Epiphyseal Plate Development in the Zinc-Deficient Rat

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ABSTRACT The effect of zinc deficiency on shearing strength, histological changes and proline utilization of the epiphyseal plate of the tibia of the weanling male rat was studied. A diet was fed based on sprayed egg white and containing less than 1 mg of zinc per kilogram. Over 27 days, the force required to displace the epiphysis of the zinc-deficient (ZD) rats was always less than that required for pair-fed (PF) controls. After 18 days, approximately 15% more force was required to displace the epiphysis of the PF rats than was required in the ZD rats. The thickness of the outside compact bone next to the epiphyseal plate region as determined by scanning electron microscopy was thicker in the ZD rats than in the PF controls of comparable age. The epiphyseal plates narrowed as the rats aged, and were clearly discernible in PF controls but not in ZD rats. The incorporation of L-[U-^{14}C]proline into the epiphysis was significantly less in rats deprived of zinc for 16–22 days than in PF controls. J. Nutr. *112:* 1320–1328, 1982.

INDEXING KEY WORDS zinc deficiency · shearing strength · histological appearance · epiphyseal plate · rat

The essentiality of zinc for the rat was established in 1934 by Todd et al. (1). The adverse effect of zinc deficiency on skeletal development was reported by Follis et al. (2) in 1941. Follis et al. (2) observed that in tibiae the epiphyseal cartilages were narrower, hypertrophied cells and osteoblasts were less numerous, and trabeculae of the cartilage-shaft interface were thinner and shorter in zinc-deficient than in zinc-adequate rats. Haumont (3) found high concentrations of zinc at the sites of calcification in the long bones of dogs. Skeletal abnormalities were a conspicuous feature of zinc-deficiency in growing birds (4–6). In pullets, the long bones were shortened and thickened in proportion to the degree of zinc-deficiency (6).

While it is evident that zinc is essential for growth, its role in bone formation is not fully understood (7). It was reported that the chondrocytes near the blood vessels were apparently normal in the epiphyseal region of the zinc-deficient chicks, while chondrocytes remote from blood vessels were shaped differently, were surrounded by more cellular matrix and did not stain normally for alkaline phosphatase activity (4). In addition, the differentiation of cells remote from the blood vessels was apparently delayed and erratic (8). Depressed osteoblastic activity in the bony collar of the long bone occurred in zinc-deficient chicks, together with a reduction in chondrogenesis and an associated elevation in the amount of cartilage matrix (4, 9). Westmoreland (10) suggested that these abnormalities may adversely influence bone calcification.

In rats, zinc deficiency profoundly im-

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paired the synthesis of collagen and possibly its cross-linkage (11–13). The decreased breaking strength of healing surgical wounds in zinc deficient rats may be related to these abnormalities (14). Because the epiphyseal growth zone of long bones is collagenous, we decided to evaluate the effects of zinc deficiency on growth and maturation of this tissue in rats. The shearing force required to displace the epiphysis was measured, and the physical status of the tibial epiphysis was examined by scanning electron microscopy in rats of several ages. The uptake and incorporation of labeled proline into the epiphyseal plate were measured to provide a biochemical correlation to the physical and microscopic findings.

MATERIALS AND METHODS

Design of experiment

In two separate experiments, male Long-Evans rats aged 16 to 22 days (Charles River Breeding Laboratory, Wilmington, MA)[1] were divided into two groups. The pair-fed (PF) group was individually weight-matched to the zinc-deficient (ZD) rats at the beginning of each experiment. Each group was fed a 20% sprayed egg white, biotin-enriched diet with a zinc content of less than 1 mg/kg (15). The diet was modified by omission of chlortetracycline hydrochloride and addition of 1.0 g of inositol per kilogram diet (Teklad Mills, Madison, WI). The first group (ZD) consumed distilled deionized water, and the second (PF) consumed zinc-supplemented water (25 ppm zinc in the form of zinc acetate added to the drinking water). Zinc-adequate ad libitum controls were not used because the body weight of such animals increases much more rapidly than zinc-deficient rats of the same age. To minimize trace metal contamination, silicon stoppers (Rodhelm-Reiss Inc., Ronsell Rubber Products Division, Belle Mead, NJ) were used in the water bottles.

