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STIMULATORY EFFECT OF ZINC ON BONE FORMATION IN TISSUE CULTURE

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Abstract—The present investigation was undertaken to clarify the *in vitro* effect of 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^{-7} to 10^{-3} M zinc sulfate. All cultures were incubated at 37° in 5% CO_2/95% air. Zinc uptake by bone was increased significantly in cultures with concentrations of zinc greater than 10^{-6} M. Bone calcium content was increased significantly by the presence of 10^{-4} M zinc. This increase was blocked by the presence of 10^{-6} M cycloheximide. Bone alkaline phosphatase activity was elevated in the presence of zinc (10^{-6} to 10^{-3} M), but the effect was inhibited by 10^{-7} M cycloheximide or 10^{-8} M actinomycin D. Zinc (10^{-4} M) also significantly increased ATPase activity in the bone, whereas it did not alter significantly by pyrophosphatase, acid phosphatase and β-*N*-acetylglucosaminidase activities. Furthermore, bone collagen content was raised by 10^{-6} to 10^{-4} M zinc. This elevation was prevented by 10^{-7} cycloheximide or 10^{-8} M actinomycin D. Bone DNA content and [^{3}H]thymidine incorporation by the bone were not altered significantly by 10^{-4} M zinc. These findings indicate that the zinc had a direct stimulatory effect on bone mineralization *in vitro*, and that bone protein synthesis was a necessary component of this response. Zinc may stimulate bone formation in tissue culture.

Zinc has been demonstrated to have a wide variety of roles in the mammalian system, and this metal is essential for growth in humans and many animals [1]. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [2, 3]. Recently, it was reported that a comparatively low dose of zinc has a stimulatory effect on bone growth and mineralization in weanling rats [4, 5]. Of the many essential trace metals, zinc can effectively stimulate bone growth and mineralization, and zinc has been suggested to have a nutritional significance [6]. Additionally, it has been found that zinc synergistically enhances bone metabolism stimulated by 1,25-dihydroxyvitamin D_3, a calcium-regulating hormone [7, 8]. These investigations suggest a physiologic role for zinc in the regulation of bone metabolism in weanling rats. The action of zinc on the cellular metabolism of bone *in vitro*, however, has not been clarified thus far. The present study was undertaken, therefore, to investigate the mechanism of zinc action on bone metabolism in tissue culture using the calvaria from weanling rats.

MATERIALS AND METHODS

Chemicals. Dulbecco's Modified Eagle Medium (high glucose) was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Penicillin–streptomycin solution (5000 units/ml penicillin; 5000 μg/ml streptomycin) was obtained from Gibco Laboratories. Bovine serum albumin (Fraction V), cycloheximide, and actinomycin D were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]Thymidine (79.9 μCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). 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 Nippon Bio Supply Center Co., Tokyo. 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 sagittal 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 medium consisting of Dulbecco's Modified Eagle Medium (high glucose) supplemented with 0.25% bovine serum albumin (Fraction V) plus antibiotics, with either zinc (10^{-7} to 10^{-3} M) or vehicle (distilled water). 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 zinc or vehicle, were changed at 48 hr, and cultures were maintained for an additional 48 hr. In the separate experiments, the respective media contained cycloheximide or actinomycin D.

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Analytical procedures. Calvaria were cultured in the medium containing zinc 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.

Zinc content in the bone tissues was determined by atomic absorption spectrophotometry after digestion with nitric acid. Bone zinc content was expressed as μg of zinc per g wet bone tissue.

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

Alkaline phosphatase activity in the bone tissues was determined by the method of Walter and Schutt [9]. 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 μmol of *p*-nitrophenol liberated per min per mg protein. Acid phosphatase [9], β-*N*-acetylglucosaminidase [10], ATPase and pyrophosphatase [11] activities were measured by methods described in the respective references. Protein was determined by the method of Lowry *et al.* [12].

