H] proline test described by Marks (19) was used to evaluate the rate of matrix formation. This test is based on the assumption that [^{3}H] proline injected in vivo is incorporated into matrix that has been synthesized de novo by osteoblasts. Marks (19) demonstrated that measurements of [^{3}H] proline per mg bone are consistent with the data provided by the quantitative assay for labeled hydroxyproline, and suggested that the [^{3}H] proline test provides a

specific index of the rate of matrix formation. ^{45}Ca and [^{3}H] proline were both used in the current studies to provide measures of the rates of mineralization and matrix formation in the same animals. Incorporation of both [^{3}H] proline and ^{45}Ca into the unloaded tibiae was decreased in comparison with controls at 1 week, suggesting that the initial defect in bone formation in response to unloading was a decrease in both matrix formation and mineralization. ^{45}Ca and [^{3}H] proline incorporation returned to normal between the 7th and 15th day of continued unloading. These results are consistent with the results of experiments that used tetracycline to measure bone formation.

By separating the metaphyseal and diaphyseal regions of the long bones, we were able to show that unloading exerts quantitatively different effects on regions of bone with different proportions of cancellous and cortical tissue. The decreases in bone mass and calcium content were greater in the unloaded metaphyseal tissue than in unloaded diaphyseal tissue. In addition, matrix formation and mineralization in the metaphysis significantly exceeded control levels at 15 days of unloading, whereas the increase in the diaphysis was not statistically significant. In both regions, the resumption in radiolabel uptake to control levels or above was temporally associated with the cessation of bone loss in the unloaded tibiae and L1 vertebrae and suggested that adaptation had been achieved by a reinitiation of bone formation.

Although both matrix formation and mineralization returned toward control levels between the 7th and 15th day of unloading, there was evidence that the bones had become hypomineralized. The total bone mass of both the L1 vertebrae and tibiae contained a lower percentage of calcium on the 15th day of unloading than did bones from controls. In addition, unloaded bone

incorporated less ^{45}Ca than [^{3}H] proline at 15 days of unloading. These results suggest that recovery of matrix formation exceeded that of mineralization. The possibility that skeletal unloading causes a reduction in the percent of mineralized bone as well as in total mineral content is supported by the finding that following space flight the mandibles of rats show an increase in immature, hypomineralized bone and a corresponding decrease in mature, fully mineralized bone (5).

These studies support the hypothesis by Albright et al. (24) that the absence of mechanical forces causes a reduction in bone formation. The inhibition of bone formation in growing rats demonstrated in these studies may be comparable to the decrease in bone formation in immobilized adults that has been documented by Bronner et al. (14) and Minaire et al. (4). Although results from growing animals cannot be extrapolated directly to adult humans, it appears that unloading inhibits bone formation in both the modeling and remodeling skeletons. Such data suggest that skeletal unloading may have a primary, if transitory, effect on the number or activity of osteoblasts. However, at least in the growing rat, bone formation returns to normal despite continued unloading. In the absence of a pronounced change in bone resorption, a resumption in the rate of bone formation could reasonably be expected to play the major role in maintenance of a threshold for bone mass during prolonged unloading of the modeling skeleton.

In the suspended rat the time required for both the inhibition and recovery of bone formation during skeletal unloading is short and predictable. The model has the additional benefit of permitting direct comparisons between loaded and unloaded bones in the same animal. Thus, we believe that this model will be useful in studies of the factors that regulate bone formation in vivo.

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LEGENDS

FIGURE 1. Temporal Effects of Unloading on Bone Mass during 4 Weeks of Suspension.

Male Sprague-Dawley rats were randomized into control and experimental groups. The latter were suspended at 6.5 weeks of age. Control and experimental animals were sacrificed in groups of 5-6 at weekly intervals. Tibiae and humeri (A) and C1 and L1 vertebrae (B) were removed, defatted, and weighed. Data are presented as mean ± S.E. * denotes significant decrease in bone mass in comparison with controls.

FIGURE 2. Comparison of the Effects of Unloading on Bone Mass in Cancellous and Cortical Bone.

The tibiae and humeri from the rats described in the legend to Figure 1 were divided into metaphyseal (M) and diaphyseal (D) fragments and weighed again. Data are presented as mean ± S.E. * denotes significant decrease in bone mass in comparison with controls.

FIGURE 3. Temporal Effects of Unloading on Calcium Content of Bone during 4 Weeks of Suspension.

The bones depicted in Figures 1 and 2 were hydrolyzed and then analyzed for calcium content. Data are presented as mean ± S.E. * denotes significant decrease in bone mass in comparison with controls.

FIGURE 4. Temporal Effects of Unloading on Calcium Content in Cancellous and Cortical Bone during 2 Weeks of Suspension.

