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Distribution of Type X Collagen in Tibiotarsi of Broiler Chickens with Vitamin D Deficiency

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Summary: Type X collagen is a significant component of the extracellular matrix of the hypertrophic zone of physeal cartilage, but its precise role in endochondral ossification has not been determined. The concentration of type X collagen increases in physeal cartilage in chicks with vitamin D deficiency. The purpose of our study was to determine whether defective endochondral ossification due to vitamin D deficiency was associated with abnormalities in the distribution of type X collagen in the proximal tibiotarsus of chicks. To accomplish this, we induced vitamin D deficiency in broiler chicks and sequentially evaluated the pattern of type X collagen immunoreactivity in the proximal tibiotarsus using a monoclonal antibody specific for chicken type X collagen. Type X collagen immunoreactivity was present in the matrix of the prehypertrophic zone, hypertrophic zone, cartilage cores of the primary spongiosa, and within the chondrocytes of the prehypertrophic and early hypertrophic zones in vitamin D-deficient and D-replete chicks. However, rachitic chicks exhibited two consistent differences in type X collagen immunoreactivity: hypertrophic chondrocytes in the late hypertrophic zone and primary spongiosa contained intracellular type X collagen; and type X collagen was concentrated into laminated aggregates in the pericellular and territorial matrices in the late hypertrophic zone and primary spongiosa. We conclude from these findings that (1) normal serum concentrations of $1,25(OHD)_2D_3$ and $25OHD_3$ are not required for type X collagen production; (2) type X collagen production does not decrease in the most mature zones of the physes in chicks with vitamin D deficiency; and (3) newly secreted type X collagen accumulates in the pericellular and territorial matrices of the late hypertrophic zone and primary spongiosa of rachitic chicks, perhaps because it is not readily incorporated into the interterritorial matrix.

Key words: Type X collagen—Rickets.

Type X collagen is a component of the extracellular matrix in physeal cartilage [1–3]. It is present within and around chondrocytes that are beginning to hypertrophy and persists in the extracellular matrix until the physeal cartilage is resorbed during endochondral ossification [4]. As intracellular type X collagen is not evident in the distal aspects of the hypertrophic zone, its production by chondrocytes may decrease in the more mature areas of the physis [4]. Because type X collagen is uniquely localized to the prehypertrophic and hypertrophic zones of the physis, it is believed to play a role in endochondral ossification; however, its role has not been elucidated. Initially it was hypothesized that type X collagen may have a role in initiation of matrix mineralization [1, 5], but Poole and Pidoux [2] have recently questioned that view because they could find no relationship between type X collagen and matrix vesicles or initial foci of mineral deposition in mineralizing cartilage [2]. Using immunoelectron microscopy, they determined that type X collagen is associated with type II collagen fibrils and suggested that type X may focus initial mineralization to interfibrillar areas [2]. A third hypothesis is that type X collagen targets physeal cartilage for resorption during endochondral ossification [3].

Abnormalities in the production of type X collagen occur in two important diseases of poultry—tibial dyschondroplasia and vitamin D deficiency-induced rickets [6, 7]. In both these conditions, endochondral ossification is delayed, but the molecular events responsible for delay have not been identified [8–11]. Bashey et al. [6] reported that cartilage that accumulates in tibial dyschondroplasia contains less type X collagen than normal hypertrophic cartilage. This finding indicates that chondrocytes in dyschondroplastic physes fail to mature properly. Reginato et al. [7] determined that physeal cartilage from chicks with vitamin D deficiency contain an increased concentration of type X collagen relative to collagen types II, IX, and XI, and suggested that this may be a response to hypocalcemia. However, Lian et al. [12] found that $1,25(OHD)_2D_3$ increased the production of type X collagen by chondrocytes *in vitro,* so the effect that vitamin D has on production or metabolism of type X collagen is not yet clear.

The purpose of this study was to determine whether defective endochondral ossification due to vitamin D deficiency was consistently associated with abnormalities in the distribution of type X collagen in the proximal tibiotarsus of chicks; this could provide insight into the role type X collagen has in endochondral ossification. To accomplish this, we induced vitamin D deficiency in broiler chicks and sequentially evaluated the pattern of type X collagen immunoreactivity in the proximal tibiotarsus using a monoclonal antibody specific for chicken type X collagen.

