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EFFECT OF 1,25-DIHYDROXYVITAMIN D_3 ON BONE METABOLISM IN TISSUE CULTURE

ENHANCEMENT OF THE STEROID EFFECT BY ZINC

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Abstract—The present investigation was undertaken to clarify the interaction of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and zinc on bone metabolism in tissue culture. Calvaria were removed from weanling rats (3-week-old males) and cultured for periods up to 96 hr in Dulbecco's Modified Eagle Medium (high glucose, 4500 mg/dl) supplemented with antibiotics and bovine serum albumin. The experimental cultures contained 10^{-10} to 10^{-6} M 1,25$(OH)_2D_3$. All cultures were incubated at 37° in 5% CO_2/95% air. Bone calcium content was increased significantly by the presence of 10^{-9} to 10^{-7} M 1,25$(OH)_2D_3$. The steroid (10^{-9} to 10^{-7} M) also significantly increased alkaline phosphatase activity in the bone, whereas it did not alter significantly acid phosphatase activity. [^{3}H]Leucine incorporation by the bone was raised significantly by 10^{-8} to 10^{-7} M 1,25$(OH)_2D_3$. Furthermore, bone DNA content was increased significantly by 10^{-9} to 10^{-7} M 1,25$(OH)_2D_3$. Meanwhile, the presence of 10^{-4} M zinc, which can stimulate bone formation, significantly enhanced the effect of 10^{-7} M 1,25$(OH)_2D_3$ to increase alkaline phosphatase activity and DNA content in rat calvaria. The present study demonstrates that 1,25$(OH)_2D_3$ has a direct stimulatory effect on bone metabolism in tissue culture and that zinc can enhance the steroid effect.

Vitamin D_3 is converted by the liver and kidney to a physiologically active hormonal form which has effects on bone and mineral metabolism. 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the most active metabolite of vitamin D_3, stimulates the intestinal absorption of calcium [1, 2], and increases bone resorption [1, 3]. *In vivo*, vitamin D_3 metabolites are important for the promotion of bone formation and mineralization [4–6]. In contrast to the widely accepted *in vivo* effects of vitamin D_3 on bone formation, there is no conclusive *in vitro* evidence that vitamin D_3 stimulates bone formation directly [7]. However, the presence of a high affinity receptor for 1,25$(OH)_2D_3$ in bone cells has been demonstrated [8, 9]. The receptor has been localized in oesteoprogenitor cells, osteoblasts, osteocytes, chondroblasts, and certain populations of chondrocytes [10, 11]. These observations support the possibility that 1,25$(OH)_2D_3$ may elicit a direct stimulatory effect on bone formation and mineralization.

Recently, it has been reported that 1,25$(OH)_2D_3$ elevates alkaline phosphatase activity in early cultures of ROS 17/2 cells, but that the steroid reduces the enzyme activity in later cultures, suggesting that its effect may depend on the state of differentiation of the cells [12]. Furthermore, it has been demonstrated that 1,25$(OH)_2D_3$ has a direct specific anabolic effect on osteoblastic cells *in vitro* during the growth phase and this effect is related to receptor concentration [13]. 1,25$(OH)_2D_3$ may be an important modulator of the growth and differentiation of human bone cells *in vitro* [14]. These findings are consistent with the possibility that 1,25$(OH)_2D_3$ has direct effects on bone formation *in vivo*. Further investigation is needed, however, to elucidate, whether 1,25$(OH)_2D_3$ has a direct stimulatory effect on bone formation and mineralization in the bone tissue culture system. The present investigation, therefore, was undertaken to clarify the effect of 1,25$(OH)_2D_3$ on bone formation in rat calvaria *in vitro*.

More recently, it has been reported that zinc, which is an essential metal for the growth of animals [15], has a stimulatory effect on the bone growth and mineralization of weanling rats [16]. Zinc can enhance markedly 1,25$(OH)_2D_3$-stimulated bone formation *in vivo*, suggesting a physiologic significance of zinc in the stimulation of bone formation [17, 18]. Therefore, the present report also describes the interaction of 1,25$(OH)_2D_3$ and zinc on bone metabolism in tissue culture.

