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Direct measurement of bone resorption and calcium conservation during vitamin D deficiency or hypervitaminosis D

([^{3}H]tetracycline/collagen/chicks/growth modeling/kinetics)

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ABSTRACT **When bone is remodeled during the growth of a given size bone to a larger size, some bone is resorbed and some is deposited. Much of the resorbed bone mineral, calcium, can be reutilized during bone formation. The net and absolute effects of normal growth, vitamin D deficiency, or vitamin D excess were compared on bone resorption, bone formation, and calcium reutilization. Growing chicks were prelabeled extensively with three isotopes: ^{45}Ca, [^{3}H]tetracycline, and [^{3}H]proline. Data were obtained weekly during 3 weeks of control growth, vitamin D deficiency, or vitamin D overdosage while on a nonradioactive diet. Bone resorption as measured by increases in the marrow (inner) diameter of the midshaft of the femur and humerus and by the weekly losses of total [^{3}H]tetracycline and [^{3}H]collagen per whole bone was not significantly different among any of the groups studied. The data indicated that the high rate of cortical bone resorption in experimental chicks was not increased above that observed in control chicks. Vitamin D deficiency had little effect on the total ^{45}Ca in whole bones, whereas vitamin D-treated chicks lost 40% of their ^{45}Ca. Thus, vitamin D overdosage resulted in a decrease of ^{45}Ca reutilization, whereas vitamin D deficiency resulted in an apparent increase of ^{45}Ca reutilization. Both vitamin D-deficient and vitamin D-treated chicks had a decreased accumulation of dietary calcium per whole bone. The insufficient mineral mass in vitamin D-deficient chicks resulted from the indirect inhibition of bone mineralization due to the low intestinal absorption of calcium rather than from a change in bone resorption. In vitamin D-treated chicks the apparent bone atrophy and net loss of ^{45}Ca from bone resulted from inhibiting bone matrix formation and mineralization instead of increasing bone resorption. The constancy of bone resorption under these experimental conditions suggests that bone mineralization is the major regulator of bone mass.**

Calcium is conserved markedly in the adult animal. Steenbock concluded (1, 2) that vitamin D (D) did not affect calcium conservation* in the adult rat during pregnancy and lactation but was active in the replacement of the lost calcium. In 1955 Hevesy (3) demonstrated that 50% of the ^{45}Ca incorporated during fetal growth was conserved during the lifespan of the mouse. Subsequently, Lacroix and Ponlot (4, 5) pointed out that conservation of calcium could not be measured by the classical methods of histology and kinetics. From autoradiographic data in immature dogs, he concluded that incorporated calcium was conserved systemically rather than locally. Similar conclusions were drawn from kinetic studies in which the specific radioactivity of ^{45}Ca approached equality in various parts of the rat skeleton (6, 7) and in redistribution of ^{90}Sr in uniformly labeled beagles (8). However, the rapid ionic exchange of ^{45}Ca between bone and body fluids after pulse labeling has complicated the transient kinetics of isotopes in young rats (9, 10). Due to this complication, various workers (11, 12) have expressed the need for a more direct measurement of bone turnover. In addition, tracer methods for calcium kinetics involve only plasma tracer concentrations and focus on *net* pool turnover rather than on more absolute measurements of bone turnover (13).

Bone resorption† has been observed directly by using histological measurements of the increase in diameter of the bone marrow space in rapidly growing rats (14). Resorption has been measured kinetically by either ^{45}Ca under steady-state conditions in adult rats (15) or ^{40}Ca dilution of the natural isotope ^{48}Ca in human subjects (16). Yet none of these approaches quantified the conservation of bone calcium, although it was recognized that such conservation was occurring (15).

The purpose of the present study is to determine whether D deficiency or hypervitaminosis D has a disproportional effect on bone formation or bone resorption up to and above what would be expected to occur due to the growth process alone. The major effect of the two experimental conditions is to inhibit bone mineralization indirectly or bone formation directly without increasing bone resorption. The constancy of bone resorption under these experimental conditions suggests that bone mineralization is the major regulator of bone mass.