Materials and Methods

One-day-old male broiler chicks (Cornish giant, Peterson Arbor Acre) were separated into two groups, housed in stainless steel cages, and kept in a lightproof room. One group was fed a commercially prepared diet devoid of vitamin D (Teklad #170245, Teklad, Madison, WI; 1.13% calcium, 0.79% phosphorus); the other group was fed the same diet to which 1,400 IU of vitamin D_3/kg was added. Both groups were maintained on their respective diets for 24 days.

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Table 1. Mean ± SEM plasma $1,25(OH)_2D_3$, $25OHD_3$, calcium, and phosphorus concentrations in chicks fed vitamin D-deficient or vitamin D-replete diet

No. of days & diet	$1,25OH_2D_3$ (pg/ml)	$25OHD_3$ (ng/ml)	Ca (mg/dl)	Phos (mg/dl)
Day 7				
D_3 deficient	39 ± 9	Not detectable[b]	7.68 ± .44	—
D_3 replete	61 ± 7	11.0 ± .8	8.58 ± .29	—
Day 14				
D_3 deficient	28 ± 8[d]	Not detectable[b]	6.55 ± .32[b]	4.7 ± .5
D_3 replete	66 ± 10	8.0 ± .9	9.83 ± .32	5.0 ± .1
Day 21				
D_3 deficient	5 ± 2[c]	Not detectable[b]	6.33 ± .58[c]	4.0 ± .5
D_3 replete	57 ± 12	16.3 ± 2.8	10.20 ± .21	4.7 ± .6
Day 24				
D_3 deficient	10 ± 2[d]	Not detectable[b]	6.68 ± .15[b]	4.2 ± .3[d]
D_3 deficient & oral D_3[a]	285 ± 36[b]	14.7 ± 1.3	10.10 ± .19	4.6 ± .2[d]
D_3 replete	23 ± 4	17.6 ± 1.8	11.02 ± .49	6.6 ± .6

N = 6 chicks per group
[a] Treated with 100 U of D_3 orally from day 21–24
Significantly different from D_3 replete: [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$

In addition, 6 chicks that had been fed the vitamin D-deficient diet for 21 days were treated with 100 U D_3 orally daily through day 24. Six chicks from each of the two groups were randomly selected and killed for examination at 7, 14, 21, and 24 days of age.

The chicks were anesthetized with a 1:1 mixture of CO_2:O_2, weighed, and decapitated. Blood samples were collected following decapitation and subsequently assayed for $1,25(OHD)_2D_3$ (pg/ml) [13], $25OHD_3$ (ng/ml) [14], calcium (mg/100 ml) [15], and phosphorus (mg/100 ml) [16]. The left tibiotarsi were harvested, fixed, demineralized, and processed for immunohistochemistry as described elsewhere [4]. The primary antibody, AC9 (monoclonal mouse anti-chicken type X collagen provided by Dr. Thomas Schmid, Rush Presbyterian-St. Luke's Medical Center, Chicago), is specific for chicken type X collagen, as positive staining is completely blocked by adsorbing AC9 with purified chicken type X collagen [1, 17–19]. Negative controls, in which the primary antibody was replaced with media that contained a nonsense antibody harvested from hybridoma cultures (used at 1:500 dilution), were run with each group of slides. Additional specimens of tibiotarsi from the D-deficient and D-replete chicks killed at 21 and 24 days of age, plus specimens from the D-deficient chicks that were treated orally with vitamin D_3 were not demineralized and were embedded in glycol methacrylate (JB-4, Dupont-Sorvall), sectioned at 3 μm, mounted on acid-cleaned poly-L lysine-coated slides, allowed to dry, and then stained with the Von Kossa technique to demonstrate mineral.

Each tibiotarsus was evaluated microscopically for changes typical of rickets. The proximal tibiotarsal physis was evaluated for type X collagen immunoreactivity as described elsewhere [4].