The separation of collagen from the bone tissues was done by the method of Flanagan and Nichols [13]. The bone tissues were shaken with 2.0 ml of 0.1 N NaOH solution for 16 hr at 4°. The bone tissues, remaining after alkaline extraction, were further extracted at 4° with continuous shaking in three washes of 10% sodium EDTA solution (pH 7.5) for a total of 48 hr to remove the mineral components. After demineralization, the tissues were washed in water and acetone, and finally reextracted with a mixture of ethanol–ether (1:1) for 24 hr. Following removal of these solvents, the sediments were evaporated to dryness at 105°, and 5.0 ml of 6 N HCl solution was added to the dried materials. Hydrolyzed samples were evaporated to dryness and taken up in a suitable volume of distilled water. Hydroxyproline was determined by the method of Kivirikko *et al.* [14]. Collagen content was expressed as the amount of hydroxyproline (nmol) per g of wet bone tissue.

To measure DNA content, the bone tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr [13]. After alkali extraction, samples were centrifuged at 10,000 *g* for 5 min, and the supernatant fraction was collected. DNA content in the supernatant was determined by the method of Ceriotti [15] and expressed as the amount of DNA (mg) per g of wet bone tissue.

DNA synthesis was studied by examining the effect on [^{3}H]thymidine incorporation into the bone tissue. Calvaria were cultured for 48 and 96 hr, and [*methyl*-^{3}H]thymidine (5.0 μCi/ml of medium) was added during the last 60 min of the culture period [16]. At the end of the incubation, the bones were extracted with ice-cold 5% trichloroacetic acid, acetone, and ether; the acid-insoluble residues were then dried and weighed. The radioactivity in the acid-insoluble residues was determined after digesting them with 0.2 N NaOH solution. Data are expressed as dpm per mg dry weight of the acid-insoluble residues.

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

RESUULTS

Uptake of zinc by bone. The uptake of zinc by rat calvaria cultured in the presence of zinc for 48 and 96 hr is shown in Fig. 1. Increasing the concentrations of zinc, in the range of 10^{-7} to 10^{-3} M, progressively increased zinc uptake by the bone, a significant increase in zinc uptake was seen at concentrations greater than 10^{-6} M zinc. When calvaria were cultured in the presence of 10^{-4} M zinc for 48 hr, the bone zinc uptake was remarkable; this uptake was further enhanced at 96 hr of culture. In the presence of 10^{-3} M zinc, bone zinc uptake was extremely elevated (data not shown). After culture for 48 hr in the presence of 10^{-4} M zinc in the medium, the bone was transferred to medium without zinc and cultured for 48 hr. The changing medium, the bone released only 15% of the accumulated zinc ($P < 0.001$). Using 10^{-5} M zinc, no release of zinc from the bone was observed after changing the medium (data not shown); the culture medium used did not contain zinc.

Effect of zinc on calcium content in bone. The alteration of calcium content in rat calvaria cultured for 48 and 96 hr is shown in Table 1. Calcium content in bone cultured for 96 hr in control medium without zinc was increased markedly. The presence of 10^{-6} and 10^{-5} M zinc significantly increased the calcium

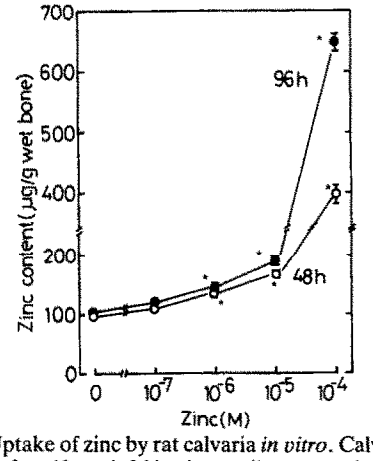

Fig. 1. Uptake of zinc by rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing either vehicle or 10^{-7} to 10^{-4} M zinc. Each point is the mean of five calvaria per group. The vertical lines represent the SEM. The bone zinc content at 0 hr of culture was 103 ± 7 μg/g wet bone. Key: (*) $P < 0.01$, compared with control group; (○) 48-hr culture; and (●) 96-hr culture.