MATERIALS AND METHODS

Animals. From 5 to 14 days of age, male chicks were injected every other day (five times) with increasing doses of ^{45}Ca, [^{3}H]tetracycline, and [^{3}H]proline (17, 18). The dose was 3.5, 35, and 175 μCi of each labeled compound, respectively, per chick. At 18 days of age, five chicks were bled and sacrificed as controls; of the remaining chicks two-thirds were placed on a standard chick starter feed containing D, 0.92% calcium, and 0.73% phosphorus. The remaining chicks were given a D-deficient diet containing 1.4% calcium and 1.1% phosphorus. Half of the chicks receiving standard feed were given vitamin D_3 daily (50,000–100,000 units intramuscularly). Groups of control, D-deficient, and D-treated chicks (5 per group) were bled and sacrificed weekly for 3 weeks. Three days prior to sacrifice, two chicks in each group were given nonradioactive oxytetracycline intramuscularly (20 mg/kg of body weight).

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Abbreviation: D, vitamin D.

* Conservation—i.e., skeletal retention and reutilization of incorporated calcium—is the result of a complex set of physiologic interactions among bone, kidney, and intestine.

† During growth modeling, bone resorption is the sum of resorption of metaphyseal cancellous bone and diaphyseal cortical bone. Cancellous bone is a minor component of the total skeletal mass and its rapid resorption will result in a major release of the incorporated [^{3}H]tetracycline and ^{45}Ca from cancellous bone prior to the initiation of a study. Therefore, the loss of [^{3}H]tetracycline from whole bones is a measure of cortical bone resorption.

Isolation of Bones and Physical Measurements. Immediately after the chicks were killed, the femur and humerus on one side were separately dissected out as intact units and cleaned of all soft tissues. The whole bones were measured for length, volume [by using Archimedes' principle (19, 20)], and inner and outer diameter at the midshift (21). The increase of the inner diameter at the midshaft is an approximation of cortical bone resorption, whereas an increase of the outer diameter is an approximation of the sum of formation and resorption. After the bones were defatted twice with $CHCl_3$/MeOH, 2:1 (vol/vol), and dried *in vacuo* over NaOH pellets, dry weights were obtained. The bone mineral was extracted repeatedly with 0.5 M HCl at 4°C (17, 18) until no significant amount of radioactivity was removed. Whole bones from the opposite side were fixed in 100% ethanol and viewed under ultraviolet light.

Chemical and Isotopic Analyses of Serum and Bone. Serum and acid extracts of bones were assayed for calcium by using an automatic titrator (18). The organic residue remaining after acid extractions was hydrolyzed with 6 M HCl at 121°C and analyzed for hydroxyproline (22).

The radioactivity of serum (^{45}Ca) and of acid extracts of long bones (^{45}Ca and [^{3}H]tetracycline) was analyzed by differential counting (23) and expressed as radioactivity per mg of calcium or per whole bone. The radioactivity in bone collagen was determined on acid hydrolysates by removing hydroxyproline chromatographically (22) and expressing and measuring its radioactivity.

The total weight of calcium and collagen and the total radioactivity of each isotope per whole bone from control and experimental chicks were compared to the respective baseline values obtained 4 days after the last injection of the labeled ion and compounds. It was previously shown that all the animals were equally radioactive with respect to each isotope at the end of the labeling period (17, 18). Calcium conservation can be estimated by comparing the relative losses of [^{3}H]tetracycline and [^{3}H]collagen to that of ^{45}Ca in terms of total radioactivity per whole bone.

Because whole bone is a composite of growth cartilage and bone, preliminary quantitative measurements were made on whole chick femurs and humeri similarly to those done on rat newborn (24) and fetal (25) whole bones for collagen (24), ^{45}Ca, and [^{3}H]tetracycline (25). The radioactivity in the calcified fraction of chick bone as a percentage of its total radioactivity (calcified plus uncalcified) is: [^{3}H]collagen, 60%; [^{3}H]tetracycline, 76%; and ^{45}Ca, 92%.

RESULTS

Physical Increments of Bone During Growth Modeling. The increases in length and volume of whole femurs (Fig. 1 *A* and *B*) and humeri (not shown) were similar for control and D-deficient chicks, whereas increases for the D-treated chicks were significantly smaller. The increase in the marrow (inner) diameter of the midshaft of long bones was similar for control and D-treated chicks (Fig. 1*C*) but somewhat greater with D-deficient chicks. The outer diameter of bone from D-treated chicks was significantly smaller than that of D-deficient chicks (Fig. 1*C*), but no significant difference existed between the experimental groups and the control group. Significant differences in whole bone volumes were more readily discernible than those in outer diameters because measurements of volume can be made with substantially greater precision.

Tetracycline Fluorescence. Gross observations of tetracycline fluorescence in whole bones demonstrated no significant difference between control and vitamin D-deficient chicks. No tetracycline fluorescence was observed in D-treated chicks after the first week of treatment.