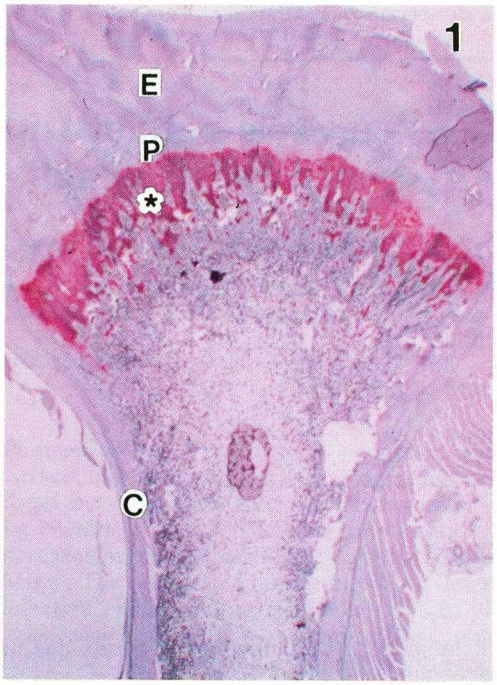
Serum concentrations of calcium, phosphorus, $1,25(OHD)_2D_3$, and $25OHD_3$ from vitamin D-deficient and vitamin D-replete chicks were compared statistically using Student's paired t test; P values <0.05 were considered significant [20].

Results

Chicks that had been on the vitamin D-deficient diet for 7 days had no gross or microscopic changes indicative of rickets. Their serum levels of calcium, $1,25(OHD)_2D_3$, and $25OHD_3$ were decreased but only $25OHD_3$ was significantly decreased (Table 1). Serum phosphorus values were not obtained in this group because of a laboratory error.

All chicks that were fed the vitamin D-deficient diet for 14 days or longer had typical gross and microscopic features of rickets, which included marked thickening of the proliferative zone of the physis, irregular thickening of the hypertrophic zone of the physis, thickening of the cortex due to accumulation of osteoid, excessive bone resorption, and fibrosis of the metaphyseal marrow [8]. In addition, tibiotarsi were soft and poorly mineralized; the late hypertrophic zone was poorly mineralized in rachitic chicks maintained for 21 or 24 days. Serum levels of calcium, $1,25(OHD)_2D_3$ and $25OHD_3$ were significantly decreased in all chicks fed the vitamin D-deficient diet for 14 days or longer; serum phosphorus levels were decreased but only significantly decreased in chicks fed the vitamin D-deficient diet for 24 days. Chicks that had been fed the vitamin D-deficient diet for 21 days and then treated with D_3 orally for 3 days had serum calcium and $25OHD_3$ levels similar to vitamin D-replete chicks. However, serum phosphorus levels were significantly decreased and $1,25(OHD)_2D_3$ levels were significantly increased in this group. Also, this group had gross and microscopic lesions typical of rickets, but in contrast to the age-matched D-deficient chicks, the late hypertrophic zone contained foci of mineralization.