Table 1. Effect of zinc on calcium content in rat calvaria *in vitro*

Treatment	Calcium content (mg/g bone ash)		
	0 hr	48 hr	96 hr
Control	380.3 ± 8.7	382.4 ± 5.7	413.4 ± 3.2
10^{-6} M Zinc		381.7 ± 6.2	437.3 ± 4.8*
10^{-5} M Zinc		396.0 ± 6.6	442.1 ± 3.4*
10^{-4} M Zinc		419.9 ± 7.5*	416.1 ± 1.6

Each value is the mean ± SEM of five calvaria. Calvaria were cultured in the presence of 10^{-6} to 10^{-4} M zinc for 48 and 96 hr.
* $P < 0.01$, compared with control group.

content of the bone cultured for 96 hr, but not for 48 hr. After culture for 48 hr in 10^{-4} M zinc, bone calcium content was increased significantly. This increase, however, was not observed after 96 hr of culture. When the bone was cultured for 48 hr in the presence of 10^{-4} M zinc and/or 10^{-6} M cycloheximide, no increase in bone calcium content occurred (Fig. 2).

Effect of zinc on enzyme activity in bone. The changes in alkaline phosphatase activity in rat calvaria cultured in the presence of increasing concentrations of zinc (10^{-7} to 10^{-3} M) are shown in Fig. 3. When the bone was cultured for 48 hr in medium without zinc, the basal activity of alkaline phosphatase did not increase significantly in comparison with the value at 0 hr of culture. This basal activity, however, had decreased by 96 hr of culture in comparison with the value obtained when the bone was cultured for 48 hr. Alkaline phosphatase activity in bone cultured in the presence of 10^{-6} M zinc for 96 hr increased significantly; the presence of 10^{-7} M zinc had no effect. With higher concentrations of zinc, the effect was greater; at 10^{-4} M zinc the effect was maximal. When the bone was cultured for 48 hr in the presence of 10^{-4} M zinc and/or 10^{-7} M cycloheximide or 10^{-8} M actinomycin D, the zinc increase in bone alkaline phosphatase activity did not occur (Fig. 4).

The effect of the addition of zinc on alkaline phosphatase activity in enzyme extracts obtained from uncultured rat calvaria was examined; the result is shown in Fig. 5. Alkaline phosphatase activity was not altered in the presence of 10^{-7} or 10^{-6} M zinc. At zinc concentrations greater than 10^{-5} M, the enzyme activity clearly decreased; zinc had an inhibitory effect on the enzyme.

The changes in ATPase, pyrophosphatase, acid phosphatase and β-*N*-acetylglucosaminidase activities in rat calvaria cultured in the presence of 10^{-4} M zinc for 48 hr are shown in Table 2. Of these enzymes, only ATPase activity was enhanced significantly by the zinc.

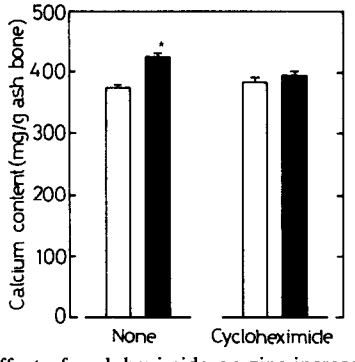

Fig. 2. Effect of cycloheximide on zinc-increased calcium content in rat calvaria *in vitro*. Calvaria were cultured for 48 hr in medium containing: vehicle alone; 10^{-4} M zinc; 10^{-6} M cycloheximide; or 10^{-4} M zinc plus 10^{-6} M cycloheximide; or 10^{-4} M zinc plus 10^{-6} M cycloheximide. Each bar is the mean of five calvaria per group. The vertical lines represent the SEM Key: (*) $P < 0.01$, compared with control group; (□) none; and (■) zinc.