Male Sprague-Dawley rats were randomized according to weight into control and experimental groups, and experimental rats were suspended for 2, 5, 7, 10, 12, or 15 days. On the date of sacrifice all rats were 8.5 weeks old. Each tibia was divided into metaphyseal and diaphyseal regions before hydrolysis and measurement of calcium content. Data are calculated as mg calcium per bone or bone segment and expressed as mean $\pm$ S.E. * denotes significant difference in comparison with controls. Data from control group A (see Table 1) are used for the 0 day time point.

FIGURE 5. Effect of Unloading on ^{45}Ca Uptake by Cortical and Cancellous Bone.

Animals were injected 24 h prior to sacrifice with ^{45}Ca and [^{3}H] proline in a saline vehicle. The L1 vertebrae and tibiae were extracted, and tibiae were divided into metaphyseal and diaphyseal regions. Each bone or bone segment was weighed, hydrolyzed in HCl, and counted for [^{3}H] proline and ^{45}Ca content. Data are depicted as dpm ^{45}Ca per bone or bone segment and expressed as mean $\pm$ S.E. * denotes significant difference in comparison with controls. Data from control group A (see Table 2) are used for the 0 day time point.

FIGURE 6. Effect of Unloading on Specific Activity of ^{45}Ca in Bone.

The experimental protocol is described in the legend to Figure 5. Data are calculated as dpm ^{45}Ca per mg calcium and expressed as mean $\pm$ S.E. * denotes significant difference in comparison with controls. Data from control group A (see Table 2) are used for the 0 day time point.

FIGURE 7. Effect of Unloading on [^{3}H] Proline Uptake by Cortical and Cancellous Bone.

The experimental protocol is described in the legend to Figure 5. Data are calculated as dpm [^{3}H]proline per bone or bone segment and expressed as mean $\pm$ S.E. * denotes significant difference in comparison with controls. Data from control group A (see Table 2) are used for the 0 day time point.

FIGURE 8. Effect of Unloading on [^{3}H] Proline Incorporation per mg Bone Mass.

The experimental protocol is described in the legend to Figure 5. Data are calculated as dpm [^{3}H] proline per mg fat-free bone and expressed as mean $\pm$ S.E. * denotes significant difference in comparison with controls. Data from control group A (see Table 2) are used for the 0 day time point.