Distribution of type X collagen in the proximal tibiotarsus of the vitamin D-replete chicks was similar to that recently described for broiler chickens and turkeys [4] (Figs. 1, 2). Type X collagen was present in the matrix of the prehypertrophic zone, hypertrophic zone, and cartilage cores in the primary spongiosa of the physis. Intracellular type X collagen was limited to chondrocytes in the prehypertrophic and early hypertrophic zones (Fig. 2a). Chicks that had been fed the vitamin D-deficient diet for 7 days had a pattern of type X collagen immunoreactivity similar to age-matched vitamin D-replete chicks. However, there were several differences in type X collagen distribution in chicks that had developed rickets (i.e., chicks on vitamin D-deficient diet for 14, 21, and 24 days) (Figs. 3–6). In the late hypertrophic zone and primary spongiosa of rachitic chicks, type X collagen was concentrated in the pericellular and territorial matrices forming thick, irregular layers around chondrocytes (Figs. 4b, 5). Many chondrocytes, especially at the periphery of hypertrophic zone and primary spongiosa, contained granular to globular intracellular type X collagen. In addition, there were several foci of type X collagen immunoreactivity in the osseous trabeculae of the proxima tibiotarsal cortex in rachitic chicks (Fig. 6). Rachitic chicks treated with vitamin D_3 for 3 days after day 21 had a distribution of type X immu-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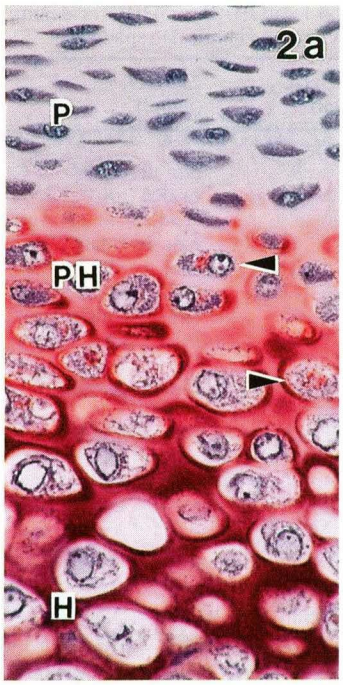
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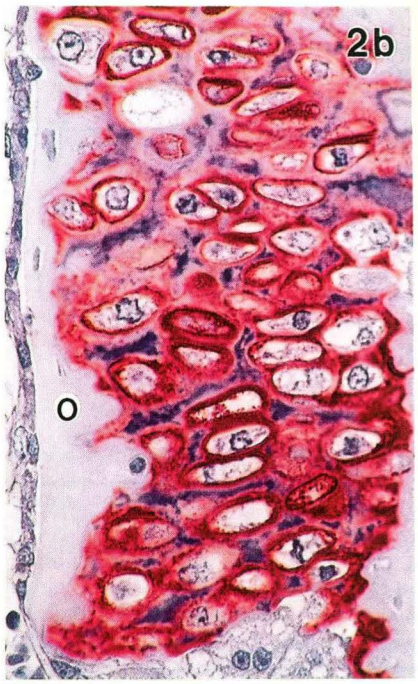
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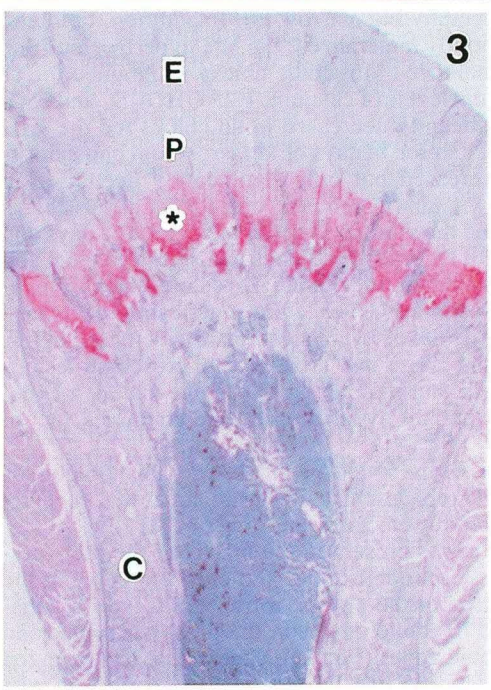
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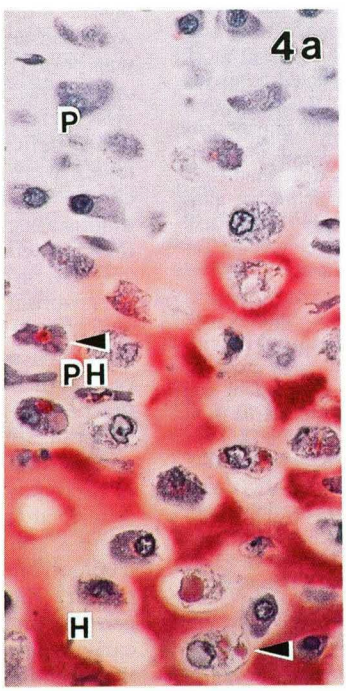
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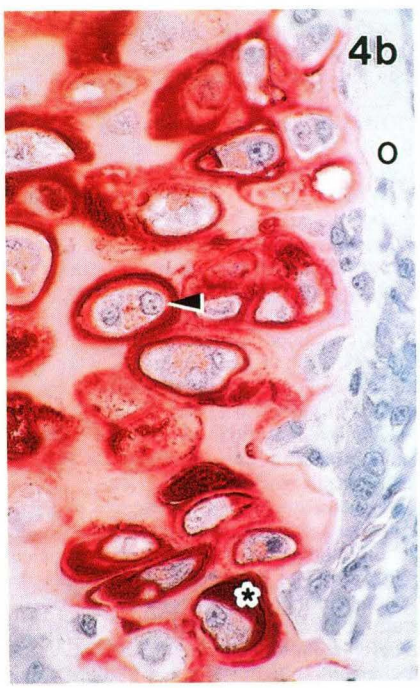
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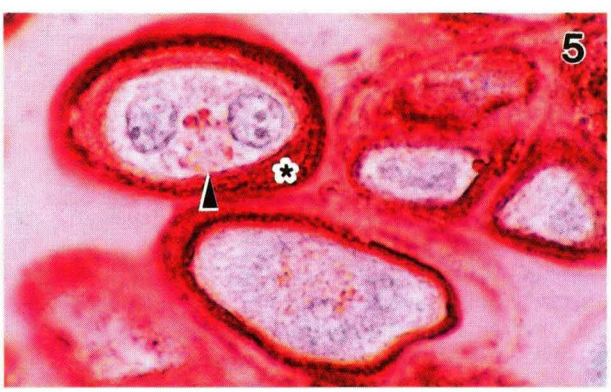
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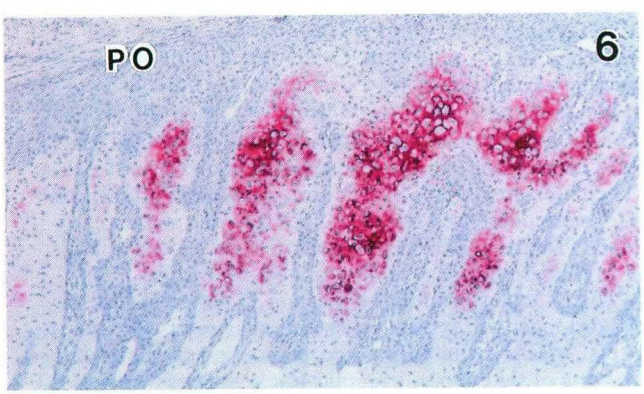