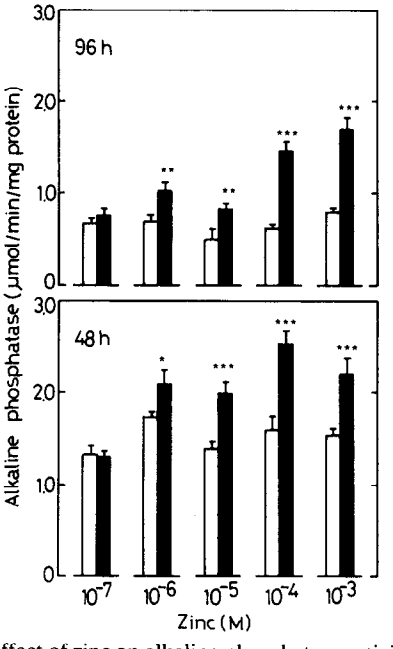

Fig. 3. Effect of zinc on alkaline phosphatase activity in rat calvaria *in vitro*. Calvaria were cultured for 48 and 96 hr in medium containing either vehicle or 10^{-7} to 10^{-3} M zinc. Each bar is the mean of five calvaria per group. The vertical lines represent the SEM. The bone enzyme activity at 0 hr of culture as 1.209 ± 0.005 μmol/min/mg protein). Key: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$, compared with control group; (□) control; and (■) zinc.

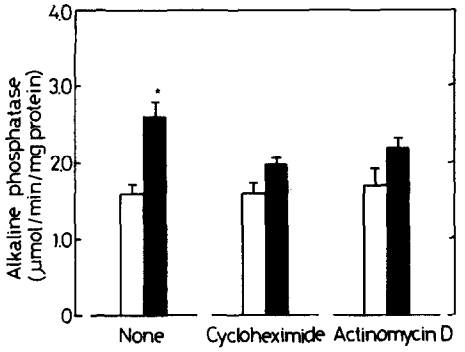

Fig. 4. Effect of cycloheximide or actinomycin D on zinc-increased alkaline phosphatase activity in rat calvaria *in vitro*. Calvaria were cultured for 48 hr in medium containing: vehicle alone; 10^{-4} M zinc; 10^{-7} M cycloheximide; 10^{-4} M zinc plus 10^{-7} M cycloheximide; 10^{-8} M actinomycin D; or 10^{-4} M zinc plus 10^{-8} M actinomycin D. Each bar is the mean of five calvaria. The verticle line represent the SEM. Key: (*) $P < 0.01$, compared with control group; (□) control; and (■) zinc.

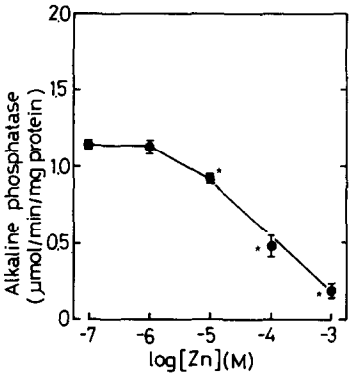

Fig. 5. Effect of zinc addition on alkaline phosphatase activity of enzyme extracts from rat calvaria. Zn^{2+} was added to the incubation mixture to final concentrations of 10^{-7} to 10^{-3} M. The calvaria tissue homogenate was prepared from normal rat. The enzyme control value was 1.209 ± 0.050 μmol/min/mg protein). Each point is the mean of five calvaria. Vertical lines represent the SEM. Key: (*) $P < 0.001$, compared with no addition values.

Effect of zinc on collagen content in bone. The alteration of collagen content in rat calvaria cultured in the presence of zinc for 48 and 96 hr is shown in Table 3. Collagen content in the bone was increased significantly by 48 hr of culture with 10^{-4} M zinc but not with 10^{-6} or 10^{-5} M. With culture for 96 hr in the presence of 10^{-6} to 10^{-4} M zinc, bone collagen content increased significantly. With greater zinc concentrations, however, the effect of zinc weakened. When rat calvaria were cultured in the presence of 10^{-4} M zinc and 10^{-7} M cycloheximide or 10^{-8} M actinomycin D for 48 hr, the increase of bone collagen content did not occur (Fig. 6).

Effect of zinc on DNA synthesis in bone. The effects of zinc on DNA content and [^{3}H]thymidine incorporation by rat calvaria cultured for 48 and 96 hr are shown in Table 4. At 48 and 96 hr of culture, DNA content in the bone was not altered significantly by the presence of 10^{-4} M zinc. Nor was the incorporation of [^{3}H]thymidine into bone cultured for 48 and 96 hr increased significantly by the presence of 10^{-4} M zinc. Thus, zinc did not stimulate DNA synthesis in bone culture.

