Studies on the Pathogenesis of Avian Rickets

I. Changes in Epiphyseal and Metaphyseal Vessels in Hypocalcemic and Hypophosphatemic Rickets

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Growth plate morphometry and measurements of serum chemistry were correlated to clarify the pathogenesis of hypocalcemic and hypophosphatemic avian rickets. Accumulation of proliferating and maturing cartilage in hypocalcemic chicks is accompanied by increased length and increased variation in length of perforating epiphyseal vessels, decreased number and abnormal arrangement of marrow spaces, an increased proportion of cells to blood vessels in marrow spaces, and a change in the distribution but not the total number of DNA-synthesizing chondrocytes per unit width of growth plate. Accumulation of hypertrophic cartilage in hypophosphatemic rickets is accompanied by no change in length, distribution, or number of perforating epiphyseal vessels, elongation but no change in number or arrangement of marrow spaces, an increase in the relative proportion of blood vessels to cells in the marrow spaces, and no change in distribution but a decrease in total number of DNA-synthesizing chondrocytes per unit width of growth plate. Both types of rickets have decreased amounts of calcified cartilage. These results provide further evidence that hypocalcemia and hypophosphatemia cause morphologically distinct types of rickets in birds. The data indicate that the thickness of the proliferating and maturing region and hence the distance of the hypertrophic zones from the epiphysis are anatomically and temporally related to length of perforating epiphyseal vessels and serum calcium levels. They indicate that in hypocalcemic rickets accumulation of proliferating and maturing cartilage is unlikely to be the result of increased chondrocyte replication and that the relative rates of chondrocyte hypertrophy and resorption of hypertrophic cartilage by marrow are equal. They support the concept that delayed chondrocyte hypertrophy is the major cause of growth plate thickening in hypocalcemic rickets. Data presented in this study, when considered together with data from the literature on hypophosphatemic rickets, support the long-held concept that growth plate thickening in this disease is caused primarily by a decreased rate of resorption of hypertrophic cartilage by marrow relative to the rates of chondrocyte proliferation, maturation, and hypertrophy. The data further support the concepts that growth of cartilage into marrow is a biphasic process including longitudinal growth effected mainly by blood vessels, and resorption of the lateral walls of marrow spaces effected mainly by marrow cells, and that it is the latter phase that is defective in hypophosphatemia. (Am J Pathol 1982, 109:288–301)

PATHOLOGIC CHANGES in cartilage of the growth plate of vitamin-D–deficient chickens appear to be determined by changes in the availability of calcium and phosphate rather than by vitamin D deficiency *per se*.[1] Thickening of the growth plate in hypocalcemic vitamin-D–deficient chickens is due to an accumulation of proliferating and maturing chondrocytes,[2,3] a lesion that is also seen in hypocalcemic animals receiving adequate dietary vitamin D.[2,3] Hypophosphatemic vitamin-D–deficient chickens have growth plates that are thickened by an accumulation of hypertrophic chondrocytes,[1,3] a type of lesion also seen in vitamin-D–replete chickens with hypophosphatemia.[2,3]

The mechanisms responsible for these differential responses of avian growth plate cartilage to varying levels of calcium and phosphate are unknown. Studies

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by Trueta and colleagues in mammals suggest that they may be related to differences in the responses of epiphyseal and metaphyseal blood vessels. These investigators have provided evidence that an intact epiphyseal vascular supply is necessary for the nutrition and growth of the growth plate.[4] Metaphyseal vessels (the nutrient and metaphyseal systems) are necessary for calcification, degeneration, and resorption of cartilage.[4-7] These data suggest that accumulation of proliferating chondrocytes in calcium deficiency might be associated with increased growth of the epiphyseal vascular system, whereas the accumulation of hypertrophic chondrocytes in phosphate deficiency might be related to decreased growth of metaphyseal vessels. We undertook the present study to test those hypotheses and to provide further data about the mechanisms involved.

Materials and Methods

In this study we compared the growth and maturation of epiphyseal blood vessels, marrow (metaphyseal) blood vessels and their accompanying marrow cells, and growth plate cartilage in the proximal tibial growth plate of 2-3-week-old normal, hypocalcemic, and hypophosphatemic White Rock chicks. Normal chicks (Group A) were fed a diet composed of 900 g of Teklab Rachitogenic Chick Test Diet AOAC, 60 g Teklab Brigg's Chick Salts A, Teklab Vitamin Mix with Vitamin D_2 (2000 IU/kg) AOAC, and US Biochemical Vitamin D_3 (500 IU/kg). Preliminary studies showed that without the vitamin D_3 supplement, chicks developed hypocalcemia and rachitic cartilage lesions characteristic of that condition. Hypocalcemic vitamin-D-deficient animals (Group B) were obtained by use of the same diet, but the vitamin mix contained no vitamin D_2, and no vitamin D_3 was added. Hypophosphatemic animals were produced by two types of diets. Vitamin-D-deficient hypophosphatemic animals (Group C) were obtained by use of the same diet as used for Group B but with 88.6 g $CaCO_3$ USP (Malinckrodt) added. Vitamin-D-replete hypophosphatemic animals were obtained by use of the same diet as used for Group A but with 151 tablets/960 g feed of ground Amphojel (Wyeth Laboratories, Philadelphia, Pa) added.