The animals were housed individually in stainless-steel cages located in a humidity- and temperature-controlled room (25°, 40–50% relative humidity) with 12-hour periods of light and dark. Food intake, water consumption, and weight changes were measured daily between 0800 and 0900 hours. Wasted food was determined for pair-feeding, because ZD rats were significantly more wasteful than PF controls ($P < 0.01$). Each rat in the PF group was individually pair-fed an amount of diet that equaled the amount of diet eaten by a ZD rat on the previous day.

After the first 8 days, an animal was killed daily, alternating between ZD and PF control rats. The rats were anesthetized with diethyl ether and bled by cardiac puncture with heparin-coated needle and syringe (Eli Lilly & Co., Indianapolis, IN). Plasma, liver and femurs were removed for determination of zinc. Tibiae were removed for measurements of epiphyseal shearing strength (16) and for scanning electron microscope examination.

After a rat was killed, the tibia was dissected within 5 minutes to prevent autolysis. The tibia was separated from the femur shaft by careful disarticulation of the knee and was mounted with the anterior aspect up in the apparatus for shearing force determination (fig. 1). This apparatus consisted of a tensiometer (Institute of Physiology, University of Lund, Lund, Sweden), a Y-shaped yoke and a vise. After the tibia was clamped in the vise, 0.5-mm diameter needles were inserted 3 mm into the tibial epiphysis, and tension in the string was increased until the epiphysis was displaced.

Only one tibia per animal was used to determine the shearing force required to displace the epiphysis. The contralateral tibia was used for histologic study. If there was cutting by the pins because of improper placement or other disruptions, that tibia was saved for histologic study, and another attempt was made to shear the contralateral epiphysis.

The whole tibia end, the epiphyseal cap, and the shaft (from the shearing force experiment) were immersed for 2 to 3 hours in a primary fixative solution composed of Hank's balance salt solution (Sigma Chemical Co., St. Louis, MO) containing 2% glutaraldehyde (Ted Pella, Inc., Tustin, CA) at pH 7.4. While in the fixative solution, the whole

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

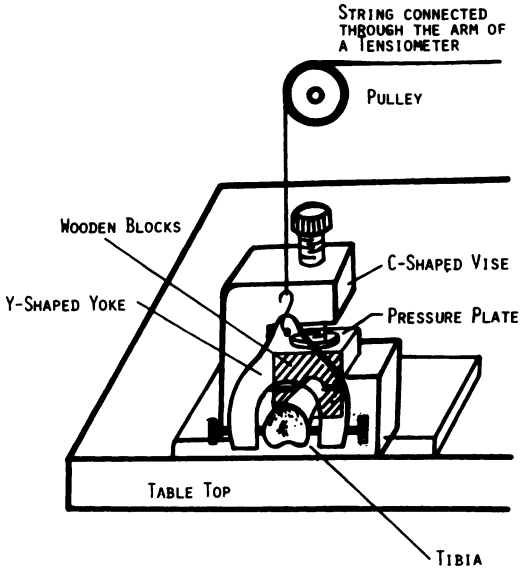

Fig. 1 The apparatus used to measure the force required to displace the proximal epiphysis. The apparatus consisted of: a C-shaped vise with a threaded pressure plate, which positioned the tibial shaft between two carved wooden blocks; a Y-shaped yoke through which screws were threaded in the ends of the arm; needles of 0.5-mm diameter maintained along the central axis by opposing screws and projected (approximately 3 mm) to hold the tibial epiphysis of a rat; a tensiometer modified for use in these measurements. A string from the extending arm of the device was crossed over a pulley and hooked to the Y-shaped yoke. The tension in the string could be varied by the device. The epiphysis was pulled by the string which passes over the pulley in the direction perpendicular to the bone shaft.

tibia was cut into samples 50- to 75-mm long. All samples were transferred to a 0.2 M cacodylate buffer (Sigma Chemical Co.) for 15 minutes and subsequently placed in a postfixative solution composed of 4% OsO_4 (Stevens Metallurgical Corp., New York, NY) and 0.144 M cacodylate buffer 1:1 for 1 hour. After postfixation, the samples were rinsed with 0.144 *N* cacodylate buffer for 10 minutes and then dehydrated through immersion in solutions from 30% to 100% acetone in 8 steps as follows: the samples were immersed for 5 minutes in each increasing concentration of acetone. The acetone concentrations were 30, 40, 50, 60, 70, 80 and 90%. The samples were subsequently immersed in 95% acetone for 10 minutes followed by final dehydration steps of two consecutive 10-minute periods in 100% acetone. All the fixation and postfixation steps were carried out at room temperature.