Chemical Changes in Bone and Serum During Growth. The rate of increase in total bone calcium was markedly depressed for both D-deficient and D-treated chicks compared to control values (Fig. 2*A*). At the end of the third week, the D-treated chicks contained significantly less calcium than did the D-deficient chicks. In contrast to calcium, the collagen mass in D-deficient chicks was similar to that of controls (Fig. 2*B*), whereas the D-treated chicks had significantly less collagen than did the controls. The levels of serum calcium increased significantly from the normal range of 10.9–11.5 mg/100 ml to 15.8 mg/100 ml for D-treated chicks and decreased to 7.0 mg/100 ml for the D-deficient chicks (Fig. 2*C*).

Isotopic Measurements Per Whole Bone. Bone resorption as measured by the weekly loss of [^{3}H]collagen (Fig. 3*A*) and [^{3}H]tetracycline (Fig. 3*B*) was not significantly different among the control, D-deficient, and D-treated chicks. The rate of loss of [^{3}H]tetracycline (Fig. 3*B*) was greater than the loss of [^{3}H]-collagen (Fig. 3*A*). The control and D-deficient chicks lost a greater proportion of [^{3}H]collagen (Fig. 3*A*) than ^{45}Ca (Fig.

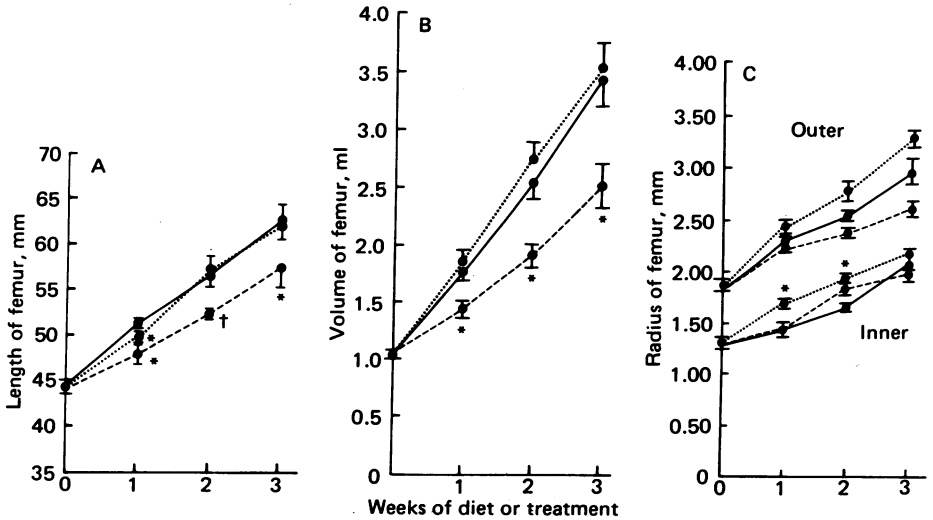

FIG. 1. Physical measurements of whole chick femur: Effect of D deficiency (·····) or hypervitaminosis D (- - -). (*A*) Length. (*B*) Volume. (*C*) Inner and outer radius. Data are presented as means ± SEM; $n = 5$ for each treatment group. For difference from the control group (—): *, $P < 0.05$; †, $P < 0.001$. In the outer radius a significant difference occurred between D-deficient and D-treated chicks at 2 and 3 weeks.