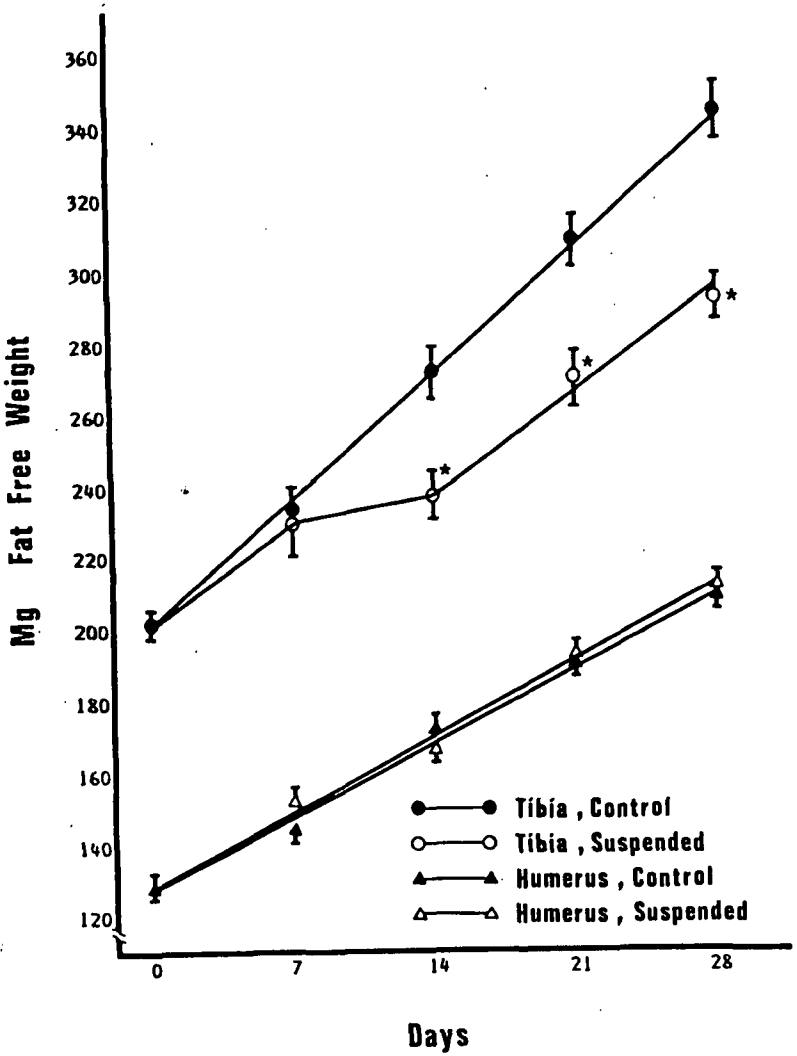


Fig 1A

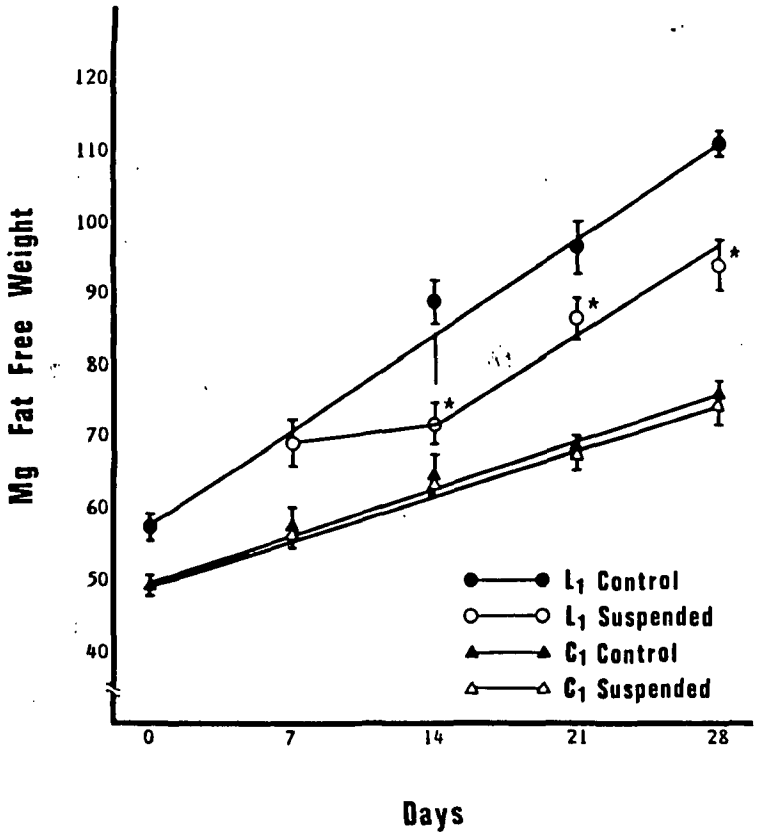
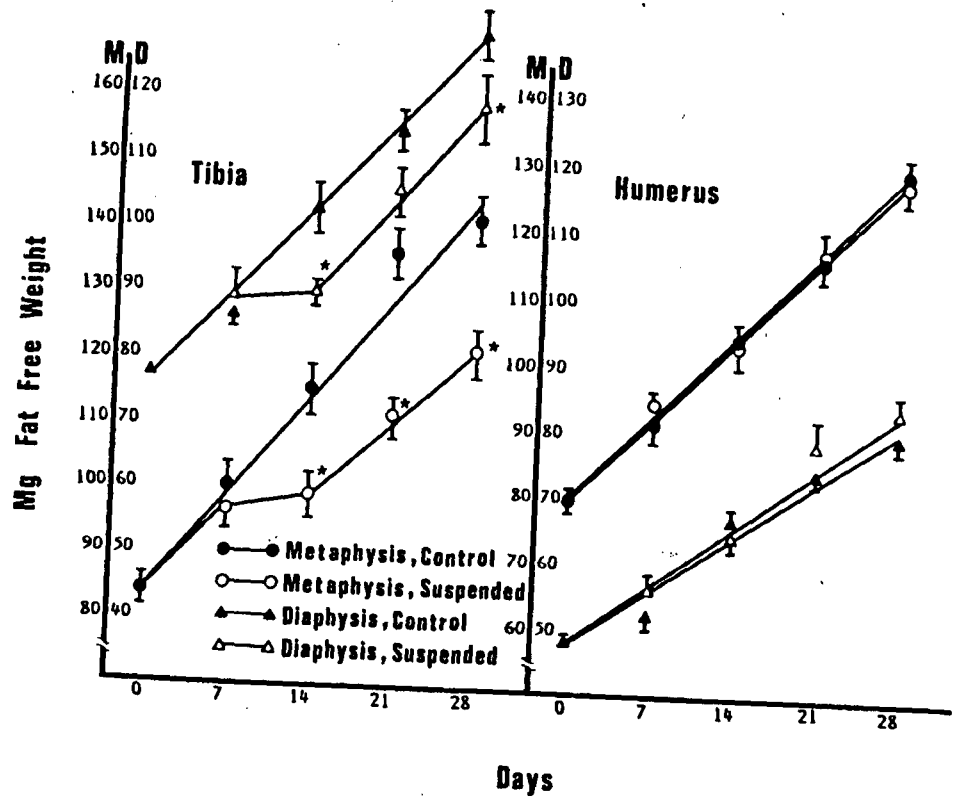