Feed samples were wet-washed and analyzed for calcium by absorption spectrophotometry[8] and phosphate by colorimetry.[9-11] The calcium and phosphate content and calcium phosphate ratio of the diets are listed in Table 1.

Table 1—Calcium and Phosphate Content of Diets

Diet	Vitamin D_3	$CaCO_3$	$Al(OH)_3$	Ca%	P%	Ca:P
A	+			2.0	1.4	1.4
B				1.8	1.3	1.4
C		+		5.1	1.3	3.8
D	+		+	1.7	1.3	1.3

Composition of experimental diets: presence of vitamin D, excess $CaCO_3$, and $Al(OH)_3$ is indicated by +. Numerical values are the percentage by weight of calcium and phosphate and the calcium/phosphate ratio of the diets.

One-day-old chicks obtained from a commercial hatchery were housed in heated brooders in the absence of ultraviolet light. All animals received chick starter mash for the first 4 days. On Day 4 they were divided into 6 groups with equivalent mean starting weights and placed on one of the diets above. The animals were weighed at weekly intervals and sacrificed by cardiac puncture (under ether anesthesia) after 2 weeks (Group D) or 3 weeks (Groups A-C). Group D animals were sacrificed earlier because of a nearly 100% mortality between Weeks 2 and 3.

Three animals from each group were used for studies of the gross distribution of blood vessels by vascular perfusion with India ink. After perfusing the vascular system with warmed heparin solution (1 IU heparin/ml of saline, 37 C) via the left ventricle, we injected warmed India ink into the vessels (1 part ink: 2 parts 2% gelatin in saline) until the fine vessels in the claws were readily visualized. Dissected tibiae were placed in ice water for 1 hour, fixed 24 hours in Zamboni's fixative,[12] and dehydrated through 100% ethanol; and 1-2-mm hand-cut sections were embedded in methacrylate.[13]

Longitudinal 0.5-1-mm slices of proximal tibial growth plate from an additional 3-4 animals from each group were fixed in Zamboni's fixative, washed with phosphate-buffered saline (PBS), and incubated 30 minutes in diaminobenzidine solution[14] for demonstration of endogenous peroxidase activity in red cells and, hence, visualization of the blood vessels. After rinsing in PBS, sections were stained with alizarin red-S[15] for demonstration of insoluble calcium deposits in relationship to the blood vessels.

The proximal left tibia of 8-9 animals in each group was cut freehand into 0.5-1-mm longitudinal sections which were incubated with gentle agitation for 3 hours at 37 C in 5 ml of Ham's F12 culture medium containing 2 μCi/ml of ^{3}H-thymidine. Following incubation, the tissues were fixed for 24 hours in Zamboni's fixative, washed with PBS, dehydrated, and embedded in methacrylate for sectioning at 4-5 μ on a Bright motor-driven rotary microtome. Sections were immersed in NTB-2 Kodak Nuclear Liquid Emulsion, exposed at 20 C for 4 weeks, and developed with Kodak D-19 developer. After drying, the specimens were counterstained with light green. In

preliminary experiments, chicks were given subcutaneous injections of 1.5 μCi/g body weight of ^{3}H-thymidine; and their proximal tibial growth plates were dissected, fixed, and processed as described above after 2 hours. The distribution of labeled chondrocytes in these *in vivo* experiments was identical to that with the *in vitro* technique.

^{3}H-Thymidine uptake of chondrocytes was quantitated in two ways. We determined the total number of cells per unit transverse width of growth plate by counting all the labeled cells in strips 500 μ wide, including the entire longitudinal thickness of the proliferating and maturing zone between its junctions with the epiphysis and the hypertrophic zone. We determined the density of labeled cells in each strip by dividing the total number of cells by the number of 500 × 500 sq μ fields in the strip within which labeled cells were found. Five strips were counted for each histologic section. In a preliminary experiment, three histologic sections from each of three separately incubated and processed tissue slices were counted in this way, and the results are indicated in Table 2. The data show that the major source of variation was uneven distribution of labeled cells within a single microscopic section of a given animal's growth plate and that the mean obtained by counting 5 adjacent 500-μ wide strips from such a section provided the same value that was obtained by counting three sections from the same tissue slice or by counting three histologic sections from each of three separate tissue slices. Counting was, therefore, done on a single section of a single slice from each animal.

