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Vitamin D deficiency in rats with normal serum calcium concentrations

(bone morphometry/bone fracture analysis/25-hydroxyvitamin D 1-hydroxylase)

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ABSTRACT **Rats were raised after weaning on a vitamin D-deficient diet which used whole wheat and casein as the major protein source. For at least the first year of life, plasma calcium concentrations of these rats were the same as those of vitamin D-replete rats, and the rate of growth was normal for at least 6 months. The following evidence establishes the vitamin D deficiency of the rats (both male and female) on this diet: (*i*) plasma levels of 1,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin D_3) became undetectable after 6 weeks on the diet; (*ii*) by 4 months of age, the epiphyseal growth plates of the tibia were significantly enlarged and disorganized; (*iii*) when subjected to fracture in a dynamic torsion machine, the femur showed marked weakening as indicated by stress analysis; (*iv*) isolated kidney cells from the deficient rats showed a 3-fold increase in 25-hydroxyvitamin D 1-hydroxylase activity. When mother rats were placed on the vitamin D-deficient diet during lactation, plasma calcium values in the pups decreased and remained low throughout life and there was a stunted body growth pattern. It is concluded that hypocalcemia is not a necessary manifestation of vitamin D deficiency, that the onset of vitamin D deficiency during neonatal life influences the calcium homeostatic system, and that the normocalcemic, vitamin D-deficient animal provides an experimental model in which the effects of vitamin D deficiency can be studied independently of hypocalcemia.**

Prior to the availability of reliable assays for 1,25-dihydroxycholecalciferol [1,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_3$], a deficiency in this vitamin in chicks and rats was identified by measuring the decrease in plasma calcium and phosphorus concentrations that resulted in time from the use of classical rachitogenic diets (1, 2). Various dietary manipulations such as the addition to the diet of large quantities of glucose (3) or manipulation of the calcium-to-phosphorus ratio (4) were usually used to give the desired end result—i.e., a low plasma calcium concentration, thereby indicating a deficiency of 1,25-$(OH)_2D_3$.

The rat chow routinely provided animals to be used in all experiments by our research groups is that developed by Toverud and associates (5). This diet includes ground whole wheat and casein as protein sources with a total phosphorus content of 0.4% in the form of organic phosphate. Normally, the calcium content is also 0.4% but can be varied from near zero to 1%. Recent studies in our laboratories required that both male and female rats be maintained for up to 1 year on a vitamin D-deficient diet. Therefore, the same diet, without vitamin D, was used. Data presented here provide evidence for the disassociation of hypocalcemia from vitamin D deficiency.

MATERIALS AND METHODS

Reagents. Collagenase and hyaluronidase for tissue digestion were obtained from Sigma. Salt solutions and media were obtained from GIBCO. 25-OH[26,27-*methyl*-^{3}H]D_3 (9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from Amersham. 25-OHD_3 was a gift from UpJohn. Other D_3 metabolites—24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, 1,25-$(OH)_2D_3$, and 1,24,25-$(OH)_3D_3$—were gifts from M. Uskokovic (Hoffmann–LaRoche). All organic solvents used were the grade required for HPLC. Rat diets were prepared for us by ICN Biochemicals.

Table 1. Contents of diet provided experimental rats*

Component	Amount, % (wt/wt)
Whole wheat flour	75
Casein (vitamin-free)	15
Corn oil	4
Salt mixture (calcium and phosphorus-free)	2
Vitamin mix†	1.2
Corn starch	1
Phosphorus	0.4‡
Calcium	0.4§

* Modified from Boass *et al.* (5).
† For vitamin D-deficient diet, vitamin mix was void of vitamin D.
‡ Includes phosphorus contained in whole wheat.
§ Calcium content controlled by use of $CaCO_3$.

Animals. Sprague–Dawley (ARS Laboratories, Madison, WI) rats were maintained under standardized conditions of temperature, humidity, and light. The female rats were fed ad lib. In the major experiment utilizing males, the rats were trained to a 0900 feeding schedule and were fed 16–18 g daily. Rats were obtained either as 10-day-old litters (with mothers) or were purchased to be shipped after weaning on the 21st–23rd days of life. Rats to be made vitamin D deficient were fed our vitamin D-deficient diet (Table 1) between days 23 and 25 and maintained on this diet thereafter. Both deficient rats and their vitamin D-replete controls were maintained in a room illuminated with filtered fluorescent lighting.