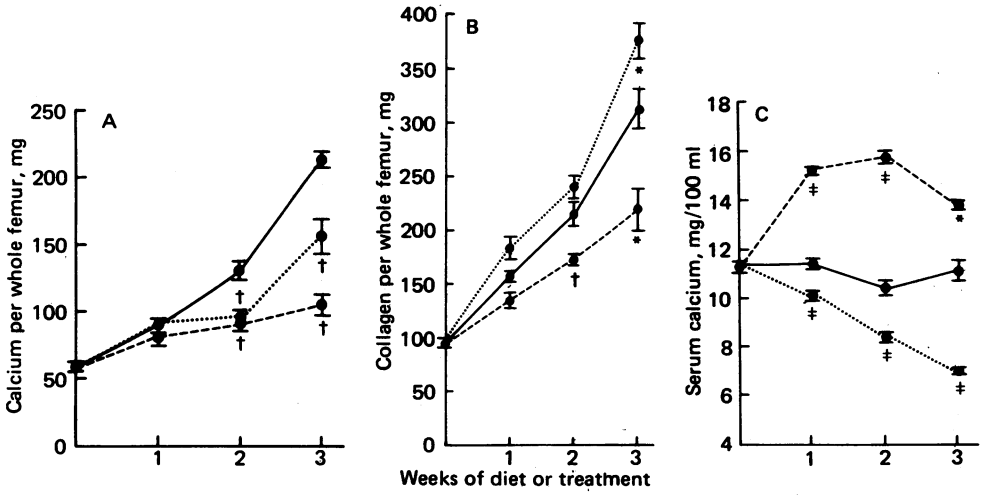

FIG. 2. Chemical measurements per whole chick femur: Effect of D deficiency (·····) or hypervitaminosis D (- - -). (*A*) Bone calcium. (*B*) Bone collagen. (*C*) Serum calcium. Data are presented as means ± SEM; $n = 5$ for each treatment group. For difference from the control group (—): *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$.

3*C*). No significant difference in the rate of loss of total ^{45}Ca was observed between D-deficient and control chicks (Fig. 3*C*), whereas the D-treated chicks showed a marked loss of ^{45}Ca (40%) which was similar to the loss of [^{3}H]collagen (Fig. 3*A*). In previous experiments chicks that were D-deficient for 6 weeks showed a marked translocation of ^{45}Ca from humerus to femur.§

Specific Radioactivity of ^{45}Ca in Bone and Serum. A comparison of the specific radioactivity of ^{45}Ca in serum and bone (Table 1) demonstrated that a simultaneous dilution occurred in serum and bone with serum always having a lower specific activity than bone. A similar dilution occurred in the specific radioactivity of bone ^{45}Ca of control and D-treated chicks, and the least dilution occurred in D-deficient chicks. By 3 weeks of diet or treatment the highest specific radioactivity of serum ^{45}Ca was observed in the D-deficient state and the lowest, in the D-treated state.

§ Klein, L. & Ungier, M. (1977) in *Abstracts of Sixth Parathyroid Conference, Vancouver*, p. 121 (abstr.).

Relative Specific Radioactivity of ^{45}Ca. The ratio of the specific radioactivity of serum calcium to that of bone calcium was constant (within a range of 0.60–0.66) for the control chicks over a 3-week period (Fig. 4). The ratio of serum ^{45}Ca to bone ^{45}Ca for D-deficient chicks rose markedly to 0.83, whereas the ratio markedly declined to 0.44 for the D-treated chicks (Fig. 4).

DISCUSSION

Rationale of Experimental Design. The quantification of bone resorption requires direct and nearly absolute measurements derived from steady-state kinetics of previously incorporated isotopes. Because any biologically available mineral may be rapidly and extensively reincorporated (3, 26), the use of a foreign organic compound like tetracycline (27) which is poorly reused (18) became the means for quantifying resorption. Thus, the resorptive phase of bone calcium metabolism could be quantified independently by using [^{3}H]tetracycline as a calcium surrogate that was not subject to significant reutilization (18).

Vitamin D_3, rather than any of the vitamin D_3 metabolites, was used to induce hypervitaminosis D because of the uncer-