All samples were dried to the critical point in CO_2 (Sorvall critical point–dry system, #49300, Sorvall Co., Newtown, CT). After drying, the samples were attached to specimen stubs with silver paste and coated with gold-palladium (60:40) in a Hummer II Sputter Coater (Technics, Alexandria, VA). Specimens were examined by using a scanning electron microscope (Model S-4, Cambridge Scientific Instrument Ltd., Cambridge, England), and images were recorded on photographic film.

Blood samples drawn by cardiac puncture were centrifuged in plastic disposable tubes for 15 minutes at 18,900 × *g*. The plasma was transferred to a plastic disposable tube, diluted 1:1 with distilled deionized water, and the zinc content was determined by flame atomic absorption spectroscopy (Varian, Model AA-6, Varian-Techtron Pty. Ltd., Melbourne, Australia). Absorption was measured at 213.9 nm, and the zinc contents were compared with those obtained from zinc solutions prepared from a certified reference sample (Bovine Liver Standard Reference Material #1577, National Bureau of Standards, Washington, DC). Femur and freeze-dried liver samples were prepared and analyzed by flame atomic absorption spectroscopy (15).

To evaluate the effect of zinc deficiency on collagen synthesis, 20 weanling male Long-Evans rats were randomly divided into two groups, ZD and PF, housed individually, and fed as described above. The rats were maintained on this regimen for 16–22 days.

L-[U-^{14}C]Proline (New England Nuclear, Boston, MA) (391 mCi/mmol), diluted 1:4 (vol/vol) with phosphate-buffered saline (0.85%), was injected (3.25 μCi/100 g body weight) intraperitoneally into the ZD rats between 0800 and 0900 hours. The rats were then maintained without food and water for 140 minutes, anesthetized with diethylether and bled to death by cardiac puncture with heparin-coated needle and syringe. The tibial epiphyseal plates were rapidly removed. The following day, the PF control rats were injected with L-[U-^{14}C]proline and processed in the same way as their pair mates.

Plasma was separated from red blood cells,

and 0.1 ml was dissolved in a Protosol (New England Nuclear):ethanol mixture (1:2 vol/vol, 0.5 ml) in a liquid scintillation vial (New England Nuclear). The vial was capped tightly and incubated in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, IL) at 60° for 1 hour, cooled to room temperature and 30% hydrogen peroxide (0.5 ml) added. The vial was capped loosely and incubated for an additional 30 minutes. After cooling to room temperature, 15 ml of Aquasol (New England Nuclear) was added to the vial. The solution was made slightly acidic with 0.5 M HCl (0.5 ml) to prevent chemiluminescence. The vials were stored at 4° for 1 day in a liquid scintillation spectrometer (Model Mark II, Nuclear Chicago, IL) before counting.

The epiphyseal plate from each tibia was placed in a separate, preweighed liquid scintillation vial. After recording the weight, Protosol (1.0 ml) was added to the sample, and the vial was capped tightly. The vial was incubated at 80° in a Dubnoff metabolic shaking incubator for 2–3 hours until the sample was dissolved. The vial was cooled to room temperature, and 30% hydrogen peroxide (0.5 ml) was added. Then the vial was capped loosely and incubated for an additional 30 minutes. The vial was cooled again to room temperature, and 15 ml of Aquasol was added. To prevent chemiluminescence, 1 M Tris/HCl buffer (pH 4, 0.5 ml) was added. The vials were stored at 4° in the dark in a liquid scintillation spectrometer for 1 day before counting. Two values for epiphyseal ^{14}C content were obtained for each rat.

Values cited in the text are the means ± SD unless otherwise stated. Differences between analyses from ZD and PF rats were ascertained by the Student's *t*-test. Multiple regression analysis (17) was used to ascertain differences between ZD and PF compact bone thickness with time. The Johnson-Neyman technique (17) was applied to determine regions of significance.