Fig 1B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150 110
140 100
130 90
120 80
110 70
100 60
90 50
80 40
Tibia
M D
140 130
130 120
120 110
110 100
100 90
90 80
80 70
70 60
60 50
Humerus
Mg Fat Free Weight
Metaphysis, Control
Metaphysis, Suspended
Diaphysis, Control
Diaphysis, Suspended
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0 7 14 21 28
Days

Fig 2

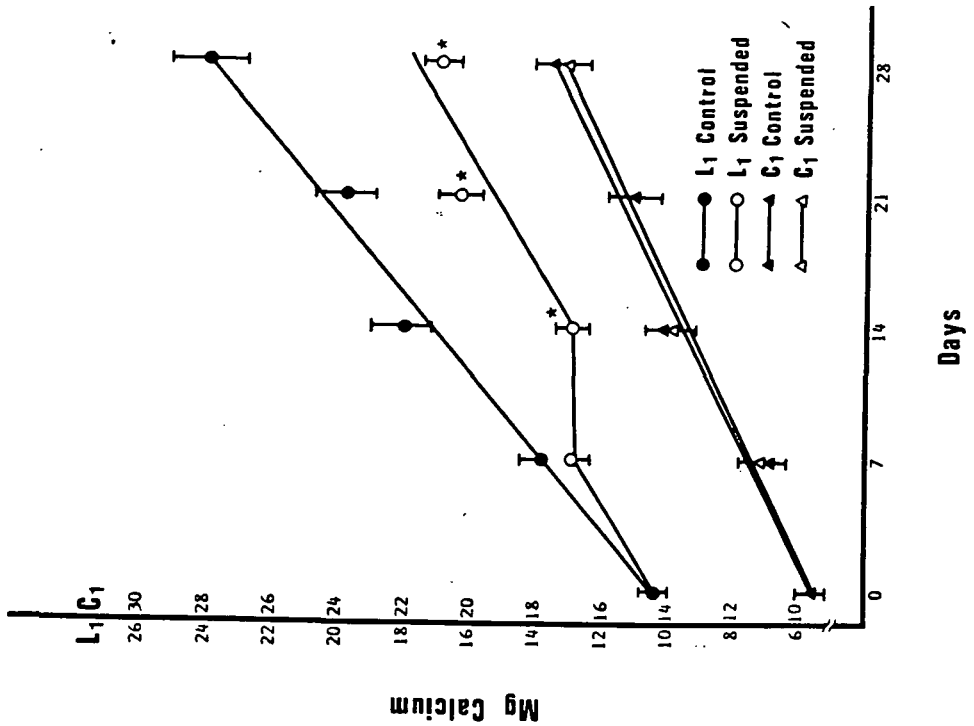

Mg Calcium
Days
L1 Control
L1 Suspended
C1 Control
C1 Suspended
L1 C1

Fig 3B

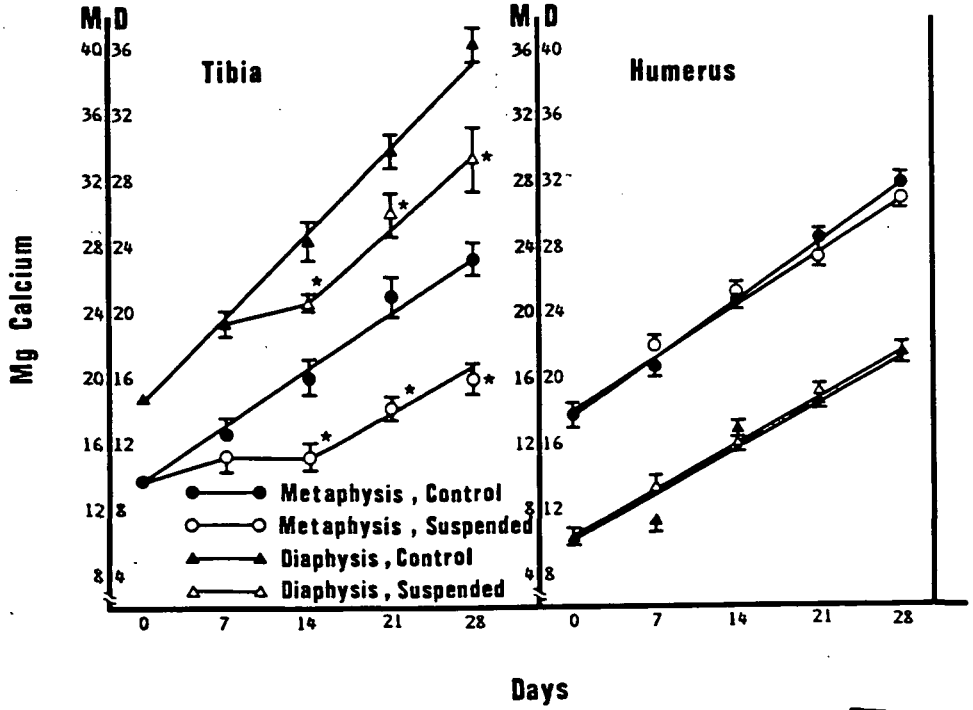

Tibia
Humerus
M D
Mg Calcium
Days
Metaphysis , Control
Metaphysis , Suspended
Diaphysis , Control
Diaphysis , Suspended