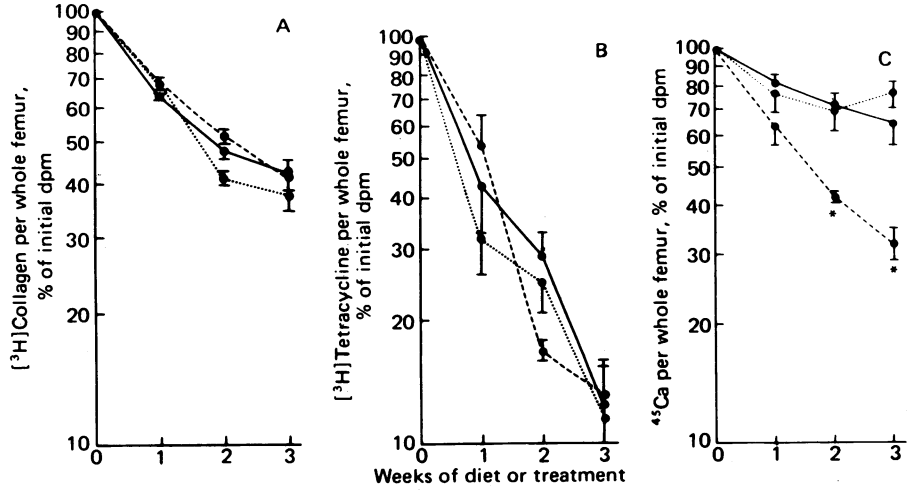

FIG. 3. Isotopic measurements per whole chick femur: Effect of vitamin D deficiency (·····) or hypervitaminosis D (- - -). (*A*) Total radioactivity of [^{3}H]collagen; 100% = 1,150,000 dpm (1 dpm = 16.7 mBq). (*B*) Total radioactivity of [^{3}H]tetracycline; 100% = 192,000 dpm. (*C*) Total ^{45}Ca per whole femur; 100% = 185,000 dpm. Data are presented as means ± SEM; $n = 5$ for each treatment group. *, For difference from the control group (—), $P < 0.01$.

Table 1. Specific radioactivity of ^{45}Ca in serum and bone from control, D-deficient, and D-treated chicks

	Age, weeks			
	3	4	5	6
Control				
Serum	2275 ± 155	1390 ± 104	775 ± 82	504 ± 36
Bone	3670 ± 250	2280 ± 170	1230 ± 130	813 ± 59
D-Deficient				
Serum	—	1661 ± 96	1718 ± 152*	1295 ± 69†
Bone	—	2340 ± 135	2290 ± 202*	1570 ± 84†
D-Treated				
Serum	—	905 ± 167‡	600 ± 75	280 ± 28*
Bone	—	1740 ± 340	1300 ± 150	670 ± 65

Specific radioactivity is expressed in dpm/mg. Data are presented as means ± SEM; $n = 5$ for each treatment group.
* $P < 0.01$.
† $P < 0.001$.
‡ $P < 0.05$.

tainty of the metabolic roles of the different D_3 metabolites (28).

^{45}Calcium in Blood and Bone. The constant ratio of serum ^{45}Ca specific activity to that of bone ^{45}Ca during normal growth modeling is evidence that the chicks had approached a steady-state relationship for the distribution of ^{45}Ca between bone and blood. This technique permits quantification of the relative contribution of intestine-derived calcium (60–66%) and bone-released calcium (34–40%) to the total calcium in blood of rapidly growing chicks. The numerical value of this ratio is 0.60–0.66 during 7 weeks in rapidly growing chicks and during 14 months in rapidly growing dogs (29). This value is similar to that reported for the ^{48}Ca abundance in serum from adult human subjects (16). During 6 weeks of prolonged D deficiency,§ the blood-to-bone ratio approaches unity, suggesting that when the dietary intake of calcium is markedly inhibited the rachitic chicks approach an isotopic equilibrium between bone and blood.

Effect of D Deficiency. The rachitic state was demonstrated by the low amounts of calcium in serum and bone. During the 3 weeks of a D-deficient diet, no significant change was observed in the weekly rate of bone resorption as measured by increase in the inner diameter of femur and humerus (data for humerus not shown) and in their weekly loss of [^{3}H]tetracycline and [^{3}H]collagen. The rachitic bones showed an increased retention of ^{45}Ca, whereas the accretion of new mineral was markedly inhibited. Part of the increased ^{45}Ca retention in the skeleton is due to the redistribution of ^{45}Ca from the parts of the skeleton.§

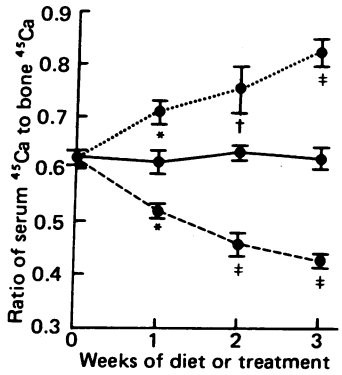

FIG. 4. Ratio of serum ^{45}Ca specific activity to bone ^{45}Ca specific activity: Effect of D deficiency (·····) or hypervitaminosis D (- - -). Data are presented as means ± SEM; $n = 5$ for each treatment group. For difference from the control group (——): *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$.

Thus, the physical, chemical, and isotopic data suggest that physiological doses of D in the growing chick do not have a direct effect on either cortical bone resorption or bone accretion of resorbed ^{45}Ca, and that D affects bone accretion of *new* mineral *indirectly* via calcium absorption by the intestine. Whether D has other direct effects on bone cannot be ruled out by our data. Maximal conservation of resorbed ^{45}Ca occurs in the absence of D, whereas decreased conservation occurs in the presence of D. These interpretations are consistent with the earlier ones of Steenbock and coworkers (1, 2).