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Fig. 1. Proximal tibiotarsus from a 21-day-old vitamin D-replete chick (control). Type X immunoreactivity is limited to the distal portion of the physis (*) epiphysis (E); proliferative zone (P); tibiotarsal cortex (C). × 7.875. **Fig. 2(a)** and **(b)** Proximal tibiotarsal physis from 21-day-old vitamin D-replete chick. **(a)** Includes the proliferative (P), prephypertrophic (PH), and hypertrophic zones (H). **(b)** The primary spongiosa. Matrix of prehypertrophic zone, hypertrophic zone, and primary spongiosa has intense type X immunoreactivity, but intracellular type X immunoreactivity (arrowheads) is limited to chondrocytes in the prehypertrophic and early hypertrophic zones. Proliferative zone and osteoid (O) are devoid of immunoreactivity. × 400. **Fig. 3.** Proximal tibiotarsus from a 21-day-old vitamin D-deficient chick. This chick has developed rickets, evidenced by the thickened proliferative zone (P) and thickened cortex (C). Type X immunoreactivity is most prominent in the distal portion of the physis (*), as in the D-replete chick. Epiphysis (E). × 7.875. **Fig. 4(a)** and **(b).** Proximal tibiotarsal physis from 21-day-old vitamin D-deficient chick. **(a)** Includes proliferative (P), prehypertrophic (PH), and hypertrophic (H) zones; chondrocytes in proliferative zone are disorganized, which is typical of rickets. **(b)** The primary spongiosa. Chondrocytes in the prehypertrophic zone, hypertrophic zone, and primary spongiosa contain abundant intracellular type X (arrowheads). Type X is also present in the matrix of the prehypertrophic and early portion of the hypertropic zones, but is concentrated into the pericellular and territorial matrices of the primary spongiosa (*). × 400. **Fig. 5.** Primary spongiosa from 21-day-old vitamin D-deficient chick (higher magnification of 4b). Granular intracellular type X immunoreactivity (arrowhead). Also, note thick lamination of type X in the pericellular matrix (*). × 1000. **Fig. 6.** Cortex of proximal tibiotarsus from 21-day-old vitamin D-deficient chick. Type X immunoreactivity in osseous trabecula subjacent to periosteum (PO). × 63.