At specified intervals after initiation of the vitamin D-deficient regimen, blood samples were obtained for serum calcium and phosphorus analysis (6, 7). For the first 6 weeks, sufficient numbers of rats were sacrificed to provide plasma for 1,25-$(OH)_2D_3$ determination. At later times, blood for metabolite analysis was obtained when animals were sacrificed. 1,25-$(OH)_2D_3$ levels were quantitated by radioimmunoassay after HPLC separation from other metabolites (8).

Biomechanics. Rat femurs were tested in a dynamic torsion-testing machine with a dual beam oscilloscope attachment. The two ends of each femur were cast in a quick-setting polyester resin in a mold that fitted into the torsion-testing machine. The distance between these two mold end pieces was kept constant so that the length of the femur exposed to fracture was also constant. One end of the bone specimen was rigidly held in place;

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Abbreviation: D_3, cholecalciferol (vitamin D_3).

the opposite end was subjected to a sudden twist. The torque applied and the resultant angular displacement of the bone ends were continuously recorded on the oscilloscope. These torque–angle relationships were calculated from Polaroid camera film copies of the oscilloscope tracings.

Four parameters of bone strength were calculated according to Panjabi *et al.* (9). The torque was measured as the y coordinate of the curve at the highest point in the peak. The angular displacement was the x coordinate. The mean torsional rigidity was calculated as the ratio of torque to angular displacement at the point of fracture. The elastic energy absorption to fracture was calculated as one-half the torque times angular displacement.

Histology of Bone. Tibia were removed immediately upon sacrifice of the animal and dissected free of soft tissue. A piece of cortical bone was removed transversely from the anterior tuberosity of the proximal tibia to allow for quicker penetration of the fixatives. Bones were then placed in a phosphate-buffered modified Millonig's fixative at pH 7.4 and 37°C. Samples were dehydrated with increasing concentrations of acetone and embedded in methyl methacrylate. Undecalcified bone sections were cut at a thickness of 5 μm on a Jung model K microtome, collected on 0.5% calfskin gelatin-coated slides, and stained with a modified Goldner stain.

Light microscopic evaluations were made with a Leitz microscope on coded slides. For each animal, 50 measurements of the growth plate width were made with ×16 objective and ×12.5 eyepiece. Growth plate width in the rat tibia is defined as beginning at the zone of reserve cartilage and passing toward the ossification center. This area consists of the resting zone, zone of cellular proliferation, zone of matrix synthesis, zone of hypertrophy, and initial location of provisional calcification.

Isolation of Renal Cells. At the time of sacrifice, cells were obtained from the kidneys of deficient and replete rats for *in vitro* metabolism studies. The cells were isolated according to a modification of the procedure of Turner *et al.* (10). Kidneys were perfused *in situ* with calcium-free salt solution to remove blood and subsequently with 100 ml of a calcium-containing magnesium-free solution of collagenase (0.5%). At this point, kidneys were removed from the animal, dissected free of capsule, and minced into flasks containing 1.0% collagenase and 2% hyaluronidase for a 15-min incubation at 37°C. After mechanical dispersion, the cells were washed three times with a calcium-free salt solution and resuspended in calcium-containing medium (McCoy's 5a) at a concentration of 5×10^6 cells per ml.

Metabolism of 25-OH[^{3}H]D_3. Flasks containing isolated renal cells and tubules were gassed with 95% O_2/5% CO_2 for 60 sec and incubated at 37°C with 25-OH[^{3}H]D_3 at varying concentrations in a shaking incubator for 1 hr. At the end of the incubations, the flasks were quick frozen in dry ice/ethanol and stored for later extraction and chromatography as described (11). Protein content per flask was determined by the procedure of Lowry *et al.* (12).

RESULTS

Growth and Metabolite Levels. By the time the vitamin D-deficient rats were 3 months old, their plasma 1,25-$(OH)_2D_3$ levels were nondetectable (<6 pg/tube). Table 2 shows a comparison of serum calcium and 1,25-$(OH)_2D_3$ levels at several times. The calcium values did not decrease despite the gradual decline in 1,25-$(OH)_2D_3$. At the time of sacrifice, 1,25-$(OH)_2D_3$ levels were nondetectable even when 10 ml of serum was extracted. Serum calcium levels were not significantly different from those of rats raised on an identical diet containing vitamin D_3. Serum phosphate levels, determined in several experiments, were not significantly different (mean ± SEM: replete, 6.5 ± 0.05 mg/dl; deficient, 6.2 ± 0.2 mg/dl). There was no difference in body weights of these two groups at least up to 6 months of age.