RESULTS

The zinc-deficient rats displayed many of the typical signs of zinc deficiency such as alopecia, growth retardation and dermal lesions. Within 3–4 days the rats exhibited anorexia and consumed food in a cyclic fashion. The food efficiency ratio was similar for the PF (0.30) and ZD rats (0.27). The plasma zinc concentration in the PF control rats was 153 ± 16, μg/dl, which was significantly ($P < 0.0001$) higher than in the ZD group (37 ± 16 μg/dl). The plasma zinc varied from day to day in the ZD group (fig. 2). The liver zinc concentration was significantly less ($P < 0.01$) in the ZD rats (66 ± 9) than in PF rats (88 ± 9 μg/g). Liver zinc levels in both groups fluctuated from day to day (fig. 2). Femur zinc levels were significantly ($P < 0.0001$) lower for the ZD rats (192 ± 41) than for the PF group (435 ± 20 μg/g). Zinc concentrations in the femurs of the ZD rats decreased as a function of longer treatment, but remained constant in the PF control rats through the period of this investigation (fig. 2). The plasma, liver and femur zinc concentrations are the means of values obtained over the 26-day period (fig. 2).

The force required to displace the epiphysis of a ZD rat was always less than that

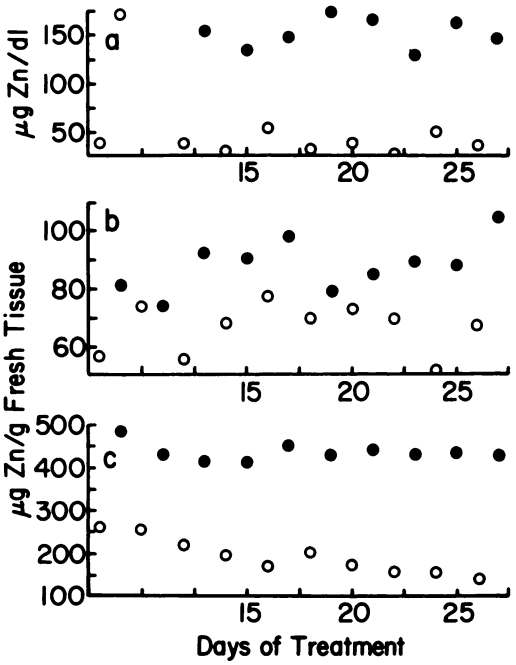

Fig. 2 The relationship between time of treatment and (a) plasma (b) liver (c) femur zinc concentrations in rats fed a zinc-deficient diet (O —— O) and in individually pair-fed control rats (● —— ●) with zinc supplementation in the water.

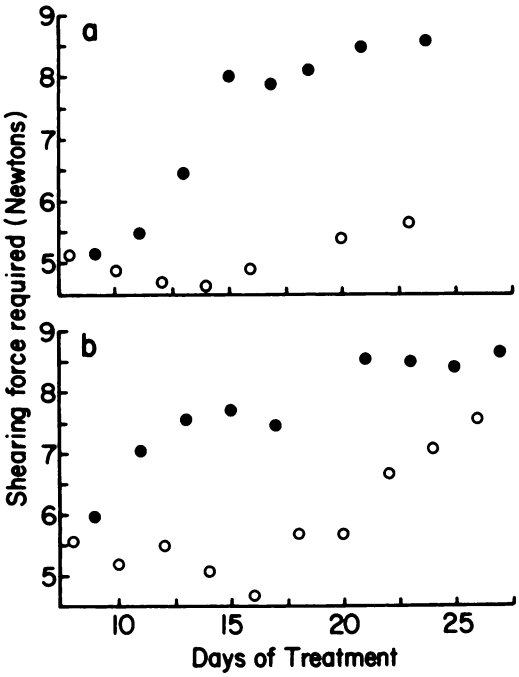

Fig. 3 The relationship between the shearing force required to displace the epiphysis and time of treatment for both zinc-deficient (○ —— ○) and pair-fed control (● —— ●) rats (a) from the first trial (b) from the second trial.

required for the respective PF control (fig. 3). In the ZD rats, this force decreased during the first few days then increased with longer treatment time. In contrast, the force required to displace the epiphysis of PF animals progressively increased and approached a value of 8.5 Newtons. After 18 days of treatment, the force that was required to displace the epiphysis was about 15% higher for the PF than ZD rats. The thickness of the outside compact bone next to the epiphyseal plate region, as determined from scanning electron micrographs, was greater ($P < 0.005$) in ZD than in PF control rats of comparable age (fig. 4). Narrowing of the epiphyseal plate of the PF rat was clearly discernible. Figure 5 illustrates the tibial epiphysis from a PF control and a ZD rat at age 39 and 38 days (after 9 and 8 days of treatment), respectively. Long columns of chondrocytes were present in the epiphyseal plate from the PF control rat (fig. 6). In contrast the epiphyseal plate from the ZD rat contained cells that were not readily identifiable (fig. 6).