The results, in tissue culture system using rat calvaria, further support the possibility that 1,25$(OH)_2D_3$ may have direct effects on bone formation *in vivo*. It was also found that the steroid effects are enhanced by zinc *in vitro*.

MATERIALS AND METHODS

Chemicals. Dulbecco's Modified Eagle Medium (high glucose) and penicillin–streptomycin solution (5000 units/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Bovine serum albumin (fraction V) and cycloheximide were obtained from the Sigma Chemical Co. (St Louis, MO). L-[4,5-^{3}H]Leucine

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(60.0 μCi/mmol) was obtained from New England Nuclear (Boston, MA). $1,25(OH)_2D_3$ was supplied by the Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Zinc sulfate and all other chemicals were reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All water used was glass distilled.

Animals. Weanling male Wistar rats weighing 60–65 g (3 weeks old) were obtained from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, 1.1% phosphorus and 0.012% zinc, and distilled water. The rats were killed by decapitation.

Bone culture. Calvaria from 3-week-old male rats were removed aseptically and cut along the sagital suture into left and right halves. One-half of each calvarium served as a control for its paired, treated half. Each half-calvarium (17–23 mg wet weight) was cultured in a 35-mm dish in 2.0 ml of serum-free medium consisting of Dulbecco's Modified Eagle Medium (high glucose) supplemented with 0.25% bovine serum albumin (fraction V) plus antibiotics, with either vehicle (1.0% ethanol as final concentration), $1,25(OH)_2D_3$, zinc or $1,25(OH)_2D_3$ and zinc. Cultures were maintained at 37° in a water-saturated atmosphere containing 5% CO_2 and 95% air for 96 hr. The respective media, containing either vehicle, $1,25(OH)_2D_3$, zinc, or $1,25(OH)_2D_3$ and zinc, were changed at 48 hr, and cultures were maintained for an additional 48 hr. In separate experiments, the respective media contained cycloheximide [19].

Analytical procedures. Calvaria were cultured in the medium containing $1,25(OH)_2D_3$ for 48 or 96 hr at 37°. After culture, the bone was removed and washed with ice-cold 0.25 M sucrose solution, blotted, and weighed.

The bone tissues were ashed for 24 hr at 640°, weighed, and then dissolved in 6 N HCl solution [19]. Calcium was determined by atomic absorption spectrophotometry. Bone calcium content was expressed as milligrams of calcium per gram of bone ash.

Alkaline phosphatase and acid phosphatase activities in the bone tissues were determined by the method of Walter and Schutt [20]. The bone tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle, and disrupted for 60 sec with an ultrasonic device. The supernatant fraction, centrifuged at 600 *g* for 5 min, was used for measurement of the enzyme activity. The efficiency of the enzyme extraction was greater than 90%, and the enzyme analysis was reproducible. The enzyme assay was carried out under optimal conditions. Enzyme activity was expressed as micromoles or nanomoles of *p*-nitrophenol liberated per minute per milligram of protein. Protein was determined by the method of Lowry *et al.* [21].

The effect on newly synthesized bone total protein was determined by studying the incorporation of [^{3}H]leucine [22]. The calvaria were pulsed with [^{3}H]leucine (5.0 μCi/ml medium) at the culture period indicated in the legends to the figures, and held for 2 hr. At the end of the period, the calvaria were removed and washed with ice-cold 0.25 M sucrose. The calvaria were extracted with ice-cold 5% trichloroacetic acid, acetone and ether, and then rinsed in ice-cold 0.25 M sucrose. The bones were dried and weighed. For determination of the amount of [^{3}H]leucine incorporated into bone total protein, the dried bone residues were dissolved in 1.0 ml of 0.2 N NaOH, and an aliquot was removed and placed in a vial for measurement of the disintegrations per minute by scintillation counting. Data are expressed as disintegrations per milligram dry weight of acid-insoluble residues.