DISCUSSION

It has been found recently that oral administration of zinc, an essential trace metal, stimulates bone growth and mineralization in weanling rats [4–8]. Zinc may play a physiological role as an activator in bone metabolism. The purpose of the present study was to clarify the cellular action of zinc on bone metabolism in tissue culture. The presence of zinc in culture medium produced appreciable increases in bone calcium content, alkaline phosphatase activity and collagen content, which are biochemical parameters of bone mineralization and formation. The present study suggests that zinc has a stimulatory effect on bone mineralization and formation in tissue culture of rat calvaria, and that bone protein synthesis is a necessary component of this response.

Although rat calvaria contain an appreciable amount of zinc, the presence of zinc concentration of more than 10^{-6} M in culture medium accelerated the accumulative of zinc by bone tissue. Only a small amount of the zinc taken up by the bone tissue was released during culture. At this time, we do not know

Table 2. Effect of zinc on the activities of various enzymes in rat calvaria *in vitro*

Enzyme	Enzyme activity (nmol/min/mg protein)	
	Control	Zinc
Alkaline phosphatase	1632 ± 142	2594 ± 196*
ATPase	533 ± 87	998 ± 179†
Pyrophosphatase	658 ± 66	768 ± 58
Acid phosphatase	51 ± 3.6	53 ± 2.4
β-*N*-Acetylglucosaminidase	51 ± 4.7	56 ± 2.3

Each value is the mean ± SEM of five calvaria. Calvaria were cultured in the presence of 10^{-4} M zinc for 48 hr.
* $P < 0.01$, compared with control group.
† $P < 0.05$, compared with control group.

Table 3. Effect of zinc on collagen content in rat calvaria *in vitro*

Treatment	Collagen content (nmol/g wet bone) 0 hr	48 hr	96 hr
Control	426.7 ± 21	456 ± 15	372 ± 3.4
10^{-6} M Zinc		495 ± 24	501 ± 21*
10^{-5} M Zinc		493 ± 17	459 ± 30†
10^{-4} M Zinc		522 ± 20†	455 ± 18‡

Each value is the mean ± SEM of five calvaria. Calvaria were cultured in the presence of 10^{-6} to 10^{-4} M zinc for 48 and 96 hr.

* $P < 0.001$, compared with control group.

† $P < 0.05$, compared with control group.

‡ $P < 0.01$, compared with control group.

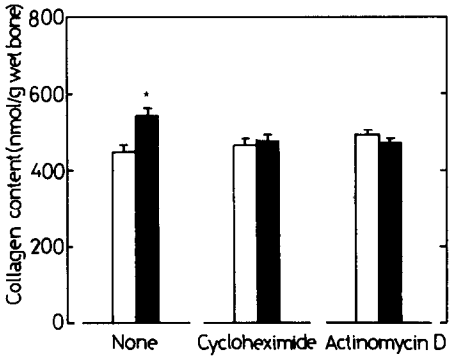

Fig. 6. Effect of cycloheximide or actinomycin D on zinc-increased collagen content in rat calvaria *in vitro*. Calvaria were cultured for 48 hr in medium containing: vehicle alone; 10^{-4} M zinc; 10^{-7} M cycloheximide; 10^{-4} M zinc plus 10^{-7} M cycloheximide; 10^{-8} M actinomycin D; or 10^{-4} M zinc plus 10^{-8} M actinomycin D. Each bar is the mean of five calvaria. Vertical lines represent the SEM. Key: (*) $P < 0.05$, compared with control group.

which cell type took up the zinc. The uptake of zinc by the bone did not cause significant changes in the activities of acid phosphatase and β-*N*-acetylglucosaminidase, which are lysosomal enzymes in bone cells. This may indicate that zinc does not influence the function of osteoclasts in bone, since activation of osteoclasts increases lysosomal enzyme activities [16]. Presumably, therefore, zinc may be localized largely to the osteoblasts in bone.

