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EFFECT OF DIPICOLINATE, A CHELATOR OF ZINC, ON BONE PROTEIN SYNTHESIS IN TISSUE CULTURE

THE ESSENTIAL ROLE OF ZINC

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Abstract—The present investigation was undertaken to clarify the essential role of zinc on bone protein synthesis in tissue culture. Calvariae were removed from 3-week-old male rats and cultured for periods up to 72 hr in Dulbecco's Modified Eagle Medium (high glucose, 4500 mg/dl) supplemented with antibiotics and bovine serum albumin. The calvariae were incubated for 24 hr at 37° in 5% CO_2/95% air in medium containing 10^{-6}–10^{-3} M dipicolinate, a chelator of zinc, and then the bones were transferred into medium containing either 10^{-4} M zinc sulfate or vehicle without dipicolinate. Zinc content in bone tissues was decreased when the culture was treated with 10^{-4} and 10^{-3} M dipicolinate for 24 hr. When calvariae treated with 10^{-4} M dipicolinate for 24 hr were further cultured in medium without dipicolinate for 24 and 48 hr, bone alkaline phosphatase activity was decreased by about 40% ($P < 0.01$) of untreated bone enzyme activity. The decreased alkaline phosphatase activity was increased markedly by the presence of 10^{-4} M zinc (about 2.5-fold of control value). This effect of zinc was blocked completely by the presence of 10^{-7} M cycloheximide, but 10^{-8} M actinomycin D caused only a partial inhibition. When calvariae treated with 10^{-4} M dipicolinate were pulsed with [^{3}H]proline, the incorporation of [^{3}H]proline into the acid-insoluble residues of bone tissue was decreased by about 40% ($P < 0.01$) of the value obtained from calvariae not treated with dipicolinate. The presence of 10^{-4} M zinc caused an increase of about 2-fold in [^{3}H]proline incorporation. Bone DNA content was not altered significantly by treatment with 10^{-4} M dipicolinate or 10^{-4} M zinc. These results clearly indicate that endogenous zinc induces the stimulation of protein synthesis at the translational process in bone cells. The present study further supports the view that zinc plays an essential role for protein synthesis in bone cells.

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 has been reported that a comparatively low dose of zinc can stimulate bone growth and bone mineralization in weanling rats [4, 5], and that zinc has a stimulatory effect on bone formation in tissue culture using the calvariae from weanling rats [6]. More recently, it has been demonstrated that the cellular mechanism of zinc action on bone formation is to stimulate bone protein synthesis; zinc can activate aminoacyl-tRNA synthetase in bone cells [7]. Although these investigations suggest a physiologic role for zinc in the regulation of bone formation in weanling rats, it has not been clarified whether endogenous zinc in bone cells can stimulate protein synthesis. The present study was undertaken, therefore, to clarify the role of endogenous zinc on bone protein synthesis in tissue culture of calvaria from weanling rats. We used dipicolinate, a chelator of zinc, to deplete zinc in bone cells by tissue culture. It was found that endogenous zinc plays an essential role in stimulating bone protein synthesis.

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MATERIALS AND METHODS

Chemicals. Dulbecco's Modified Eagle Medium (high glucose) and a penicillin–streptomycin solution (5000 units/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Bovine serum albumin (Fraction V), cycloheximide and actinomycin D were obtained from the Sigma Chemical Co. (St Louis, MO). Dipicolinate (2,6-pyridinedicarboxylic acid; neutralized with sodium hydroxide), zinc sulfate and all other chemicals were reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-[2,3-^{3}H]Proline (34.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All water used was glass distilled.

Animals. Weanling male Wistar rats weighing 60–65 g (3-week-old) were obtained from the Japan SLC, Inc. (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid), containing 1.1% calcium, 1.1% phosphorus and 0.012% zinc, and were given distilled water freely until used.

Bone culture. Calvariae from 3-week-old male rats were removed aseptically and cut along the sagittal suture into left and right halves [6]. One-half of each calvaria served as a control for its paired, treated half. Each half-calvaria (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

dipicolinate (10^{-6}–10^{-3} M) or vehicle which did not contain any zinc. Cultures were maintained at 37° in a water-saturated atmosphere containing 5% CO_2 and 95% air for 72 hr. The respective media, containing either zinc sulfate or vehicle, were changed at 24 hr, and cultures were maintained for an additional 48 hr. In separate experiments, the respective media contained cycloheximide or actinomycin D.