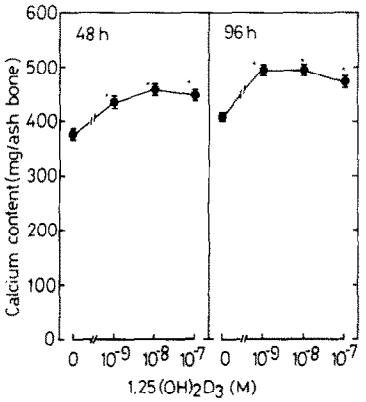

Fig. 1. Effect of $1,25(OH)_2D_3$ on calcium content in rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing either vehicle (1% ethanol) or 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$. Each point is the mean of five calvaria per group. The vertical lines represent the SE. The bone calcium content at 0 hr of culture was 380.3 ± 8.7 mg/g bone ash. Key: (*) $P < 0.01$, compared with the control (vehicle) value.

To measure DNA content, the bone tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr [23]. After alkali extraction, samples were centrifuged at 10,000 *g* for 5 min, and the supernatant fraction was collected. DNA content in the supernatant fraction was determined by the method of Ceriotti [24] and expressed as the amount of DNA (mg) per gram of wet bone tissue.

Zinc content in the culture medium and the bone tissues was determined by atomic absorption spectrophotometry after digestion with nitric acid [19]. The medium did not contain zinc.

Statistical analysis. Data are expressed as the mean ± SE. Statistical differences were analyzed using Student's *t*-test. P values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of $1,25(OH)_2D_3$ *on bone metabolism.* The alteration of calcium content in rat calvaria cultured for 48 and 96 hr is shown in Fig. 1. Calcium content in the bone cultured for 96 hr in control medium without $1,25(OH)_2D_3$ was increased appreciably. The presence of 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$ significantly increased the calcium content in the bone

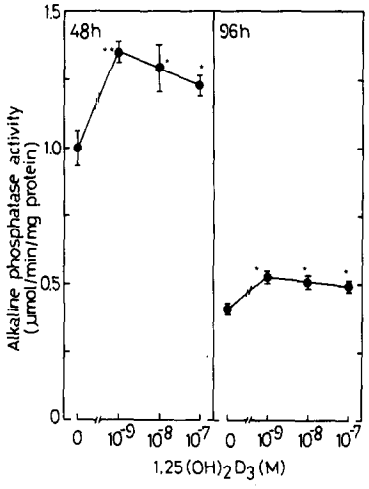

Fig. 2. Effect of $1,25(OH)_2D_3$ on alkaline phosphatase activity in rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing either vehicle or 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$. Each point is the mean of five calvaria per group. The vertical lines represent the SE. The bone enzyme activity at 0 hr of culture was 1.009 ± 0.045 μmol/min/mg protein. Key: (*) $P < 0.05$ and (**) $P < 0.01$, compared with the control value.

Table 1. Effect of $1,25(OH)_2D_3$ on acid phosphatase activity in rat calvaria *in vitro*

Treatment	Acid phosphatase (nmol/min/mg protein)	
	48 hr	96 hr
Control	40.8 ± 3.6	59.6 ± 2.8
10^{-9} M $1,25(OH)_2D_3$	39.6 ± 1.1	57.0 ± 2.4
10^{-8} M $1,25(OH)_2D_3$	40.6 ± 2.0	59.0 ± 3.8
10^{-7} M $1,25(OH)_2D_3$	43.8 ± 0.8	51.8 ± 4.2

Each value is the mean ±SE of five calvaria. The bones were cultured for 48 and 96 hr in medium containing 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$. Data were not significant.

cultured for 48 hr. After culture for 96 hr in 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$, bone calcium content was increased markedly in comparison with the value at 48 hr of culture.

