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The Effect of Vitamin D Deficiency on Bone Repletion (41214)

CHUNG-CHING LIU, JOEL L. IVEY, AND DAVID J. BAYLINK

Department of Medicine, University of Washington, Seattle, Washington 98195, and American Lake Veteran's Administration Medical Center, Tacoma, Washington 98493

Abstract. The present study was undertaken to determine the effects of vitamin D deficiency, serum calcium, and PTH on the two major bone changes associated with bone repletion: the increase in endosteal matrix formation and the decrease in endosteal bone resorption. Following a period of bone depletion induced by calcium deficiency, vitamin D-deficient repleting rats demonstrated a marked increase in endosteal bone matrix formation over that seen in pair-weighted nonrepleting control rats. Further, in repleting rats deficient in vitamin D, bone matrix deposition was equivalent to that seen in pair-weighted vitamin D-treated repleting rats, indicating that vitamin D is not essential for matrix repletion. Analysis of serum parameters showed that the repleting vitamin D-deficient rats were severely hypocalcemic and exhibited an eightfold elevation in serum iPTH. Thus, neither the normalizing of the hypocalcemia nor of the high PTH levels incurred during bone depletion is necessary for matrix repletion. On the other hand, in the absence of vitamin D, there was a severe impairment of mineralization of the repleting bone matrix. Bone resorption was depressed during repletion in both vitamin D-deficient and vitamin D-treated rats. The depression of resorption in the vitamin D-deficient rats occurred despite a sustained increase in serum PTH, suggesting that some aspect of the repletion state is capable of suppressing PTH-mediated bone resorption.

Bone functions not only as a mechanical support system but also as a storage depot of calcium and phosphate. The most readily available mineral of this reservoir is located in the endosteal region of bone. At times of low calcium stress, such as would occur with a low calcium diet or with pregnancy and lactation, there is a hormonally mediated dissolution of the endosteal region of bone to provide calcium in an attempt to maintain the serum level within physiologic limits. Once the low calcium stress is removed, excessive resorption halts and formation increases to replace the endosteal bone that was lost during the stress period. This repair process is termed *bone repletion* and is considered to be a bone volume regulatory mechanism of some importance, since the lack of such a mechanism would, with successive exposures to a low calcium stress, lead to a depleted skeleton to the extent that spontaneous fractures would occur and bone could not perform its other function, namely, mechanical support. That bone repletion does occur is well documented (1, 2). However, how this overall process is mediated remains unclear. Our studies to date (2, 3) on the mechanism of bone repletion are for the most part negative, showing that bone repletion does not depend upon calcitonin, parathyroid hormone (PTH), or normal serum calcium. Inasmuch as there is a loss of endosteal bone during the low calcium stress, we reasoned that during this period of time there would be a greater mechanical stress on this depleted skeleton which might somehow mediate the repletion process. In order to evaluate this possibility, we sought to determine if immobilization of a limb would prevent bone repletion. This, too, provided negative results in that there was no impairment of the repletion process despite decreased mechanical stress in the immobilized limb (2). One important set of hormones which have not yet been evaluated with respect to regulation of bone repletion are vitamin D metabolites. Inasmuch as vitamin D metabolites are known to play a role in the regulation of both the resorption and mineralization processes of bone (4), the present study was undertaken to determine if adequate vitamin D is required for bone repletion.

Materials and Methods. *Experimental protocol.* Male weanling Holtzman rats

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(Holtzman Co., Madison, Wis.) with a mean initial body weight of 55 g were used in all experiments. They were housed individually in suspended cages in the dark, and, in addition, the cages housing vitamin D-deficient (−D) rats were covered with heavy cloth sheets to avoid exposure to light during routine care. All experiments were divided into two phases, depletion and repletion. Bone depletion was induced by feeding rats a semisynthetic calcium-free (−Ca) diet as previously described (5) for 14 days. Repletion was initiated by changing the diet to one containing 0.6% calcium. All diets contained 0.6% phosphorus. To achieve a vitamin D-deficient state by the time repletion was to begin, the vitamin was excluded from the −Ca diet (−Ca−D) of the appropriate animals during depletion. Control animals (described below) were pair-weighted with test animals except as otherwise noted. Tetracycline (10−15 mg/kg body wt) was injected ip every 4−5 days during the repletion period to label the newly formed bone. At the end of each experiment blood was obtained under light ether anesthesia by cardiac puncture, the animals were sacrificed, and the tibias were removed and fixed for quantitative histologic measurements.