Fig 3A

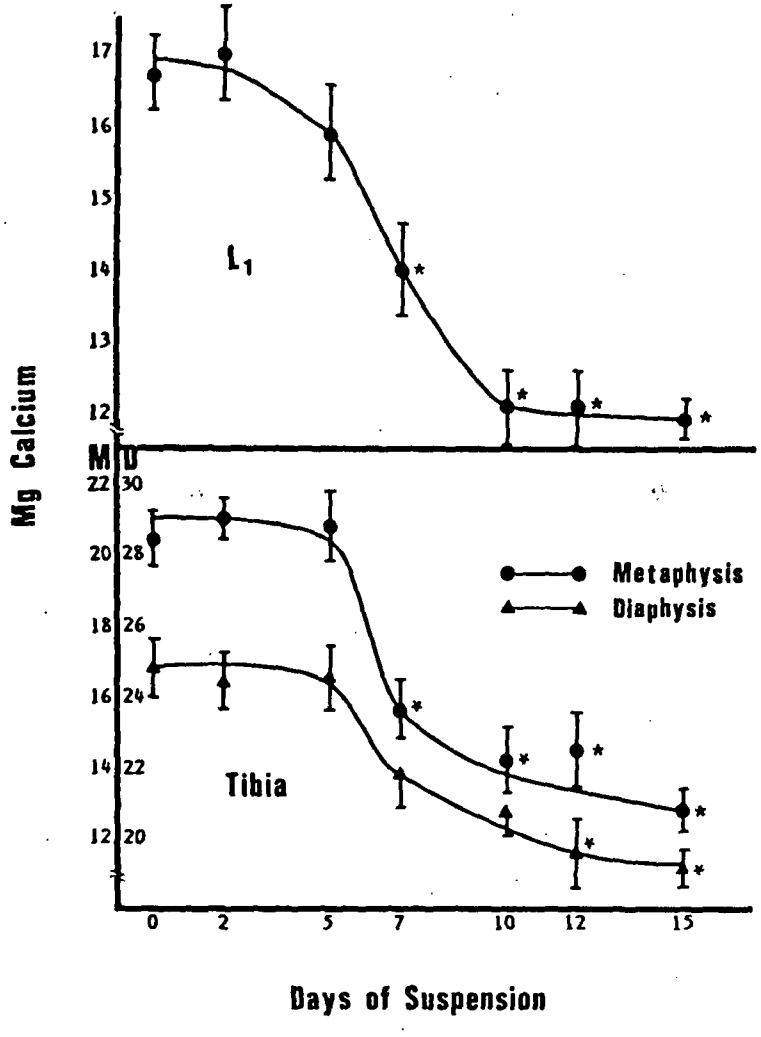


Fig 4

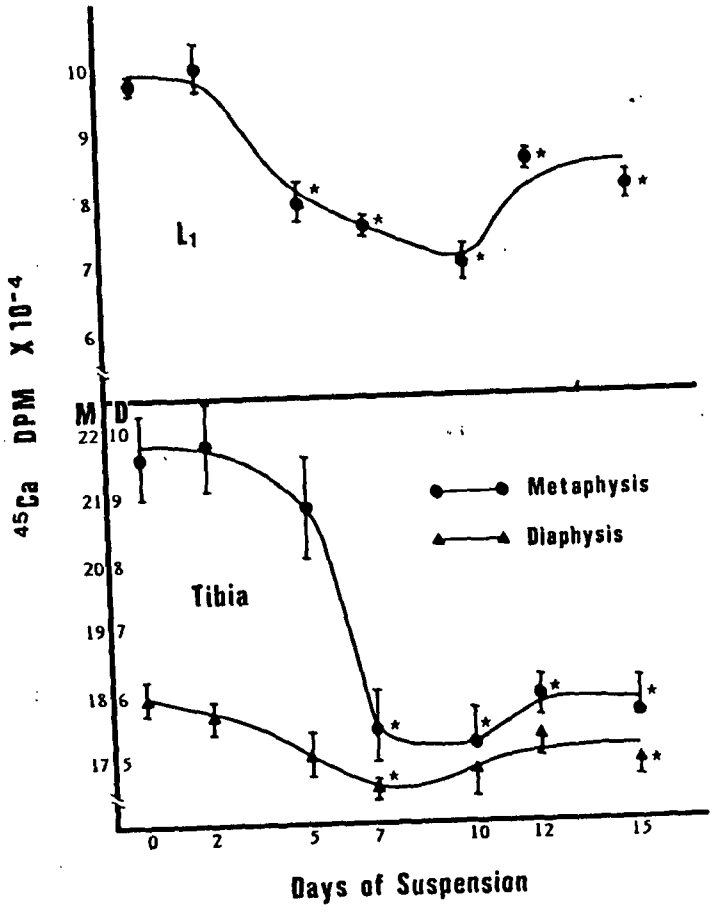
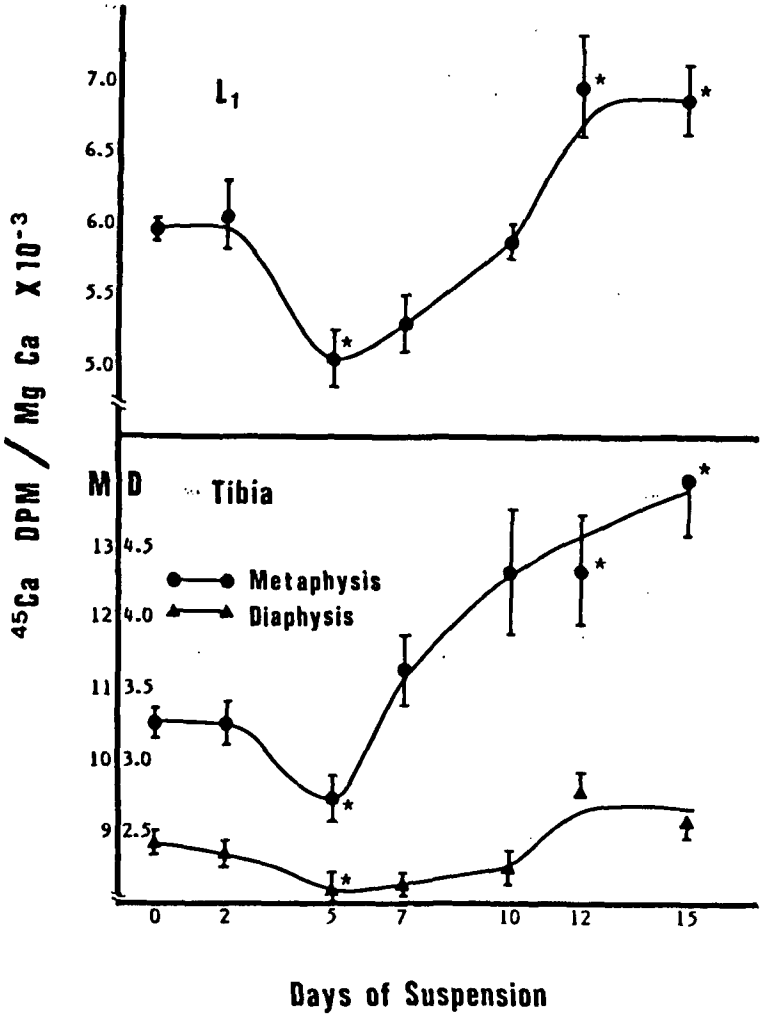


Fig 5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5.5
5.0
M D