Bone zinc content. Calvariae were cultured in the medium containing dipicolinate (10^{-5}–10^{-3} M) for 24 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 tissue was determined by atomic absorption spectrophotometry after digestion with nitric acid [6]. Bone zinc content was expressed as micrograms of zinc per gram of wet bone tissue. Zinc was not present in the control medium.

Bone alkaline phosphatase. Alkaline phosphatase activity in the bone tissues was determined by the method of Walter and Schutt [8]. 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 [6]. 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. Protein concentration was determined by the method of Lowry *et al.* [9]. Enzyme activity was expressed as micromoles of *p*-nitrophenol liberated per minute per milligram of protein.

Bone DNA content. The bone tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr at 4° [10]. 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 [11] and expressed as the amount of DNA (mg) per gram of wet bone tissue.

Bone protein synthesis. Effects on newly synthesized bone total protein were determined by measuring the incorporation for 2 hr of [^{3}H]proline (2.5 μCi/ml of medium) [12] added to culture for the periods indicated in the legends to the figures. At the end of the culture, the calvariae were removed and washed with ice-cold 0.25 M sucrose. The calvariae were extracted with ice-cold 10% 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]proline 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 minute per milligram dry weight of acid-insoluble residues.

Statistical analyses. 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.

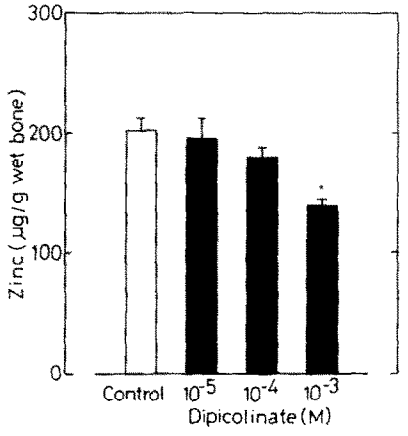

Fig. 1. Effect of dipicolinate, a chelator of zinc, on zinc content in rat calvariae *in vitro*. Calvariae were cultured for 24 hr in medium containing 10^{-5}–10^{-3} M dipicolinate. Each bar represents the mean of five calvariae. The vertical lines represent the SE. Key: (*) $P < 0.01$, compared to the control group.

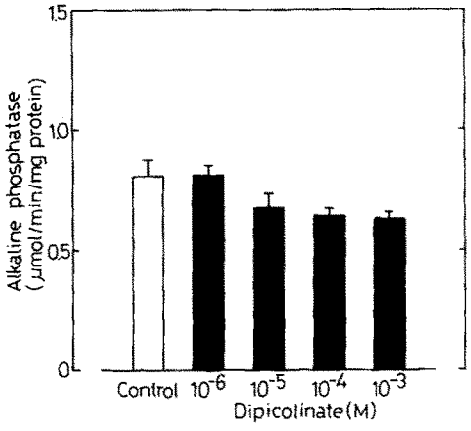

Fig. 2. Effect of dipicolinate, a chelator of zinc, on alkaline phosphatase activity in rat calvariae *in vitro*. Calvariae were cultured for 24 hr in medium containing 10^{-6}–10^{-3} M dipicolinate. Each bar represents the mean of five calvariae. The vertical lines represent the SE. Data were not significant.

RESULTS

Effect of dipicolinate on bone zinc content. The alteration of zinc content in rat calvariae cultured in medium containing dipicolinate (10^{-5}–10^{-3} M) for 24 hr is shown in Fig. 1. Zinc content in the bone tissue was reduced by increasing concentrations of dipicolinate in culture medium. At 10^{-3} M dipicolinate, bone zinc content was decreased markedly. There was no zinc in the control medium. Also, calvariae did not release zinc into the medium with 48-hr culture. Thus, dipicolinate, a chelator of zinc, could cause the release of zinc from the bone tissues.