Experiment 1a: Bone matrix formation in repleting rats deprived of vitamin D. Test animals were fed a −Ca−D diet for 2 weeks and subsequently placed on a +Ca−D diet to study the effect of −D on bone matrix repletion (Fig. 1). At the end of depletion, 26 animals (6 control and 20 −Ca−D) were sacrificed to obtain base line serum and bone parameters. These base line bone parameters were needed for subsequent calculation of certain mean rates, i.e., bone formation, resorption, and osteoid maturation, as previously described (6). The remaining test animals were fed the +Ca−D diet during the 14-day repletion period. Nonrepleting control animals received a normal (+Ca+D) diet throughout the experiment.

Experiment 1b: Parathyroid activity in repleting rats deprived of vitamin D. This experiment, whose protocol was identical to that of Experiment 1a, provided blood for the determination of serum-immunoreactive PTH (iPTH) levels in repleting rats deprived of vitamin D.

Experiment 2: Bone matrix formation in

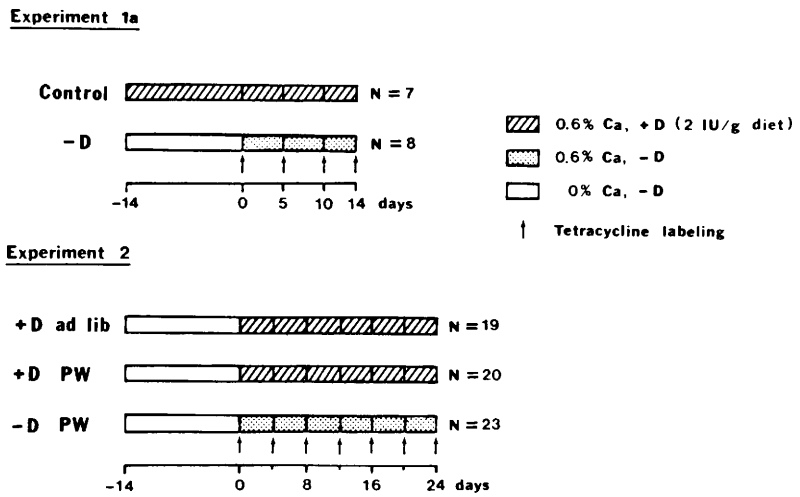

FIG. 1. The experimental protocol. Weanling rats were fed a calcium- and vitamin D-free (−Ca−D) diet for 14 days and subsequently placed on a control diet with or without vitamin D for 14 or 24 days to study the effect of −D on bone repletion. Control animals were kept on a normal diet throughout the experimental period. All animals, except those in the *ad lib* group during repletion in Experiment 2, were pair-weighted (PW).

repleting rats with and without vitamin D. This experiment was designed to more clearly delineate the effect of vitamin D on the repletion response. In order to make vitamin D the only variable, all the animals were subjected to depletion and were made vitamin D deficient (−Ca−D). At the end of the depletion period, they were divided into three groups, (Fig. 1). Two groups were subsequently fed a diet containing both calcium and vitamin D, one *ad lib*, the other pair-weighted with Group III (the test group), during the 24-day repletion period. Group III animals were given a diet with a normal calcium content but without vitamin D.

Serum chemistry. Serum calcium was measured by atomic absorption spectroscopy (7), and serum phosphorus by the method of Fiske and Subbarow (8) using a technicon autoanalyzer. Serum iPTH was determined by the method of Arnaud *et al.* (9) using chicken-12 anti-bovine antiserum as described earlier (10, 11), which detects the region of bovine PTH that is carboxy terminal to amino acid residue 34. The intra- and interassay variations of serum iPTH were ±8 and 15%, respectively.

Quantitative histology of bone. Each tibia was cut perpendicular to its long axis at the fibular junction. Transverse sections about 50-μm thick were sawed by means of a Gillings-Hamco thin-sectioning machine. The bone specimen holder was mounted on a goniometer to facilitate sawing sections. The sawed sections were then hand ground to a final thickness of about 30 μm.