The change in alkaline phosphatase activity in rat calvaria cultured in the presence of $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) is shown in Fig. 2. When the bone was cultured for 48 hr in medium without $1,25(OH)_2D_3$, the basal activity of alkaline phosphatase increased significantly in comparison with the value at 0 hr of culture (data not shown). This basal activity, however, was decreased by a 96-hr culture in comparison with the value obtained when the bone was cultured for 48 hr. Alkaline phosphatase activity in the bone cultured in the presence of 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$ for 48 and 96 hr increased significantly. Meanwhile, bone acid phosphatase activity was not altered significantly, when the bone was cultured in the presence of 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$ for 48 and 96 hr (Table 1).

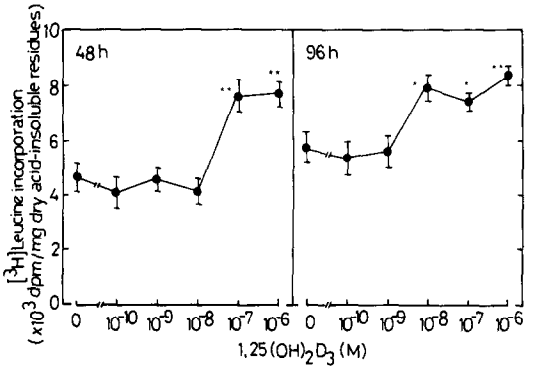

Fig. 3. Effect of $1,25(OH)_2D_3$ on the incorporation of [^{3}H]leucine into the acid-insoluble residues of rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing either vehicle or 10^{-10} to 10^{-6} M $1,25(OH)_2D_3$, and pulsed with [^{3}H]leucine (5.0 μCi/ml of medium) 2 hr before removing the bone. Each point is the mean of five calvaria per group. The vertical lines represent the SE. Key: (*) $P < 0.05$ and (**) $P < 0.025$, compared with the control value.

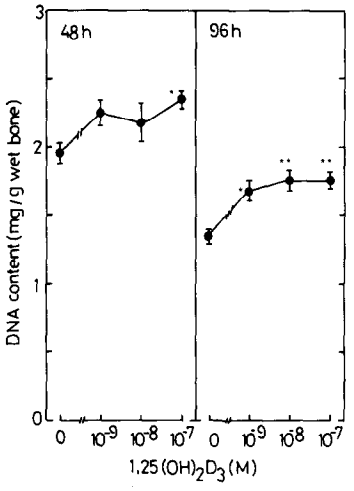

Fig. 4. Effect of $1,25(OH)_2D_3$ on DNA content in rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing either vehicle or 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$. Each point is the mean of five calvaria per group. The vertical lines represent the SE. Key: (*) $P < 0.01$ and (**) $P < 0.001$, compared with the control (vehicle) value.

The effect of $1,25(OH)_2D_3$ on [^{3}H]leucine incorporation by rat calvaria is shown in Fig. 3. The incorporation of [^{3}H]leucine into the bone cultured for 48 and 96 hr was increased significantly by the presence of 10^{-7} and 10^{-6} M $1,25(OH)_2D_3$. At 96 hr of culture, 10^{-8} M $1,25(OH)_2D_3$ significantly increased [^{3}H]leucine incorporation. Thus, $1,25(OH)_2D_3$ stimulated protein synthesis in the tissue culture of rat calvaria.

The effect of $1,25(OH)_2D_3$ on DNA content in rat calvaria cultured in the presence of $1,25(OH)_2D_3$ for 48 and 96 hr is shown in Fig. 4. In culture without $1,25(OH)_2D_3$, bone DNA content at 0 hr of culture

Table 2. Effect of zinc on $1,25(OH)_2D_3$-increased calcium content in rat calvaria *in vitro*