Tibia
Metaphysis
Diaphysis
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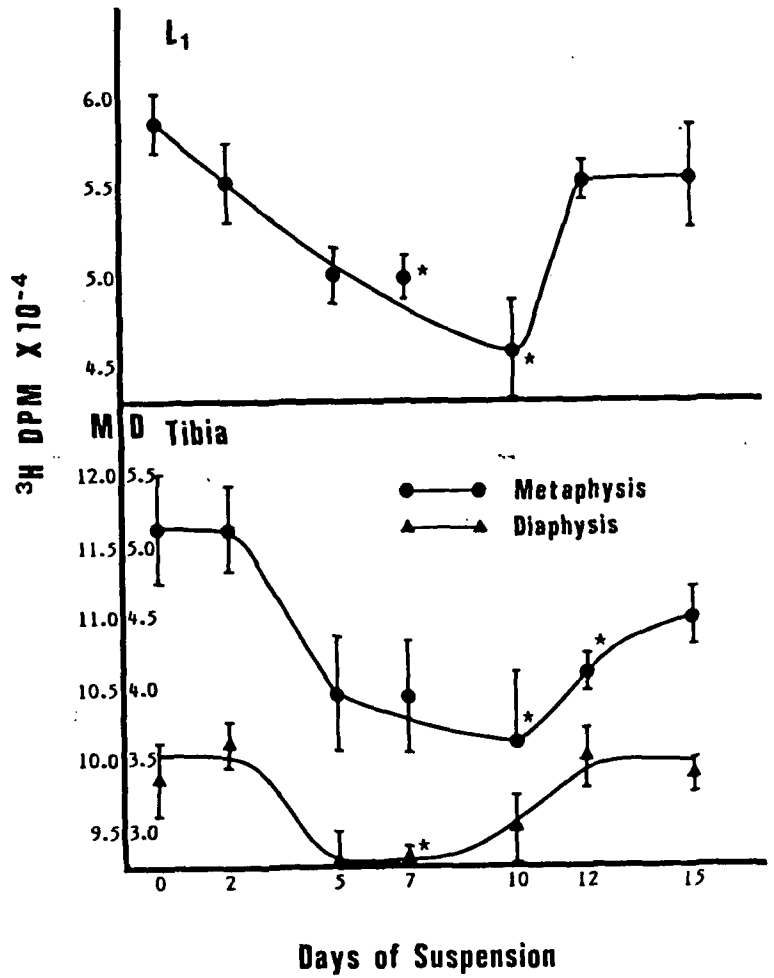
12
11
10
9
4.5
4.0
3.5
3.0
2.5
0
2
5
7
10
12
15
45Ca DPM / Mg Ca X10-3
Days of Suspension