The method used for quantitation of bone parameters has been described elsewhere (6) and is briefly described below:

(*i*) *Matrix formation rate* (mm^3/day). The amount of matrix formed per day, including unmineralized as well as mineralized matrix, was measured. It is identical to bone formation rate, except that it exceeds the bone formation rate when osteoid maturation is impaired.

(*ii*) *Matrix apposition rate* ($\mu m/day$). The width of new matrix added per day at a bone forming surface was calculated by dividing the amount of bone matrix formed by the length of the forming surface. This parameter usually reflects osteoblast cell activity (A_{ob}, the amount of matrix deposited/day) in contrast to the forming surface, which reflects osteoblast cell number (N_{ob}); forming surface in mm^2 × apposition rate in mm or $N_{ob} \times A_{ob}$ = bone formation rate, mm^3.

(*iii*) *Osteoid maturation rate*, R_{om} (*%/hr*). A measure of the onset of mineralization was performed. A certain amount of time elapses between the deposition of osteoid and initiation of mineralization in this osteoid. This *mineralization lag time* can be calculated by dividing the width of osteoid by the matrix apposition rate. If one assumes that osteoid is 0% mature when formed and 100% mature when mineralization is initiated, R_{om} can be calculated simply by dividing 100% by the mineralization lag time.

(*iv*) *Endosteal bone resorption rate* (mm^3/day). Since no resorption occurs at the periosteum in our sampling site in the tibial diaphysis, this represents essentially the total resorption rate and is equal to the change in medullary area plus the endosteal bone formation rate. Initial medullary area was predicted from the measured initial total area, since the latter is highly correlated with the initial medullary area (12).

Results. *Body weight and serum parameters.* Because bone formation is proportional to weight gain and because −D impairs weight gain, control animals, except those in the *ad lib* group of Experiment 2, were pair-weighted with the animals of the respective test groups (Tables I and IV). Serum calcium was decreased in −Ca−D rats during depletion and remained low during repletion (+Ca−D) (Table I). This is consistent with the vitamin D-deficient status of the rats. Serum phosphorus was not changed by −Ca−D, but was significantly increased during repletion in −D rats when compared with that of the nonrepleting control group of Experiment 1a or, in Experiment 2, with that of the control rats fed the +Ca+D diet *ad lib* during repletion (Groups III vs I, $P < 0.001$; Table IV). However, when repleting rats were pair-weighted with −D rats, these repleting rats had lower serum phosphorus compared to the −D rats (Groups III vs II, Table IV).

The hypocalcemia found in the repleting

TABLE I. BODY WEIGHT AND SERUM PARAMETERS (EXPERIMENT 1)

Parameter	Start of repletion		End of repletion	
	Control	−Ca−D	Control	−D
Final body wt (g)	68 ± 4[a]	61 ± 2	135 ± 4	130 ± 3
Serum calcium (mg/dl)	11.02 ± 0.21	6.79 ± 0.24[b]	11.90 ± 0.10	6.50 ± 0.15[b]
Serum phosphorus (mg/dl)	9.27 ± 0.45	9.51 ± 0.38	9.54 ± 0.10	12.03 ± 0.28[b]

[a] Mean ± SE (The number of animals/group in this and other tables is shown in Figs. 1 and 2).
[b] $P < 0.001$ as evaluated by Student's t test.

+Ca−D rats of Experiment 1b was accompanied by an eightfold elevation of serum iPTH over that of the nonrepleting control animals (2588 ± 258 vs 278 ± 43 pg/ml, $P < 0.001$; $N = 9$ in the control group and 8 in the test group).

Bone formation parameters. The data presented in Tables II and V show that endosteal matrix formation is increased during repletion in −D as well as in +D rats. At the end of depletion the endosteal-forming surface was significantly less in the −D group than in the nonrepleting control group of Experiment 1a (baseline values). However, after 2 weeks of repletion this parameter in the −D group increased to a level similar to that of the control group, suggesting that osteoblast proliferation was stimulated. Since the endosteal matrix apposition rate was also increased, 52% greater than the control rate, the results suggests that the activity of osteoblasts was also increased during repletion. As a result of the increase in the endosteal-forming surface and in the rate of matrix apposition, the amount of endosteal matrix produced was significantly increased, 38% greater than the control.