Table 2. Circulating 1,25-$(OH)_2D_3$ in serum of rats on vitamin D-deficient diet since weaning

Week	Serum calcium, mg/dl	Serum 1,25-$(OH)_2D_3$, pg/ml
2	10.75	43
4	9.93	39
6	—	18
10	10.32	Nondetectable
Vitamin D supplemented	10.56	133

Biomechanics. Upon sacrifice, it was noted that the deficient animals had shorter femurs than the replete rats. Precise length and geometric shapes of these bones were not measured at that time. The results of the biomechanical torsion testing are listed in Table 3. Calculated values of fracture torque and torsional rigidity were significantly decreased in the deficient animals. These properties are dependent upon the geometrical structure of the bone. Therefore, these measurements do not reflect directly the quality of bone but are related more to the gross properties of the femur. The torsional rigidity measurements indicate that the femurs from the replete rats had more resistance to deformation than did those from the deficient rats. The latter femurs were weaker—less torque was required to fracture the bone. The amount of energy absorbed prior to the fracture of the femur was almost 50% less in the deficient animals. However, due to the small number of vitamin D-deficient bones prepared for this study, the statistical significance of this difference was marginal.

These biomechanical studies indicate that there is a significant difference in the properties of bone from replete and deficient animals. This difference may be due to the size and shape of the bone, to its internal and external geometric structure, or to its intrinsic mechanical properties.

Histology of Bone. Vitamin D-replete and vitamin D-deficient rats were sacrificed at 5 months old. Gross examination of the bone revealed that, in the deficient rats, the tibias were extensively bent and the femurs were shortened. More precise measurements of bone length were not made at this time. The epiphyseal growth plate of the tibia appeared to be wider in the deficient rats compared to the replete rats (mean ± SD: replete, 181.13 ± 17.71 μm^3, $n = 3$; deficient, 296.59 ± 33.80, $n = 3$; $P < 0.05$). Histological examination of the tibia confirmed the presence of an enlarged epiphyseal growth plate with extremely irregular boundaries. Also, the various zones of cellular activity in the growth plate were not as distinct as those seen in the replete rats. There was also an increase in the number of chondrocytes in the zone of cellular hypertrophy in the deficient rats (Fig. 1).

Table 3. Biomechanical data from vitamin D-replete and vitamin D-deficient rats

Measurement	With vitamin D ($n = 23$)	Without vitamin D ($n = 8$)
Fracture torque, Nm	0.62 ± 0.05	0.38 ± 0.09*
Angular displacement, °	8.71 ± 0.86	8.10 ± 1.40
Torsional rigidity, Nm/°	2.21 ± 0.19	1.17 ± 0.26*
Fracture energy, Nm	333.6 ± 45.6	171.6 ± 64.7

Results are shown as mean ± SD.
* For difference between regimens, $P < 0.025$.