Effect of Hypervitaminosis D. No significant difference between control and D-treated chicks was found in the rate of cortical bone resorption as measured by the inner diameter of the femur and humerus (data for humerus not shown) and in their weekly loss of [^{3}H]tetracycline and [^{3}H]collagen during the 3 weeks of treatment. In the D-treated chicks most of the serum calcium was derived from nonradioactive, presumably dietary calcium rather than from skeletal radioactive calcium. In the D-deficient chicks, and even more so in the D-intoxicated chicks, bone mineralization decreased markedly by the second week of treatment, as indicated by a smaller increase of calcium mass. This was confirmed by grossly observing for tetracycline fluorescence and acute uptake of [^{3}H]tetracycline (30) in whole bones, which were diminished by the second week of treatment and were totally absent by the third week. Similar histological observations have been made in hypervitaminotic D chicks (31). In contrast, markedly D-deficient chicks (30) or dogs (32) never show a complete absence of tetracycline uptake like that observed in D-treated chicks. Our experiments do not demonstrate whether the toxic effect of D on bone is due to D directly or to the resultant hypercalcemia. However, although the initial effect of hypervitaminosis D is hyperabsorption of calcium by the intestine, the crucial defect is the impairment of the uptake of calcium by bone.

Hypervitaminosis D in growing chicks did not increase the normally high rate of bone resorption but impaired the conservation of ^{45}Ca resorbed during growth modeling by inhibiting bone mineralization derived from preexisting calcium and dietary calcium. Ultimately, the *net* loss of ^{45}Ca from bone and the body is the result of the calciuric action of excessive vitamin D, which is the result of an increase in the glomerular filtered load of calcium (33) as well as of the inhibition of calcium reabsorption by the renal tubules (34). The end result is marked spillage of ^{45}Ca and ^{40}Ca into urine.

Mineralization Derived from Preexisting Calcium Versus New Calcium. We have made a distinction between bone mineralization derived from preexisting calcium and that derived from dietary calcium, because dietary calcium requires vitamin D for intestinal absorption whereas incorporated calcium is already within the organism. However, once the incorporated ^{45}Ca is released into circulation, it is subjected to all of the turnover processes of the miscible calcium pool and to the dilutional effect caused by the absorption of nonradioactive calcium from the diet. This, of course, assumes that the released ^{45}Ca is in the same physical state (ionic) as that of the dietary calcium.

The kinetic data emphasize that the impairment of bone mineralization derived from preexisting ^{45}Ca (as in the D-intoxicated chicks) results in an absolute decrease in calcium conservation. This change can occur without a necessary decrease in total mass if the lost ^{45}Ca is replaced with dietary calcium. In contrast, an impairment of bone mineralization utilizing dietary ^{40}Ca (as in the D-deficient chicks) results in

a failure of mineral accretion without a loss of preexisting mass and calcium conservation. Our interpretation is that in impaired bone mineralization accumulation of new calcium is not keeping up with a normal but rapid rate of bone resorption—i.e., the incorporation of an insufficient quantity of new calcium over a larger bone volume appears as increased bone atrophy by exhibition of increased bone porosity (31).

Increments in Bone Resorption. Although an increase in bone resorption did not occur in the present experiments, bone resorption was *decreased* with age in maturing rats (29), markedly decreased in acutely parathyroidectomized dogs (35), in microphthalamic mice (L. Klein & K. Wong, unpublished observation), and almost totally abolished in chicks and young dogs treated with high doses of disodium ethane-1-hydroxy-1,1-diphosphonate (36). Thus, although bone resorption was not increased in our present experiments above what may be a maximal level, it can be decreased with other experimental maneuvers.

The present technique of studying resorption *in vivo* cannot be related to studies of resorption with fetal bones in organ culture (37) because the fetal bones are cultured under experimental conditions that induce bone resorption without bone formation. In the absence of bone formation and mineralization, conservation of incorporated ^{45}Ca could not exist, and thus bone atrophy and bone resorption are equivalent (25).

Conservation of Incorporated Calcium. The quantification of the recycling and conservation of resorbed calcium during normal growth modeling and experimentally induced states in chicks is consistent with the early findings in adult rats that D does not play a major role in calcium conservation during pregnancy and lactation (1, 2). Our data support the hypothesis (38) that during bone resorption bone salts would be solubilized and reutilized in bone formation in a cyclic fashion. Under these conditions the skeleton would play a major role as a "calcium buffer" (39, 40) and regulator for blood calcium.

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