Data on all endosteal bone formation parameters (forming surface, matrix apposition rate, and matrix formation rate) measured during repletion in Experiment 2 showed that no significant differences were found in these parameters between the two pair-weighted repleting groups, one with and the other without vitamin D (Table V), indicating that as much bone matrix was formed in −D rats as in +D rats. The re-

TABLE II. MATRIX FORMATION AND MINERALIZATION PARAMETERS DURING 2 WEEKS OF REPLETION (EXPERIMENT 1)

Parameter	Control	−D
Formation		
Endosteal-forming surface (mm)	3.22 ± 0.17 (3.26 ± 0.29)[a]	3.54 ± 0.18 (2.27 ± 0.16)[b]
Endosteal matrix formation rate (mm^3/day)	0.0080 ± 0.0005	0.0110 ± 0.0006[c]
Endosteal matrix apposition rate (μm/day)	2.50 ± 0.17	3.80 ± 0.20[b]
Periosteal matrix formation rate (mm^3/day)	0.029 ± 0.003	0.026 ± 0.002
Periosteal matrix apposition rate (μm/day)	4.59 ± 0.37	4.24 ± 0.35
Mineralization		
Endosteal osteoid width (μm)	3.43 ± 0.34	16.12 ± 1.13[b]
Endosteal osteoid maturation rate (%/hr)	3.02 ± 0.17	1.52 ± 0.10[b]
Periosteal osteoid width (μm)	7.49 ± 0.69	22.33 ± 2.01[b]
Periosteal osteoid maturation rate (%/hr)	4.14 ± 0.48	1.03 ± 0.11[b]

[a] The endosteal-forming surface was measured at the start and the end of repletion; parentheses indicate this parameter at the start of repletion.
[b] $P < 0.001$.
[c] $P < 0.005$.

TABLE III. ENDOSTEAL BONE RESORPTION PARAMETERS (EXPERIMENT 1)

Parameter	Control	−D
Endosteal resorbing surface (mm)		
Start of repletion	1.05 ± 0.25	2.22 ± 0.17[a]
End of repletion	0.77 ± 0.12	0.28 ± 0.14[b]
Difference	−0.28	−1.94
Endosteal bone resorption rate (mm^3/day)	0.0056 ± 0.0038	−0.0056 ± 0.0036[c]

[a] $P < 0.001$.
[b] $P < 0.02$.
[c] The value is not significantly different from 0.

sults from these two experiments suggest that the stimulation of matrix formation associated with repletion is independent of vitamin D.

Results from Experiment 2 also showed that the decreased food intake, i.e., decreased growth, associated with pair-weighting reduced the effect of repletion to stimulate endosteal matrix formation (Groups I vs II, Table V). To determine the extent to which bone repletion was growth dependent, we investigated the relationship between periosteal matrix formation (which itself correlates well with other growth parameters) and endosteal matrix formation (which is a measure of bone repletion). Based on the correlation between these two parameters ($r = 0.32$, $P < 0.05$), it was found that growth could only account for 10% of the observed increase in bone matrix repletion.

Matrix mineralization parameters. Compared to the respective control rats, R_{om} were significantly decreased at both the periosteum and endosteum in repleting −D rats in both experiments (Tables II and V). As previously described, the rate of matrix apposition at the endosteum was increased in the repleting rats of Experiment 1a and equal to that of the repleting +Ca+D pair-weighted rats of Experiment 2. Together, the increased rate of matrix apposition and the delay in the onset of mineralization resulted in a significant increase in the width of osteoid seams. The increase in periosteal osteoid width was primarily due to the decrease in R_{om}. Coefficients of correlation support this conclusion: at the periosteum $r = -0.86$ and -0.90, respectively, for Experiments 1a and 2; $P < 0.001$. However, at the endosteum the increase in osteoid width (X_1) observed in Experiment 1a was found to be due to both a decrease in R_{om} (X_2) and to an increase in the matrix apposition rate (X_3); partial correlation: $r^2(12.3) = 0.86$ and $r^2(13.2) = 0.76$. Thus, during repletion, vitamin D deficiency delayed osteoid maturation while matrix apposition was not impeded.