Treatment	Calcium content (mg/g bone ash)	
	48 hr	96 hr
Control	386.6 ± 3.9	410.1 ± 4.7
10^{-7} M $1,25(OH)_2D_3$	446.4 ± 7.8*	472.0 ± 11.1*
10^{-4} M Zinc	421.1 ± 7.1†	420.0 ± 3.1
10^{-7} M $1,25(OH)_2D_3$ + 10^{-4} M zinc	440.3 ± 7.3*	452.8 ± 8.4*

Each value is the mean ± SE of five calvaria. The bones were cultured for 48 and 96 hr in medium containing either vehicle (control), 10^{-7} M $1,25(OH)_2D_3$, 10^{-4} M zinc, or 10^{-7} M $1,25(OH)_2D_3$ plus 10^{-4} M zinc.

*† Significantly different from control: *$P < 0.001$ and †$P < 0.01$.

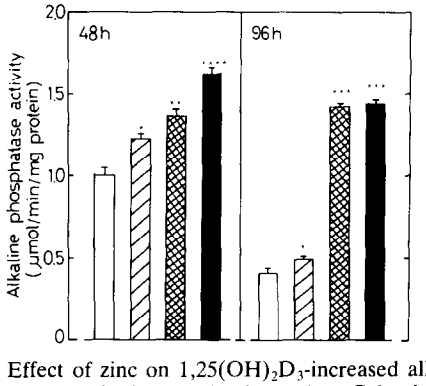

Fig. 5. Effect of zinc on $1,25(OH)_2D_3$-increased alkaline phosphatase activity in rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing: vehicle alone (□); 10^{-7} M $1,25(OH)_2D_3$ (▨); 10^{-4} M zinc; (▩); or 10^{-7} M $1,25(OH)_2D_3$ plus 10^{-4} M zinc (■). Each bar is the mean of five calvaria per group. The vertical lines represent the SE. Key: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$, compared with the control (vehicle) value; and (****) $P < 0.01$, compared with the 10^{-7} M $1,25(OH)_2D_3$ or 10^{-4} M zinc alone.

was 1.546 ± 0.148 mg/g wet bone. At 48 hr of culture, DNA content had increased but the increase was not significant (Fig. 4A). Bone DNA content at 96 hr of culture, however, was 1.421 ± 0.104 mg/g wet bone. Such a decrease of bone DNA content may be attributed to the augmentation of bone matrix content during culture, because the increase in bone calcium content was observed. Bone DNA content was increased significantly by 48 hr of culture with 10^{-7} M $1,25(OH)_2D_3$ but not with 10^{-9} or 10^{-8} M (Fig. 4A). With culture for 96 hr in the presence of 10^{-9} to 10^{-7} M $1,25(OH)_2D_3$, the DNA content increased significantly (Fig. 4B).

Effect of zinc on $1,25(OH)_2D_3$-stimulated bone metabolism. The effect of zinc on $1,25(OH)_2D_3$-stimulated bone metabolism was investigated in rat calvaria cultured in the presence of both $1,25(OH)_2D_3$ and zinc for 48 and 96 hr. The concentration of zinc used was 10^{-4} M; this concentration stimulates bone formation maximally in rat calvaria cultured for 48 and 96 hr [19]. $1,25(OH)_2D_3$-increased bone calcium content was not enhanced significantly by the presence of 10^{-4} M zinc (Table 2).

The effect of both $1,25(OH)_2D_3$ and zinc on alkaline phosphatase activity in rat calvaria cultured for 48 and 96 hr is shown in Fig. 5. With culture for 48 hr in the presence of 10^{-7} M $1,25(OH)_2D_3$ or 10^{-4} M zinc, bone alkaline phosphatase activity increased significantly. When rat calvaria were cultured in the presence of both 10^{-7} M $1,25(OH)_2D_3$ and 10^{-4} M zinc for 48 hr, bone alkaline phosphatase activity significantly increased in comparison with the value of the steroid alone or the metal alone. The additive effect of 10^{-7} M $1,25(OH)_2D_3$ and 10^{-4} M zinc on bone alkaline phosphatase activity was not seen in the 96-hr culture. In the presence of 10^{-7} M cycloheximide, alkaline phosphatase activity in rat calvaria cultured in the presence of 10^{-7} M $1,25(OH)_2D_3$ or both 10^{-7} M $1,25(OH)_2D_3$ and 10^{-4} M zinc for 48 hr did not increase significantly (Table 3).