Bone calcium content was increased at 96 hr of culture in the presence of 10^{-6} and 10^{-5} M zinc, which caused significant elevations of alkaline phosphatase activity and collagen content in the bone tissue. Zinc at 10^{-4} M did not produce an appreciable increase in bone calcium at 96 hr of culture, although alkaline phosphatase activity and collagen content were raised significantly. At 48 hr of culture, however, 10^{-4} M zinc had an effect on bone calcium. Zinc content in the bone was extremely elevated by 96 hr of culture. This suggests that bone calcium is reduced as zinc accumulates in bone. In fact, the calcium in bone mineral is exchanged for zinc [17]. The increase of bone calcium content at 48 hr of culture in the presence of 10^{-4} M zinc was clearly blocked by 10^{-6} M cycloheximide, an inhibitor of protein synthesis. In this case, the protein inhibitor prevented the increases of both alkaline phosphatase activity and collagen content in the bone by zinc.

Of the bone enzymes examined, the activities of alkaline phosphatase and ATPase were increased markedly by culture with 10^{-4} M zinc, whereas pyrophosphatase, acid phosphatase and β-*N*-acetylglucosaminidase activities were not altered significantly. Alkaline phosphatase and ATPase play physiological roles in bone mineralization [18]; alkaline phosphatase is a zinc-enzyme [19]. The direct addition of zinc ion to the enzyme reaction mixture, using the enzyme solution extracted from control calvaria, did not cause a significant increase in alkaline phosphatase activity. Zinc-increased alkaline phosphatase activity in bone culture, however, was reduced by the presence of cycloheximide or actinomycin D. These results suggest that zinc induced alkaline phosphatase in the bone culture.

Collagen is the main protein in bone matrix. The collagen content of the bone tissue was increased significantly at 48 hr of culture in the presence of 10^{-4} M zinc. With lower concentrations of zinc (10^{-6} and 10^{-5} M), bone collagen content was increased significantly at 96 hr of culture. Thus, zinc at physiological levels in bone cells may stimulate the synthesis of collagen. Zinc (10^{-4} M)-increased collagen content was reduced by cycloheximide or actinomycin D in bone culture. From these results, it is assumed that zinc stimulates collagen synthesis in bone cells.

DNA content in the bone was not altered sig-

Table 4. Effect of zinc on DNA synthesis in rat calvaria *in vitro*

Treatment	DNA content (mg/g wet bone) 48 hr	96 hr	[^{3}H]Thymidine incorporation (dpm/mg dry acid-insoluble residues) 48 hr	96 hr
Control	2.795 ± 0.183	2.569 ± 0.153	103.8 ± 11.2	106.2 ± 13.4
10^{-4} M Zinc	2.675 ± 0.185	2.752 ± 0.097	114.2 ± 12.6	142.6 ± 26.6

Each value is the mean ± SEM of six calvaria. Calvaria were cultured in the presence of 10^{-4} M zinc for 48 and 96 hr and pulsed with [^{3}H]thymidine (5.0 μCi/ml of medium) 60 min before removing the bone. Data were not significant.

nificantly at 48 and 96 hr of culture in the presence of 10^{-4} M zinc, which increased alkaline phosphatase activity and collagen content in the bone. Additionally, the incorporation of [^{3}H]thymidine into bone cells was not enhanced by the presence of 10^{-4} M zinc. These results indicate that zinc did not stimulate DNA synthesis of bone cells in tissue culture. It is likely that the increases of alkaline phosphatase activity and collagen content in the bone by zinc are not involved in bone cell proliferation. It should be noted that zinc administration *in vivo* causes a significant increase of DNA content in the bone of weanling rats [5, 8]. The increase of bone DNA content by zinc administration *in vivo* may be related to other factors (hormones), since the metal can synergistically enhance the stimulatory effect of 1,25-dihydroxyvitamin D_3, a calcium-regulating hormone, on bone DNA content [7, 8]. The interaction of this steroid and zinc on bone DNA content remains to be elucidated in bone tissue culture.

In conclusion, zinc had a direct stimulatory effect on bone mineralization *in vitro*; bone protein synthesis was a necessary component of this response. The present investigation supports the view that zinc, an essential trace metal, can stimulate bone formation. Zinc plays a physiologically important role as an activator in bone metabolism.

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