After injecting L-[U-^{14}C]proline into the rats, the ZD tibial epiphysis contained significantly less ($P < 0.01$) ^{14}C ($1.53 \pm 0.32 \times 10^6$ cpm/g) than PF control epiphysis ($1.82 \pm 0.16 \times 10^6$ cpm/g). The amounts of ^{14}C in the plasma of the ZD ($29{,}990 \pm 580$ cpm/ml) and PF control ($30{,}340 \pm 680$ cpm/ml) rats were similar.

DISCUSSION

The day-to-day variations in plasma and liver zinc concentrations in ZD rats were consistent with previous reports that plasma (15, 18) and liver (15) zinc concentrations correlated with cyclic feeding patterns in ZD rats. Femur zinc concentrations were not affected by cyclic feeding patterns and declined slowly as zinc deficiency progressed. This finding is consistent with previous reports (15, 19).

Zinc has been shown to be generally essential for the utilization, metabolism and excretion of amino acids in plants and animals (20–24). Also, zinc deficiency appeared to influence amino acid and protein metabolism in bones. Histidine supplementation partially alleviated bone defects that occurred in zinc-deficient chicks (25). Nielsen et al. (26) found depressed ^{35}S incorporation into the epiphyseal plate and the primary spongiosa of zinc-deficient chick tibia. These authors suggested that zinc deficiency altered the metabolism of SO_4^{2-} and consequently mucopolysaccharides in bone elongation re-

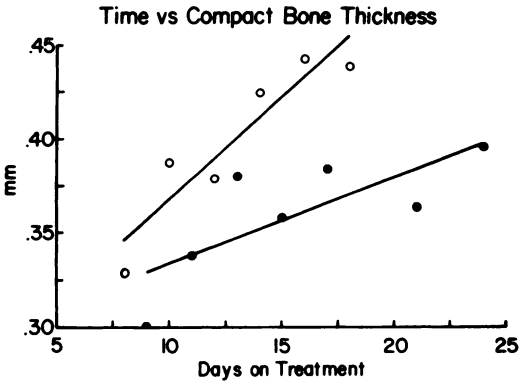

Fig. 4 The relationship between compact bone thickness and time of treatment. ○ —— ○, zinc-restricted rats, $y = 0.26 + 0.01x$; ● —— ●, zinc-adequate pair-fed control rats, $y = 0.29 + 0.005x$. The two lines are significantly different ($P < 0.005$). After $x = 13.2$ days, the compact bone thicknesses for the ZD rats are significantly ($P < 0.05$) greater than those representing the PF rats.