The change in DNA content in rat calvaria cultured in the presence of both $1,25(OH)_2D_3$ and zinc for 48 and 96 hr is shown in Fig. 6. Bone DNA content was increased significantly by culture with 10^{-4} M zinc alone for 96 hr but not for 48 hr. The presence of both 10^{-7} M $1,25(OH)_2D_3$ and 10^{-4} M zinc produced a remarkable elevation of DNA content in comparison with that of 10^{-7} M $1,25(OH)_2D_3$ or 10^{-4} M zinc alone. When rat calvaria were cultured in the presence of 10^{-7} M $1,25(OH)_2D_3$, 10^{-4} M zinc and 10^{-7} M cycloheximide for 96 hr, the increase in bone DNA content did not occur (Table 4). $1,25(OH)_2D_3$ (10^{-7} M)-increased bone DNA content was also reduced by the presence of 10^{-7} M cycloheximide (Table 4).

The bone zinc content at 0 hr of culture was 103 ± 7 μg/g wet bone. The uptake of zinc by rat calvaria cultured in the presence of 10^{-4} M zinc for 48 and 96 hr increased significantly; the zinc content with 48-hr or 96-hr culture was 401 ± 14 or 652 ± 10 μg/g wet bone (mean ± SE of five calvaria) respectively. Those zinc contents were not altered significantly by the presence of 10^{-7} M $1,25(OH)_2D_3$ (data not shown).

DISCUSSION

In vivo, vitamin D_3 metabolites are important for the promotion of bone formation and mineralization [4–6]. In contrast to the widely accepted *in vivo* effects of vitamin D_3 on bone formation, there is no conclusive *in vitro* evidence that vitamin D_3 stimulates bone formation directly [7]. The direct effects of $1,25(OH)_2D_3$ on bone alkaline phosphatase activity are controversial; they suggest that vitamin D_3 metabolites have duel stimulatory and inhibitory effects [25, 26]. Interestingly, $1,25(OH)_2D_3$ has been shown to increase the activity of phosphotyrosine phosphatase, which plays a role in the stimulation of the cell growth of isolated bone cells [27]. Also, $1,25(OH)_2D_3$ may be directly involved in the maturation of embryonic chicken chondrocytes and possibly in the calcification of growth cartilage [28]. Whether it is directly involved in the mineralization

Table 3. Effect of cycloheximide on $1,25(OH)_2D_3$ and zinc-increased alkaline phosphatase activity in rat calvaria *in vitro*

Treatment	Alkaline phosphatase (μmol/min/mg protein)
Control	1.004 ± 0.077
$1,25(OH)_2D_3$ (10^{-7} M)	
None	1.231 ± 0.043*
Zinc (10^{-4} M)	1.637 ± 0.078†
$1,25(OH)_2D_3$ (10^{-7} M) + cycloheximide (10^{-7} M)	
None	0.996 ± 0.069
Zinc (10^{-4} M)	1.121 ± 0.085

Each value is the mean ± SE of five calvaria. The bones were cultured for 48 hr in medium containing the steroid and drugs.

*$P < 0.05$, compared with the control value.

†$P < 0.01$, compared with the 10^{-7} M $1,25(OH)_2D_3$ value.