noreactivity similar to other rachitic chicks. Type X immunoreactivity was present in cartilage that was mineralized and cartilage that was not mineralized.

Discussion

In general, the pattern of type X collagen immunoreactivity in the proximal tibiotarsus of chicks fed a diet deficient in vitamin D was similar to age-matched vitamin D-replete chicks. However, we did identify several consistent differences in type X collagen distribution in chicks with rickets induced by vitamin D deficiency. It is clear from this study that physeal chondrocytes do not require normal serum concentrations of $1,25(OHD)_2D_3$ and $25OHD_3$ to produce type X collagen. This finding is consistent with a previous report in which excessive levels of type X collagen (relative to other types of collagen) were found in the growth plate of rachitic chicks [7]. We hypothesize that one or more metabolites of vitamin D directly or indirectly (by maintaining blood calcium and phosphorus levels) induce chondrocytes in the physis to hypertrophy; once chondrocytes begin to hypertrophy, they begin to produce type X collagen and secrete it into the adjacent matrix. In addition, we suggest that production of type X collagen by hypertrophic chondrocytes normally decreases in the more mature zones of the physis, as intracellular type X collagen is not evident in chondrocytes in the late hypertrophic zone and primary spongiosa [4]. This hypothesis is consistent with the observation that $1,25(OH)_2D_3$ induces chondrocytes in culture to hypertrophy and produce type X collagen [12]. Our hypothesis is also consistent with the recent findings that as hypertrophic chondrocytes begin to produce type X collagen, they have a sudden appearance of type X collagen mRNA due to an increased rate of transcription [21]; but as hypertrophic chondrocytes continue to differentiate *in vitro*, their levels of type X collagen mRNA decrease [22]. Our observation of intrachondrocytic type X collagen in the late hypertrophic zone and primary spongiosa of rachitic chicks indicates that the mechanism to cease production of type X collagen is not operational in vitamin D-deficient chicks. It is possible that intracellular type X collagen is present within chondrocytes in the late hypertrophic zone and primary spongiosa because of defective secretion rather than excessive production. However, this seems unlikely, because there is abundant, if not excessive, type X collagen in the pericellular matrix surrounding these chondrocytes. The putative signal to decrease production of type X has not been identified; however, $1,25(OHD)_2D_3$, $25OHD_3$, other vitamin D metabolites, serum calcium, and/or serum phosphorus are all candidates. Reginato et al. [7] suggested that the excessive concentration of type X collagen found in rachitic chick physes may be a response to hypocalcemia.

Concentration of type X collagen into laminated aggregates in the pericellular matrix coupled with a decreased amount of type X immunoreactivity in the interterritorial matrix of the late hypertrophic zone and primary spongiosa suggest that there may be defective incorporation of type X collagen into the extracellular matrix in rachitic chicks. Perhaps newly secreted type X collagen cannot be assimilated into the extracellular matrix of the late hypertrophic zone and primary spongiosa at the same rate it is assimilated into the matrix of the prehypertrophic and early hypertrophic zones. If the role of type X collagen is to "target" physeal cartilage for resorption as others have suggested [19], then decreased incorporation of type X into the extracellular matrix may hinder endochondral ossification. However, it is clear from our observations that overt lack of type X is not responsible for the delay in endochondral ossification that occurs in rickets due to vitamin D deficiency. Failure to alter the distribution of type X collagen in rachitic chicks by giving them vitamin D_3 orally for 3 days may indicate that chondrocytes must mature in the proper milieu in order to be responsive to the signal(s) that regulate type X collagen production and/or secretion. Alternatively, it may be that these chicks were simply not replete long enough (i.e., only 3 days) for abnormalities in type X collagen distribution to be rectified. We conclude that the mechanism that normally decreases production of type X collagen in the most mature zones of the physis is not operational in chicks with vitamin D deficiency, and that because of this, type X collagen continues to be produced and accumulates within chondrocytes and the pericellular and territorial matrices of the late hypertrophic zone and primary spongiosa.

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