Endosteal bone resorption parameters. The other major bone change associated with repletion is a decrease in endosteal bone resorption. The results presented in Table III and Fig. 2 show that this decrease

TABLE IV. BODY WEIGHT AND SERUM PARAMETERS AT THE END OF 24-DAY REPLETION (EXPERIMENT 2)

Parameter	+D *ad lib* (I)	+D PW[a] (II)	−D PW (III)	*P* I vs II	*P* II vs III
Final body wt (g)	192 ± 4	117 ± 2	119 ± 3	<0.001	NS[b]
Serum calcium (mg/dl)	11.65 ± 0.07	10.85 ± 0.09	5.52 ± 0.09	<0.001	<0.001
Serum phosphorus (mg/dl)	9.37 ± 0.12	6.66 ± 0.16	10.21 ± 0.21	<0.005	<0.001

[a] PW, pair-weighted.
[b] Not statistically significant at the 0.05 probability level.

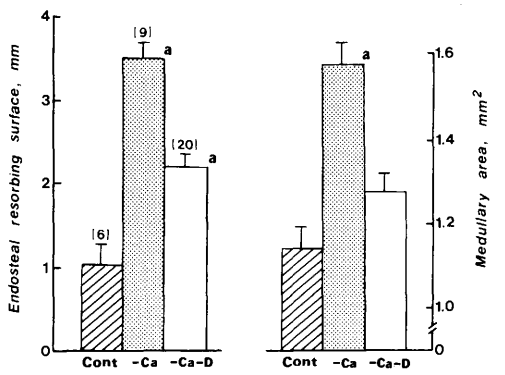

FIG. 2. Comparison of endosteal bone resorption in −Ca and in −Ca−D. In Experiment 1, a −Ca group was also included in order to compare bone resorption in −Ca and in −Ca−D. The parenthesis indicates the number of rats/group, and "a" indicates significant differences ($P < 0.001$) between the control and test groups. Data are expressed as mean + 1 SE above the mean.

can occur in −D rats as well as in +D rats. At the end of depletion, the endosteal resorbing surface (measured on the baseline animals sacrificed at this time) was significantly increased and medullary area enlarged, although not significantly (Table III and Fig. 2). After two weeks of repletion, the endosteal resorbing surface was less in repleting rats than in nonrepleting control rats. The decrease in the control rats is attributable to aging (unpublished data). The decreased endosteal resorbing surface due to repletion is even greater if one considers the change from the beginning to the end of the repletion period in the repleting animals (Table III). The marked decrease in endosteal resorbing surface was confirmed in Experiment 2, in which no resorbing surface was found in any of the repleting rats (+D or −D) at the end of the 24-day repletion period. As a consequence there was no significant difference in the endosteal bone resorption rate between repleting vitamin D-fed rats and repleting vitamin D-deficient rats.

Discussion. Quantitatively, the most important bone change associated with repletion is the marked increase in endosteal matrix formation (1, 2). The present study shows that this bone change is independent of vitamin D since even in the absence of vitamin D endosteal-forming surface and the rates of endosteal matrix formation and apposition increased as normally seen in repletion. Past work has shown that 1,25-dihydroxyvitamin D_3 (1,25-diOHD_3) inhibits bone matrix formation (4, 13); thus, decreased 1,25-diOHD_3 would not be expected to impair the stimulation of matrix formation associated with bone repletion. Indeed, under normal repletion conditions serum 1,25-diOHD_3 typically decreases from its high depletion level (14). On the other hand, it has been suggested that other vitamin D metabolites such as 24,25-dihydroxyvitamin D_3 (24,25-diOHD_3) may play a significant role in matrix formation (15). However, −D is associated with a decrease in 24,25-diOHD_3 as well (16), and thus it appears that vitamin D metabolites in general do not play a significant role in controlling the stimulation of matrix formation during repletion.

It was assumed from past studies (1−3, 14) that the increase in serum calcium from its lowered depletion phase level to normal in the repletion phase (with concomitant changes in serum levels of calcium-regulating hormones) triggered bone repletion. Since serum calcium remained significantly low (about half of the control) while animals were repleting in the absence of vitamin D, it appears that a sustained increase in serum calcium is not required for the increase in matrix formation. Our data, however, do not exclude the possibility that replenishment of calcium in the diet and the resultant increase in intestinal calcium absorption somehow triggered the increase in matrix formation independent of a change in serum calcium.