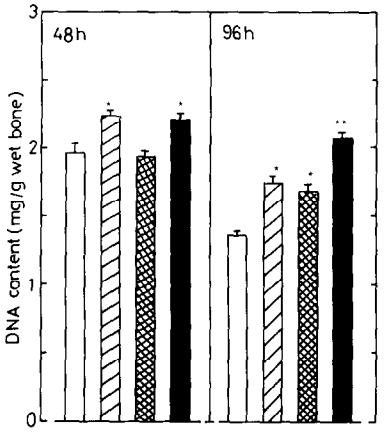

Fig. 6. Effect of zinc on $1,25(OH)_2D_3$-increased DNA content in rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing: vehicle (□); 10^{-7} M $1,25(OH)_2D_3$ (▨); 10^{-4} M zinc (▩); or 10^{-7} M $1,25(OH)_2D_3$plus 10^{-4} M zinc (■). Each bar is the mean of five calvaria per group. The vertical lines represent the SE. Key: (*) $P < 0.01$, compared with the control (vehicle) value; and (**) $P < 0.01$, compared with the 10^{-7} M $1,25(OH)_2D_3$ or 10^{-4} M zinc alone.

and formation of bone, however, is still a matter of controversy.

In the present study, we examined the effects of $1,25(OH)_2D_3$ on bone metabolism in a tissue culture system using the calvaria obtained from weanling rats (3 weeks old), although most prior calvaria work has used neonatal rats. It was found that $1,25(OH)_2D_3$ had direct anabolic effects on bone metabolism in tissue culture *in vitro*; the calcium content, alkaline phosphatase activity and DNA content increased significantly in rat calvaria cultured for 96 hr in serum-free medium containing the steroid. When the bone tissue was used, it is not clear whether the results were due to one or more of the cells (preosteoblasts, osteoblasts, osteocytes, and/or oseoclasts). $1,25(OH)_2D_3$ did not cause a significant alteration in the activity of bone acid phosphatases, which are lysosomal enzymes in bone cells. This may indicate that the steroid does not influence the function of osteoclasts in the bone, since activation of osteoclasts increases the activities of lysosomal enzymes [29]. A more recent study has provided immunocytological evidence for the localization of endogenous $1,25(OH)_2D_3$ and its receptors in osteoblasts and osteocytes from rat calvaria [11]. The present direct anabolic effects of $1,25(OH)_2D_3$ on bone tissue *in vitro* may be due to the action of the steroid on the osteoblasts and/or osteocytes.

The incorporation of [^{3}H]leucine into the acid-insoluble residues of rat calvaria cultured for 96 hr in serum-free medium containing 10^{-8} to 10^{-6} M $1,25(OH)_2D_3$ increased significantly, suggesting that the steroid can stimulate total protein synthesis in the bone cells. $1,25(OH)_2D_3$ may be able to increase the collagen content in rat calvaria. It has been reported that $1,25(OH)_2D_3$ has a stimulatory effect on collagen and noncollagen synthesis in early cultures of osteoblasts (MC 3T3-El cells) [13]. Furthermore, $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) significantly increased DNA content in rat calvaria cultured for 96 hr but not for 48 hr in serum-free medium, suggesting that the steroid can stimulate cell proliferation and DNA synthesis. Since alkaline phosphatase activity in rat calvaria increased significantly during 48- and 96-hr cultures with $1,25(OH)_2D_3$ (10^{-9} M), the steroid may have the stimulatory effect on bone alkaline phosphatase activity independently of DNA synthesis. The effect of a high concentration (10^{-7} M) of $1,25(OH)_2D_3$ to elevate bone alkaline phosphatase activity was prevented completely by the presence of cycloheximide, indicating that the steroid effect is based on protein synthesis. In addition, the steroid (10^{-9} to 10^{-7} M) produced a corresponding elevation of bone calcium content during cultures of 48 and 96 hr. Thus, $1,25(OH)_2D_3$ could reveal a direct stimulatory effect on the bone mineralization and formation of rat calvaria *in vitro* in serum-free medium. More recently, it has been reported that $1,25(OH)_2D_3$ increases epidermal growth factor receptors and transforming growth factor β-like activity in a cell line derived from fetal rat calvaria [30]. Since growth factors are localized in bone matrix, the action of $1,25(OH)_2D_3$ to stimulate bone formation may require action of growth factors.