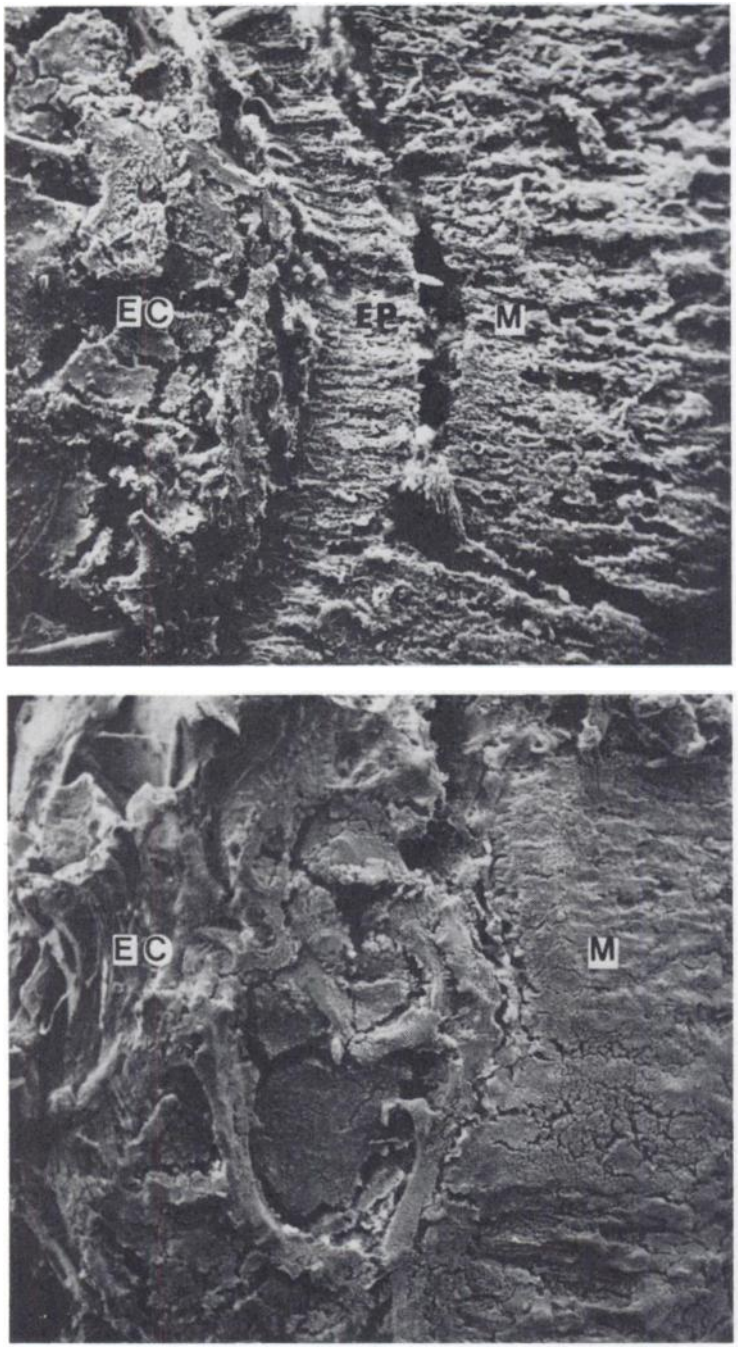

Fig. 5 The upper electron micrograph shows a representative tibial epiphyseal plate from a pair-fed control rat (39 days old). The epiphyseal plate (EP) extends between the epiphyseal cap (EC) and metaphysis (M), (21.75 × magnification). The lower electron micrograph shows a representative tibial epiphyseal plate from a zinc-deficient rat (38 days old). Between the epiphyseal cap and the metaphysis is an area of disturbed tissue which apparently corresponds to the epiphyseal plate (18.75 × magnification).

gions. Recently, zinc deficiency was shown to interfere with glycosaminoglycan metabolism in membranous bone in guinea pig alveolar implants (27). Likewise, zinc deficiency caused depressed ^{35}S incorporation into the epiphyseal plate of young rat femurs