Fig 6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4.5
M D
Tibia
Metaphysis
Diaphysis
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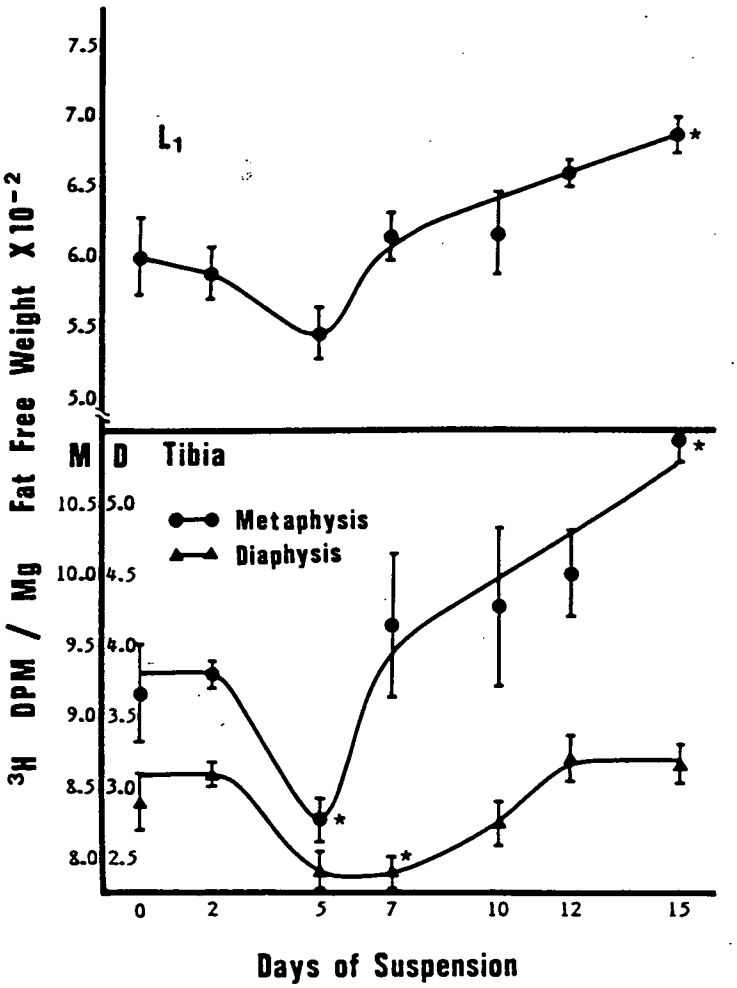
11.5
11.0
10.5
10.0
9.5
5.5
5.0
4.5
4.0
3.5
3.0
0
2
5
7
10
12
15
3H DPM X10-4
Days of Suspension

Fig 7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6.5
6.0
5.5
5.0
L1
*
M D Tibia
Metaphysis
Diaphysis
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9.5
9.0
8.5
8.0
5.0
4.5
4.0
3.5
3.0
2.5
*
*
*
0
2
5
7
10
12
15
Days of Suspension
3H DPM / Mg Fat Free Weight X10-2