Effect of dipicolinate on bone alkaline phosphatase activity. The alteration of alkaline phosphatase activity in rat calvariae cultured in medium containing dipicolinate (10^{-6}–10^{-3} M) for 24 hr is shown in Fig. 2. The presence of dipicolinate (10^{-5}–10^{-3} M) caused a slight decrease of bone alkaline phosphatase activity.

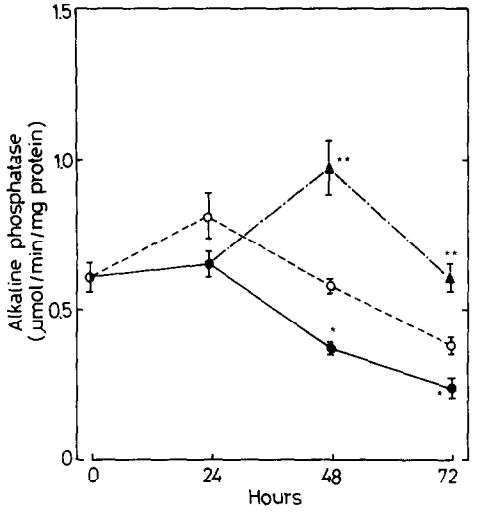

Fig. 3. Alteration of alkaline phosphatase activity in rat calvariae pre-cultured in the presence of dipicolinate *in vitro*. Calvariae were pre-cultured for 24 hr in medium containing either vehicle or 10^{-4} M dipicolinate and then transferred into medium without dipicolinate. The bones were cultured for an additional 24 and 48 hr in medium containing either vehicle or 10^{-4} M zinc sulfate. Each point is the mean of five calvariae. The vertical lines represent the SE. Key: (*) $P < 0.01$, compared to calvariae not treated with dipicolinate, and (**) $P < 0.01$, compared to calvariae in medium to which zinc was not added: (○) not treated with dipicolinate; (●) pretreated with dipicolinate; and (▲) zinc addition after pretreatment with dipicolinate.

When calvariae were cultured in medium containing 10^{-4} M dipicolinate for 24 hr, the bones were transferred into medium not containing dipicolinate and cultured for an additional 48 hr. Alkaline phosphatase activity in calvariae was decreased significantly ($P < 0.01$) by pre-culture with 10^{-4} M dipicolinate in comparison with that of control, in which the bones were pre-cultured in the absence of dipicolinate (Fig. 3). This decrease was about 35 and 40% at 24 and 48 hr of culture respectively. Calvariae, which were cultured in medium containing 10^{-4} M dipicolinate for 24 hr, were further cultured in the presence of 10^{-4} M zinc sulfate without dipicolinate for 24 and 48 hr. At 24 hr of culture, the presence of zinc caused a remarkable elevation of bone alkaline phosphatase activity (Fig. 3). This elevation was about 2.5-fold of the control in which no zinc was added. Also, at 48 hr of culture, bone alkaline phosphatase activity increased about 2.6-fold.

The effect of increasing concentrations of zinc sulfate on alkaline phosphatase activity in rat calvariae, which were pre-cultured in medium containing 10^{-4} M dipicolinate for 24 hr, is shown in Fig. 4. Calvariae were cultured in medium containing 10^{-6}–10^{-3} M zinc for 24 hr. Bone alkaline phosphatase activity was increased significantly ($P < 0.01$) by the presence of 10^{-6} M zinc. With the greater concentrations, the effect was remarkable; the enzyme activity was increased to a maximum level in the presence of 10^{-4} M zinc. However, at 10^{-3} M zinc, the effect was weakened.

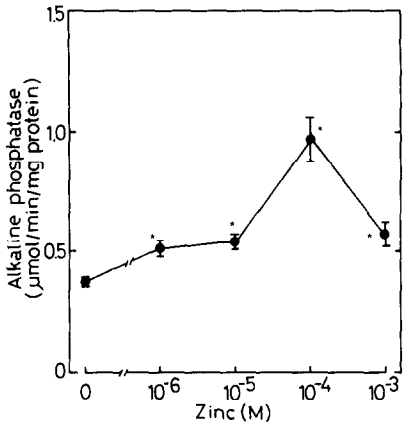

Fig. 4. Effect of zinc on alkaline phosphatase activity in rat calvariae pre-cultured with dipicolinate *in vitro*. Calvariae were pre-cultured for 24 hr in medium containing 10^{-4} M dipicolinate, and the bones were transferred into medium without dipicolinate. The bones were cultured for an additional 24 hr in medium containing either vehicle or 10^{-4} M zinc sulfate. Each point is the mean of five calvariae. The vertical lines represent the SE. Key: (*) $P < 0.01$, compared to the no addition value.