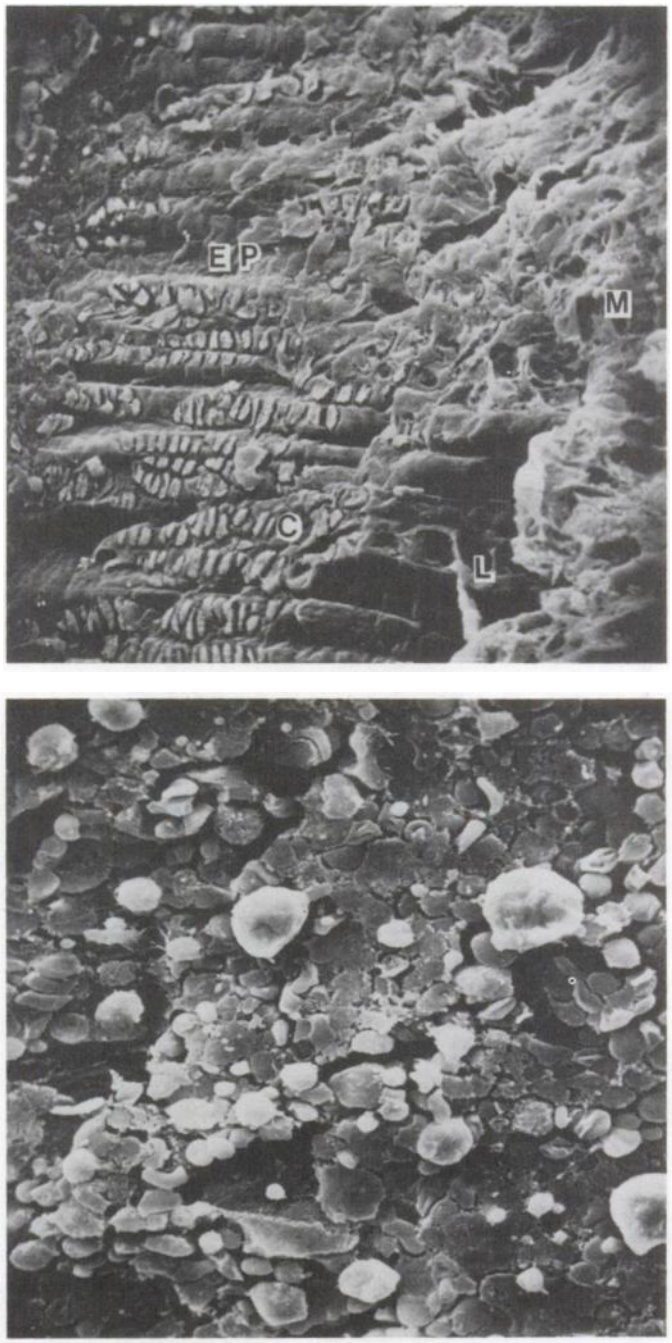

Fig. 6 The upper electron micrograph shows a representative tibial epiphyseal plate from a pair-fed control rat (36 days old). The parallel columns of chondrocytes (C) in the epiphyseal plate (EP) are typical. Metaphysis (M) and lacunae (L) are readily identified (93.75 × magnification). The lower electron micrograph shows a representative tibial epiphyseal plate from a zinc-deficient rat (36 days old). Except for their position in the epiphyseal plate, the components of this tissue cannot be positively identified (50.75 × magnification).

(28). In this study we found that less proline was incorporated into collagen from the epiphyseal plate of tibia from young zinc-deficient rats. This finding confirms a report that zinc deficiency impaired the incorporation of proline into the epiphyseal plate.

In that report the formation of hydroxyproline also apparently was suppressed (28).

Collagen synthesis of skin is apparently impaired by zinc deficiency. The biosynthesis of subcutaneous collagen and noncollagenous protein was depressed by zinc deficiency in young rats (11). McClain et al. (12, 13) observed that the incorporation of [2-^{14}C]glycine into α_1 and α_2 chains and L-[U-^{14}C]proline into the salt-soluble collagen fraction were depressed in skin from zinc-deficient rats. The effect of zinc nutriture on the collagen cross-linking process is unclear. McClain et al. (12, 13) reported an apparent enhancement of covalent intramolecular cross-link formation in skin collagen from zinc-deficient rats because of an increased content of β-components and increased aldehyde content of the salt-soluble collagen pool. Also because collagen extracted with dilute acid solutions (biologically older than salt-soluble collagen) was depressed, zinc deficiency apparently inhibited intermolecular covalent cross-linking. Fernandez-Madrid et al. (11) disagreed with these conclusions and stated that a cross-linking defect could only be established in zinc deficiency by determining cross-linking profiles of collagen from zinc-deficient tissue.

Abnormalities that might contribute to the depression of protein synthesis in bones in response to zinc deficiency are decreased activity of RNA polymerase (EC 2.7.7.6) (29), abnormal formation or stability of polysomes (30) and possibly the production of an unusual RNA polymerase that can change the base content of the RNA produced, as was observed in *E. gracilis* (31).

Abnormal collagen biosynthesis in bones as a result of zinc deficiency might contribute to the lower shearing force necessary to displace the epiphysis in ZD, compared to PF control, rats. Zinc deficiency reduced the breaking strength of healed cutaneous incisions (14, 32). Recently, an adverse effect of zinc deficiency on bone collagenase and collagen turnover in chicks was reported (33). Abnormal collagen metabolism might be a cause of the thickened outside compact bone next to the epiphyseal plate (fig. 4) and the observed irregularities in the epiphyseal plates of zinc-deficient rats (fig. 5, 6). Collagenase is necessary for remodelling of bone. The increased thickness of compact bone found in the ZD rats suggested bone remodeling was impaired.

In summary, zinc deficiency adversely affected the shearing strength, histological appearance and uptake and/or incorporation of L-[U-^{14}C]proline into the epiphyseal plate in young male rats.

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