Fig 8

Table 1. Effect of Suspension on Body Weight and Bone Mass

Groups	Body mass (g)	Tibial metaphysis (mg)	Tibial diaphysis (mg)	Humeral metaphysis (mg)	Humeral diaphysis (mg)	L1 vertebra (mg)	C1 vertebra (mg)
Experimental							
2 days suspended	266 ± 7	125.6 ± 3.3	114.0 ± 3.9	109.5 ± 2.4	75.5 ± 2.9	95.0 ± 1.6	69.0 ± 2.4
5 days suspended	246 ± 8	126.9 ± 2.9	110.5 ± 4.0	110.5 ± 4.0	74.9 ± 5.2	86.0 ± 2.4	73.0 ± 0.4
Control Group A	256 ± 3	124.3 ± 4.0	116.0 ± 3.2	111.0 ± 2.9	69.1 ± 3.5	91.0 ± 1.6	68.0 ± 0.8
Experimental							
7 days suspended	254 ± 5	104.2 ± 3.3*	108.1 ± 2.2	115.1 ± 2.6	70.5 ± 2.8	75.9 ± 1.2*	63.3 ± 1.6
10 days suspended	252 ± 7	96.7 ± 3.8*	107.6 ± 3.2	105.7 ± 3.9	69.4 ± 3.3	69.3 ± 2.3*	62.3 ± 1.6
Control Group B	262 ± 3	117.9 ± 4.4	111.1 ± 2.6	111.1 ± 2.2	74.1 ± 2.8	92.2 ± 3.2	65.6 ± 1.6
Experimental							
12 days suspended	244 ± 3*	103.7 ± 3.0*	110.7 ± 3.3*	116.5 ± 2.3	62.7 ± 2.2	75.7 ± 1.6*	67.9 ± 1.8
15 days suspended	245 ± 7	97.1 ± 1.8 *	98.7 ± 1.7*	112.8 ± 2.0	64.7 ± 1.5	73.1 ± 2.7*	66.0 ± 2.3
Control Group C	259 ± 3	124.8 ± 3.0	112.9 ± 1.3	112.3 ± 1.3	67.6 ± 1.7	90.4 ± 3.6	69.4 ± 0.9

Control rats were fed according to the mean food intake of the experimental groups. All rats were 8.5 weeks old on the date of sacrifice. Body mass was determined on the date of sacrifice. The bones were removed and defatted. Humeri and tibiae were split into diaphyseal and metaphyseal regions. All bones were then dried in a vacuum oven and weighed to obtain a total bone weight. Data are expressed as mean ± S.E. * indicates $P < 0.05$ by Student's t test in comparison with corresponding control values.

Table 2. Incorporation of ^{45}Ca and [^{3}H] Proline into Bone of Control Rats

Control group	Tibial metaphysis	Tibial diaphysis	L1 vertebra
mg Ca per total bone			
Group A	20.4 ± 2.1	24.8 ± 2.0	16.7 ± 0.7
Group B	19.6 ± 1.8	22.9 ± 1.7	17.1 ± 2.1
Group C	19.2 ± 1.6	23.7 ± 1.1	16.7 ± 2.0
$^{45}Ca \times 10^{3}$ (dpm ^{45}Ca per total bone)			
Group A	216 ± 16	60.0 ± 6.6	98.4 ± 2.9
Group B	198 ± 12	53.2 ± 6.1	92.2 ± 8.6
Group C	202 ± 15	57.2 ± 4.2	96.5 ± 9.6
$^{45}Ca/mg\ Ca \times 10^{2}$ (dpm ^{45}Ca per mg Ca)			
Group A	106 ± 6	23.9 ± 1.5	59.5 ± 1.6
Group B	111 ± 28	23.3 ± 3.1	54.4 ± 7.3
Group C	106 ± 9	24.2 ± 2.3	68.6 ± 6.8
[^{3}H] Proline $\times 10^{3}$ (dpm ^{3}H per total bone)			
Group A	116 ± 20	33.6 ± 5.2	58.5 ± 4.2
Group B	107 ± 13	32.3 ± 2.6	54.9 ± 4.5
Group C	109 ± 8	35.2 ± 6.0	60.0 ± 6.3
[^{3}H] Proline/mg bone (dpm ^{3}H per mg defatted bone)			
Group A	917 ± 86	289 ± 45	597 ± 68
Group B	911 ± 122	290 ± 26	636 ± 21
Group C	891 ± 70	318 ± 53	676 ± 72

Control groups were fed according to the mean food intake of the experimental groups. Group A was the control for rats suspended 2 or 5 days; Group B, for rats suspended 7 or 10 days; and Group C, for rats suspended 12 or 15 days. All rats were 8.5 weeks old at sacrifice. Twenty-four hours prior to sacrifice, animals were injected intraperitoneally with 10 µCi [^{3}H] proline per 100 g body weight and 1 µCi ^{45}Ca per 100 g body weight. Bones were removed and defatted, and tibiae were split into diaphyseal and metaphyseal regions. All bones were then weighed. Following hydrolysis in HCl, total calcium and ^{3}H and ^{45}Ca content were determined. Data are expressed as mean ± S.D.

Table 3: Bone Formation Rate at the Tibiofibular Junction during Initial (Days 2-8) and Later (Days 8-13) Periods of Suspension.

	Bone Formation Rate (mm^2/day)	
	Days 2-8	Days 8-13
Suspended (N=4)	0.019 ± 0.003*	0.030 ± 0.005*†
Control (N=4)	0.038 ± 0.003	0.041 ± 0.005

Rats were suspended at 6.5 weeks of age and injected subcutaneously with three tetracycline labels on days 2, 8, and 13, and then sacrificed on day 14. Bone formation rate was calculated by dividing the area of newly formed bone by the time interval between the two labels. * indicates $P < 0.05$ compared to control values; † indicates $P < 0.05$ compared to initial period. Data are expressed as mean ± S.D.