The second important bone change associated with repletion (the decrease in endosteal bone resorption) occurred in −D as well as in +D rats, suggesting that this change is independent of vitamin D. Furthermore, this decreased resorption occurred despite a low serum calcium and a markedly elevated serum iPTH, suggesting that the effect of high serum PTH to increase bone resorption can be suppressed by repletion.

Although vitamin D metabolites are not essential for stimulation of matrix forma-

TABLE V. MATRIX FORMATION AND MINERALIZATION PARAMETERS DURING 24 DAYS OF REPLETION (EXPERIMENT 2)

Parameter	+D *ad lib* (I)	+D PW (II)	−D PW (III)	*P*	
				I vs II	II vs III
Formation					
Endosteal-forming surface[a] (mm)	3.69 ± 0.04	3.89 ± 0.04	3.92 ± 0.06	<0.005	NS
Endosteal matrix formation rate (mm^3/day)	0.0118 ± 0.004	0.0098 ± 0.0003	0.0105 ± 0.0003	<0.025	NS
Endosteal matrix apposition rate (μm/day)	3.86 ± 0.14	3.06 ± 0.11	3.01 ± 0.11	<0.001	NS
Periosteal matrix formation rate (mm^3/day)	0.042 ± 0.001	0.026 ± 0.001	0.021 ± 0.001	<0.001	<0.001
Periosteal matrix apposition rate (μm/day)	6.49 ± 0.16	4.24 ± 0.14	3.45 ± 0.15	<0.001	<0.001
Mineralization					
Endosteal osteoid width (μm/day)	2.9 ± 0.2	5.0 ± 0.3	24.3 ± 0.7	<0.001	<0.001
Endosteal osteoid maturation rate (%/hr)	3.47 ± 0.14	2.26 ± 0.10	0.83 ± 0.03	<0.001	<0.001
Periosteal osteoid width (μm)	6.8 ± 0.2	7.2 ± 0.2	34.4 ± 1.8	NS	<0.001
Periosteal osteoid maturation rate (%/hr)	2.76 ± 0.07	1.76 ± 0.06	0.60 ± 0.04	<0.001	<0.001

[a] Measured at the end of repletion.

tion during repletion, they are clearly essential for mineralization of the newly synthesized matrix. Thus, during repletion, there was a severe impairment of mineralization of new matrix formed as indicated by the low endosteal R_{om} in −D compared with +D rats. Two lines of evidence suggest that the observed impairment of mineralization was due to hypocalcemia and not the low serum 1,25-diOHD$_3$ or high serum PTH: (i) hypocalcemia causes a decrease in R_{om} regardless of high (as in −Ca) or low (as in −D) serum 1,25-diOHD$_3$ (4) and (ii) R_{om} can be restored to normal in −D rats by correction of serum calcium without correction of the low serum 1,25-diOHD$_3$ (17) and in thyroparathyroidectomized rats by correction of serum calcium (18). These results suggest that vitamin D metabolites are important to the bone repairing process from the standpoint of supplying adequate mineral for deposition in bone matrix, but are not in themselves involved in the increase in matrix formation.

In a past study (2), we found that the endosteal matrix apposition rate and R_{om} were consistently coupled and suggested that the two rates may be under a similar control. In contrast, in the present study, the increase in the endosteal matrix apposition rate in repleting rats deprived of vitamin D was attended by a low R_{om}, indicating that the two rates were uncoupled. The finding of uncoupling between the two rates in repleting animals suggests that these rates can be independently regulated.

Although the main objective of the present study was to determine the effect of −D on bone matrix formation associated with repletion, an additional noteworthy observation concerns the effect of −D on −Ca-induced bone resorption. In past work, it was demonstrated that −Ca causes an increase in endosteal bone resorption and a significant increase in the medullary area (1, 2, 12). In the present study, we compared bone resorption in −Ca and in −Ca−D (Fig. 2) and found that −D significantly inhibited the endosteal bone loss associated with −Ca. We have previously attributed the increased endosteal resorption in −Ca to increases in both serum PTH and 1,25-diOHD$_3$ (14, 19). Inasmuch as −D does not impair the effect of low serum calcium to increase PTH levels (4), it is reasonable to conclude that the cause for the impairment of bone resorption in −Ca−D was a decrease in serum 1,25-diOHD$_3$. This would be consistent with the *in vitro* observations (20−22) that on a molar basis, 1,25-diOHD$_3$ is the most potent stimulator of bone resorption.

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