Table 4. Effect of cycloheximide on $1,25(OH)_2D_3$ and zinc-increased DNA content in rat calvaria *in vitro*

Treatment	DNA content (mg/g wet bone)
Control	1.337 ± 0.034
$1,25(OH)_2D_3$ (10^{-7} M)	
None	1.684 ± 0.084*
Zinc (10^{-4} M)	2.068 ± 0.072†
$1,25(OH)_2D_3$ (10^{-7} M) + cycloheximide (10^{-7} M)	
None	1.728 ± 0.078
Zinc (10^{-4} M)	1.847 ± 0.026

Each value is the mean ± SE of five calvaria. The bones were cultured for 96 hr in medium containing the steroid and drugs.

* $P < 0.01$, compared with the control value.

† $P < 0.01$, compared with the 10^{-7} M $1,25(OH)_2D_3$ value.

It has been reported that zinc, an essential metal, has a direct stimulatory effect on bone formation in rat calvaria *in vitro* [19], and that the metal has the same effect *in vivo* [16]. Zinc enhances markedly $1,25(OH)_2D_3$-increased alkaline phosphatase activity and DNA content in bone *in vivo* [17, 18]. Whether zinc can enhance $1,25(OH)_2D_3$ effects on bone metabolism *in vitro* is uncertain. To clarify this, we used 10^{-7} M $1,25(OH)_2D_3$ which had a maximum effect on the elevation of protein synthesis and DNA content. The concentration of zinc used in the experiment was 10^{-4} M, which has a maximum effect in stimulating bone formation *in vitro* [19]. This concentration corresponds to the physiological level of zinc in rat serum (1.0 to 1.2 × 10^{-4} M). The present study demonstrates clearly that zinc can enhance the effect of $1,25(OH)_2D_3$ (10^{-7} M) in elevating alkaline phosphatase activity and DNA content in rat calvaria cultured in serum-free medium. The additive effect of zinc with $1,25(OH)_2D_3$ on bone alkaline phosphatase activity was seen clearly with cultures of 48 hr. At 96 hr of culture, the combined effect was not observed, because the effect of zinc alone was remarkable and the $1,25(OH)_2D_3$ effect was slight. Meanwhile, the additive effect of zinc with $1,25(OH)_2D_3$ on bone DNA content was not seen in the 48-hr culture, whereas this effect was clear with 96-hr of culture. This may be related to the effect of zinc which did not cause a significant increase in bone DNA content at 48 hr of culture. From these results, it is assumed that the modes of action of zinc and $1,25(OH)_2D_3$ are different.

The additive effect of zinc with $1,25(OH)_2D_3$ to increase bone alkaline phosphatase activity and DNA content was prevented completely by the presence of cycloheximide. This suggests that the bone response for zinc with $1,25(OH)_2D_3$ required protein synthesis, since the metal can increase bone protein synthesis in the translational process [16, 19]. Zinc may promote the steroid effect on bone metabolism due to stimulation of protein synthesis. Presumably, zinc increases bone protein synthesis, and the increased protein would stimulate bone cell proliferation and induce the elevation of bone DNA content. More recently, the gene sequence for the receptor for $1,25(OH)_2D_3$ was determined and shown to have two zinc fingers at the site of interaction with DNA [31]. We cannot exclude the possibility, therefore, that one possible mechanism of zinc is to potentiate the interaction of the $1,25(OH)_2D_3$–receptor complex with DNA at that site.

In conclusion, in tissue culture of calvaria from weanling rats, $1,25(OH)_2D_3$ stimulated bone formation and mineralization, and the steroid effect on bone metabolism was enhanced by zinc, which can stimulate bone formation. The present investigation further supports the view that $1,25(OH)_2D_3$ has direct stimulatory effects on bone formation and mineralization *in vivo*. Zinc may regulate the steroid effect on bone formation. This suggests a physiologic role for zinc in the stimulation of bone formation.

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