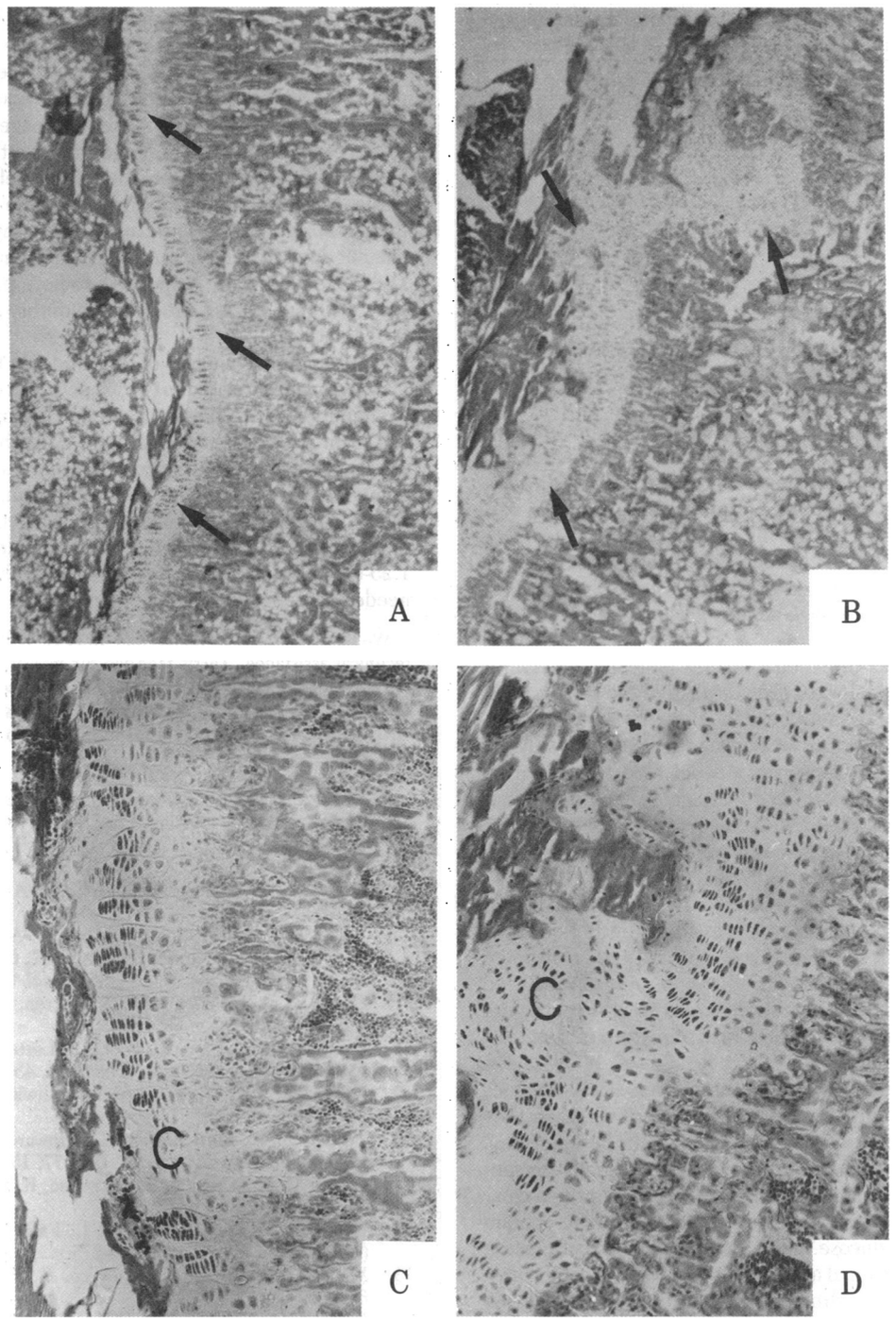

FIG. 1. Histological changes in the epiphyseal area of nondecalcified tibia from vitamin D-replete (*A* and *C*) and vitamin D-deficient (*B* and *D*) rats. Tibia from replete animals demonstrate a smooth line of cartilaginous growth plate (arrows) and chondrocytes (C) arranged in columns parallel to the bone axis. Tibia from deficient animals show an irregular growth plate line (arrows) and disorderly alignment of chondrocytes (C). (*A* and *B*, ×11; *C* and *D*, ×44.)

***In Vitro* Metabolism of 25-OHD$_3$.** Cells from the kidneys of vitamin D-deficient rats converted 25-OH[^{3}H]D$_3$ predominantly to 1,25-(OH)$_2$[^{3}H]D$_3$, whereas cells from kidneys of replete rats converted 25-OH[^{3}H]D$_3$ to other metabolites migrating in the region of 24,25-(OH)$_2$D$_3$, 25,26-(OH)$_2$D$_3$, and the 25(OH)D$_3$ 26,23-lactone with only slight formation of 1,25-(OH)$_2$[^{3}H]D$_3$. Formation of dihydroxylated metabolites increased (Table 4) in both groups as substrate level (25-OH[^{3}H]D$_3$) increased. Differences in 1,25-(OH)$_2$[^{3}H]D$_3$ formed by cells from the replete and deficient rats were significant at all three substrate levels.