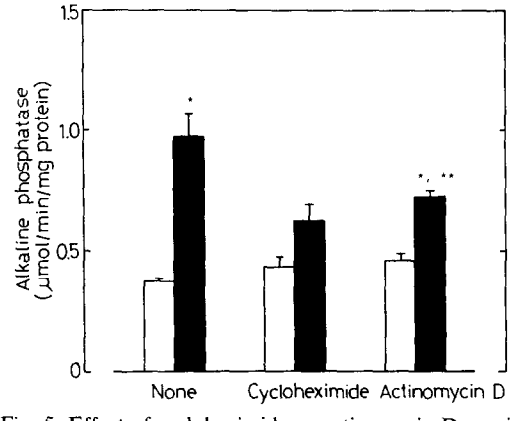

Fig. 5. Effect of cycloheximide or actinomycin D on zinc-increased alkaline phosphatase activity of rat calvariae pre-cultured with dipicolinate *in vitro*. Calvariae were pre-cultured for 24 hr in medium containing 10^{-4} M dipicolinate, and the bones were transferred into medium without dipicolinate. The bones were further cultured for 24 hr in medium containing either: 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 calvariae. The vertical lines represent the SE. Key: (*) $P < 0.01$, compared to the control group; and (**) $P < 0.05$, compared to the zinc alone group; (□) control; and (■) 10^{-4} M zinc.

When calvariae were pre-cultured in medium containing 10^{-4} M dipicolinate for 24 hr, the bones were transferred into medium containing either 10^{-7} M cycloheximide plus 10^{-4} M zinc, 10^{-8} M actinomycin D plus 10^{-4} M zinc, 10^{-4} M zinc, 10^{-7} M cycloheximide, 10^{-8} M actinomycin D or vehicle, and cultured for 24 hr. The effect of 10^{-4} M zinc to increase alkaline phosphatase activity was clearly blocked by the presence of 10^{-7} M cycloheximide, an inhibitor of protein synthesis at the translational process (Fig. 5). The presence of 10^{-8} M actinomycin

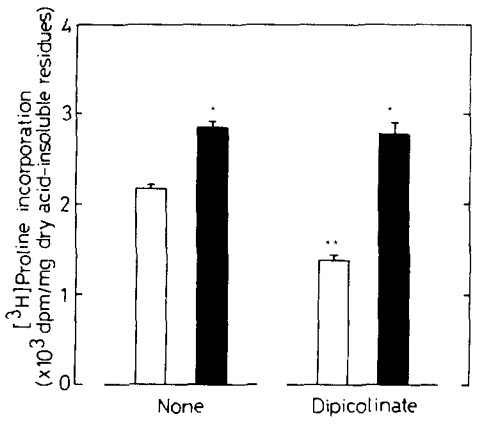

Fig. 6. Effect of dipicolinate on the incorporation of [^{3}H]proline into the acid-insoluble residues of rat calvariae *in vitro*. Calvariae were pre-cultured for 24 hr in medium containing either vehicle or 10^{-4} M dipicolinate, and then the bones were transferred into medium without dipicolinate. The bones were further cultured for 24 hr in medium containing either vehicle or 10^{-4} M zinc. The bones were pulsed with [^{3}H]proline (2.5 μCi/ml of medium) 2 hr before the end of the culture. Each bar is the mean of five clavariae. The vertical lines represent the SE. Key: (*) $P < 0.01$, compared to the control group; and (**) $P < 0.01$, compared to the control group not pre-cultured with dipicolinate; (□) control; and (■) 10^{-4} M zinc.

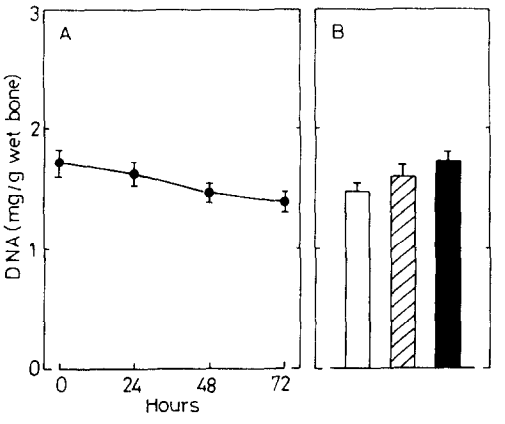

Fig. 7. Effect of dipicolinate on DNA content in rat calvariae *in vitro*. (A) Calvariae were cultured for up to 72 hr in medium containing neither dipicolinate nor zinc. (B) Calvariae were pre-cultured for 24 hr in medium containing either vehicle or 10^{-4} M dipicolinate, and the bones were transferred into medium without dipicolinate. The bones were cultured for an additional 24 hr in the presence of either vehicle or 10^{-4} M zinc. Each point or bar is the mean of five calvariae. The vertical lines represent the SE. Data were not significant. Key: (□) control; (▨) pre-culture with dipicolinate; and (■) zinc addition after pre-culture with dipicolinate.

D, an inhibitor of RNA synthesis at the transcriptional process, caused a significant ($P < 0.05$) inhibition of the increase in alkaline phosphatase activity by 10^{-4} M zinc. This inhibition, however, was about 40% of the value by which the enzyme activity was increased by zinc.

Effect of dipicolinate on bone protein synthesis. Rat calvariae, which were pre-cultured for 24 hr, were further cultured in medium containing 10^{-4} M zinc for 24 hr. The bones were pulsed with [^{3}H]proline for 2 hr before removal of the bones from the medium. The presence of 10^{-4} M zinc caused an increase of about 30% ($P < 0.01$) in newly synthesized bone total protein (Fig. 6). Meanwhile, calvariae were pre-cultured in medium containing 10^{-4} M dipicolinate for 24 hr, and the bones were further cultured in the absence of zinc for 24 hr. When the bones were pulsed with labeled proline, the protein synthesis decreased about 40% ($P < 0.01$) in comparison with that of control, which was not pre-cultured with dipicolinate. In this case, the presence of 10^{-4} M zinc produced a remarkable elevation (about 2-fold) of bone protein synthesis (Fig. 6).

Effect of dipicolinate on bone DNA content. DNA content in rat calvariae, cultured in medium without dipicolinate, is shown in Fig. 7A. Calvariae were cultured for 24 hr in medium, the medium was changed, and the bones were cultured for an additional 48 hr. Bone DNA content was not altered significantly by increasing the culture period. When calvariae were cultured for 24 hr in medium containing either vehicle or 10^{-4} M dipicolinate, the bones were transferred into medium without dipicolinate and further cultured for 24 hr in the presence of either vehicle or 10^{-4} M zinc sulfate (Fig. 7B). The pre-culture with 10^{-4} M dipicolinate did not cause a significant alteration of bone DNA content. In this case, bone DNA content was not increased significantly by the presence of 10^{-4} M zinc (Fig. 7B).

DISCUSSION

Dipicolinate (2,6-pyridinedicarboxylic acid), a chelator of zinc, has been used to prevent the effect of zinc on enzyme activity [13, 14]. The zinc chelator presumably decreases the zinc effect mainly by forming an extracellular $Zn(dipicolinate)_2^{2-}$ complex [13]. In the present studies, we used dipicolinate to estimate the role of endogenous zinc in bone protein synthesis. When calvariae obtained from weanling rats were cultured for 24 hr in medium containing dipicolinate (10^{-5}–10^{-3} M), the bone zinc content was decreased by increasing concentrations of the chelator. This indicates that the chelator can bind zinc in bone tissue. Bone alkaline phosphatase, a zinc-enzyme [15], plays a physiologic role in bone mineralization. Alkaline phosphatase activity was decreased slightly, but not significantly by culture with dipicolinate (10^{-6}–10^{-3} M) for 24 hr. Dipicolinate may not influence bone alkaline phosphatase directly. However, when calvariae were cultured with dipicolinate (10^{-4} M) for 24 hr and transferred into medium without the chelator, and then the bones were further cultured for 24 and 48 hr, bone alkaline phosphatase activity decreased about 35–40% in comparison with that of the bones which were cultured in medium not containing dipicolinate. This finding suggests that endogenous zinc in bone tissues plays a role in inducing alkaline phosphatase, since dipicolinate can deplete zinc in bone cells.