DISCUSSION

The first problem was to establish that the rats used were indeed vitamin D deficient. By vitamin D deficiency, we mean that there was insufficient 1,25-(OH)$_2$D$_3$ circulating within the animal to maintain normal bone morphology. To detect the possibility that this metabolite might still be circulating in small quantities, we extracted 10 ml of plasma obtained from rats on our deficient diet for 3 months and found no detectable 1,25-(OH)$_2$D$_3$. Our data demonstrate a gradual disappearance of 1,25-(OH)$_2$D$_3$ from the circulation, becoming undetectable between 6 and 10 weeks after start of the dietary regimen. Mallon

Table 4. *In vitro* 25-OHD 1-hydroxylase activity in renal cells isolated from vitamin D-replete and vitamin D-deficient rats

	Hydroxylase activity,* fmol/min/mg protein		
	50 nM $25\text{-}OHD_3$	100 nM $25\text{-}OHD_3$	300 nM $25\text{-}OHD_3$
Control, vitamin D replete	1.89 ± 0.12	3.37 ± 0.52	15.55 ± 1.81
Vitamin D deprived, normocalcemic	5.20 ± 0.43	13.63 ± 2.21	46.53 ± 3.38

* Mean ± SD.

et al. reported a similar pattern of decline in serum $1,25\text{-}(OH)_2D_3$ levels in rats eating a diet containing 1% calcium and 0.7% phosphorus (13).

The major metabolite formed *in vivo* upon administration of $25\text{-}OHD_3$ was $1,25\text{-}(OH)_2D_3$. Renal cells isolated from our normal calcemic, vitamin D-deficient rats converted $25\text{-}OH[^3H]D_3$ to $1,25\text{-}(OH)_2[^3H]D_3$ at a rate 2–3 times that observed in cells from vitamin D-replete rats. This acceleration of 25-OHD 1-hydroxylase activity is a further indication of the absence of vitamin D in these rats. This model allows one to measure enhanced 25-OHD 1-hydroxylase activity without the added effect of hypocalcemia.

Morphological data provided a further indication of vitamin D deficiency. The epiphyseal growth plate of the tibia was enlarged by month 4 of the regimen. In addition, the typical disarrangement of the columns of cartilage cells was readily apparent. At the present time, we are not able to confirm or dispute the report of Howard and Baylink (14) that increase of plasma calcium in vitamin D-deficient rats by use of a 1.2% calcium diet with added lactose returns the appearance of this area of the metaphysis to near normal.

By month 4 on the vitamin D-deficient diet, bones from our deficient rats were weaker than bones from the replete rats. Because all of the rats were fed 16 g of food daily and consumed their allowance, we can consider our data to represent pair-fed experiments. Under these conditions, body weight gain in deficient rats was not significantly less than in replete rats, at least for 6 months.

The significant question is why the plasma calcium values remained normal in the rats deprived of vitamin D when they decrease in most other models used for vitamin D deficiency. One component absent from our diet which is usually present in vitamin D-deficient diets is glucose. It has never been clear why many rachitogenic diets for both chicks and rodents contain high percentages of glucose. One might speculate that glucose could either alter intestinal absorption of calcium or act directly upon the 25-(OH)D 1-hydroxylase system to increase the rate at which $1,25\text{-}(OH)_2D_3$ and serum calcium decrease. To our knowledge, neither concept has been tested.

An alternate explanation relates to the time when the rats were placed on the vitamin D-deficient diet. We can produce a hypocalcemic vitamin D-deficient rat if the lactating mother is transferred to the deficient diet. When weaned to the same diet, pups from these mothers are unable to maintain normal serum calcium levels and show a permanent stunting of body growth. It appears that maternal deprivation of vitamin D during lactation produced permanent damage to those bone processes controlling calcium homeostasis in the offspring.

It has been reported that vitamin D-deficient rats with low plasma calcium concentrations are unable to respond to parathyroid hormone (15). This would be the case if there were permanent damage to bone processes. Our normocalcemic vitamin D-deficient rats were responsive to both parathyroid hormone and calcitonin during the first 6 months on this diet (unpublished data).

Finally, the advantages of this model in the study of vitamin D deficiency are related to two aspects: (*i*) it separates the effects of vitamin D deficiency from those of prolonged hypocalcemia; and (*ii*) it provides an animal model in which the progressive effects of vitamin D deficiency are more similar to the development of osteomalacia in the adult human. Investigators using this model can no longer depend on the decrease in plasma calcium values as an indicator of vitamin D deficiency. Assays for $1,25\text{-}(OH)_2D_3$ or identification of bone abnormalities will be needed to define vitamin D deficiency.

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