Dipicolinate caused an *in vitro* zinc deficiency which resulted in no zinc available to support further

growth of the bone during the additional 24–48 hr culture (after treatment with dipicolinate). Alkaline phosphatase activity decreased in both the treated and control calvariae after 24 hr. Moreover, the rate of decrease was identical in both treated and control tissues. This indicates that a continuous supply of zinc is required to support growth of this tissue *in vitro* irrespective of dipicolinate treatment. This reasoning is further supported by the fact that, if zinc were added to the control culture, a significant increase in alkaline phosphatase activity was observed also.

The presence of zinc sulfate (10^{-4} M) caused a remarkable elevation (about 2-fold) of alkaline phosphatase activity in calvariae, when the bones were cultured for 24 hr in medium containing dipicolinate and transferred into medium containing zinc without the chelator. This elevation was prevented completely by the presence of cycloheximide, an inhibitor of protein synthesis at the translational process. Meanwhile, the presence of actinomycin D, an RNA-synthesis inhibitor, caused a partial inhibition of the elevation of bone alkaline phosphatase activity induced by zinc. Zinc may be largely required for translation rather than transcription in the synthesis of alkaline phosphatase. We cannot exclude the possibility, however, that the effect of zinc occurs at the transcriptional level, since there was no significant difference between the cycloheximide- and actinomycin D-treated groups.

The effect of endogenous zinc on newly synthesized bone total protein was examined by studying the incorporation of [^{3}H]proline into calvariae cultured in medium containing dipicolinate. The preculture with dipicolinate caused a marked decrease (about 40%) of the incorporation of [^{3}H]proline into the bone acid-insoluble residues. In this case, the presence of zinc caused an increase (about 2-fold) in the incorporation of labeled proline into the bone acid-insoluble residues. However, in calvariae that were not treated with the chelator, the stimulatory effect of zinc on [^{3}H]proline incorporation into the bone cells was an increase of about 30% of control. These findings show that dipicolinate treatment causes a zinc depletion which is associated with a decrease in [^{3}H]proline incorporation in calvariae. Adding zinc to both control and dipicolinate-treated tissues led to an identical increase in [^{3}H]proline incorporation. Meanwhile, culture with dipicolinate did not cause an alteration of DNA content in bone tissues. The presence of zinc caused a slight increase in bone DNA content. Presumably, the effect of endogenous zinc to stimulate bone protein synthesis was independent of the alteration of bone DNA content.

Alkaline phosphatase is a zinc-containing metalloenzyme [15] and, therefore, the absence of zinc may prevent the synthesis of alkaline phosphatase as well as other zinc-requiring enzymes in bone. Alkaline phosphatase is present in large quantity in growing bone; thus, a block of alkaline phosphatase synthesis due to lack of zinc may lead to a significant decrease in total protein synthesis. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [2, 3]. This may be based on the decrease of bone protein synthesis by the depletion of endogenous zinc in bone cells. Presumably, endogenous zinc is an essential factor in protein synthesis of bone cells. There are many cells in bone tissues, including osteoblasts, osteoclasts and osteocytes. How much zinc is contained in those cells of bone tissue has not been determined. It is possible that osteoblasts, which are related to bone formation, contain largely zinc, since zinc can stimulate bone formation in tissue culture *in vitro* [6, 7]. This remains to be elucidated.

In conclusion, we found, by using dipicolinate, a chelator of zinc, that endogenous zinc plays an essential role in the stimulation of bone protein synthesis. Dipicolinate is a useful reagent to induce zinc deficiency *in vitro*. This reagent may be a pharmacologic tool to clarify the role of zinc in the biologic system.

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