The right tibia of all animals not injected with India ink was dissected and measured and then fixed, embedded, and sectioned at 4–5 μ as described above. Sections were stained by the von Kossa technique[16] and counterstained with hemotoxylin and eosin. These sections were used for morphometry as described below. Sections were also stained for acid phosphatase activity.[17]

We used point counting with a grid 1 × 1 cm with 121 intersections[18] to determine the relative area (volume in three dimensions) of the growth plate occupied by proliferating and maturing, uncalcified hypertrophic, and calcified hypertrophic cartilage. Counts from consecutive 1 sq mm fields along a line between the junction of the epiphysis with the growth plate and the growth plate with the metaphysis in the longitudinal axis of the bone were summed. The last grid always included at least 10 cartilage counts. The significance of differences between groups was established by use of Wilcoxon Rank Sum Test for nonparametric distributions.[19]

Point counting was also used to determine the relative volume of marrow occupied by cells and blood vessels in the terminal 500 μ of the marrow spaces. The total counts falling over cells or blood vessels was summed for all the terminal marrow spaces in a midlongitudinal section, and the ratio was calculated. The significance of differences between groups was established by the Wilcoxon Rank Sum Test.

Table 2—Tritiated Thymidine Counts in Single Histologic Sections of Separately Incubated and Processed Slices of Growth Plate Cartilage From a Phosphate Deficient Chick

Slice	Histologic section	Counts*
A	1	74 ± 18
	2	68 ± 17
	3	73 ± 12
	Mean ± 1 SD, A (1-3)	72 ± 3
B	1	68 ± 11
	2	74 ± 14
	3	77 ± 15
	Mean ± 1 SD, B (1-3)	73 ± 4
C	1	75 ± 11
	2	77 ± 20
	3	75 ± 10
	Mean ± 1 SD, C (1-3)	76 ± 1
	Mean ± 1 SD, Slices A-C	74 ± 2

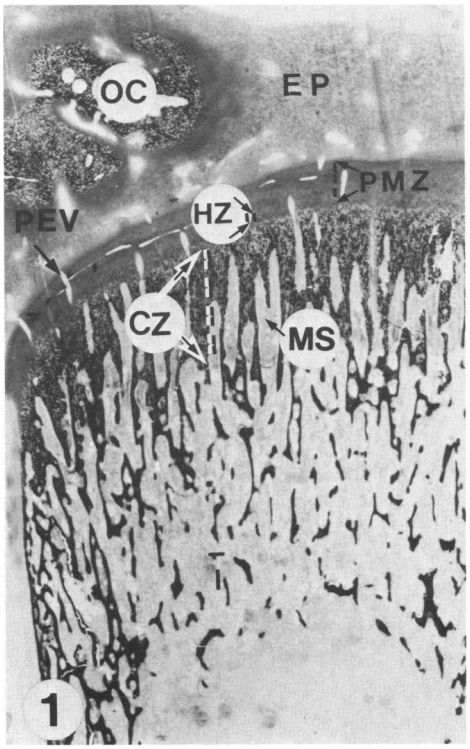
* The value for each histologic section is the mean ± 1 standard deviation of counts of the total number of labeled cells in 5 adjacent strips of proliferating and maturing cartilage 500 μ wide, extending from the junction of the proliferating and maturing zone and the epiphysis to the junction of the proliferating and maturing zone and the hypertrophic zone.

The total number of perforating epiphyseal and marrow tunnels in midlongitudinal sections of the growth plate was counted, and the ratio of the number of perforating epiphyseal to marrow spaces was calculated. Maximal and minimal lengths of these vascular spaces were measured with the use of a calibrated ocular grid. The length of the perforating epiphyseal vessels was measured from their terminal capillary loops to the epiphyseal growth plate junction. The length of marrow vessels was measured from their terminal capillary loops to the level where all calcified cartilage had been removed (ie, the junction of growth plate and metaphysis).

Serums obtained by cardiac puncture were analyzed for calcium by absorption spectrophotometry,[8] inorganic phosphate (expressed as phosphorus) by colorimetry,[9-11] and alkaline phosphatase by enzyme colorimetry.[20,21] Group comparisons were also made by the Wilcoxon Rank Sum Test.

Results

The normal proximal tibial growth plate consists of a disk of cartilage penetrated from its epiphyseal



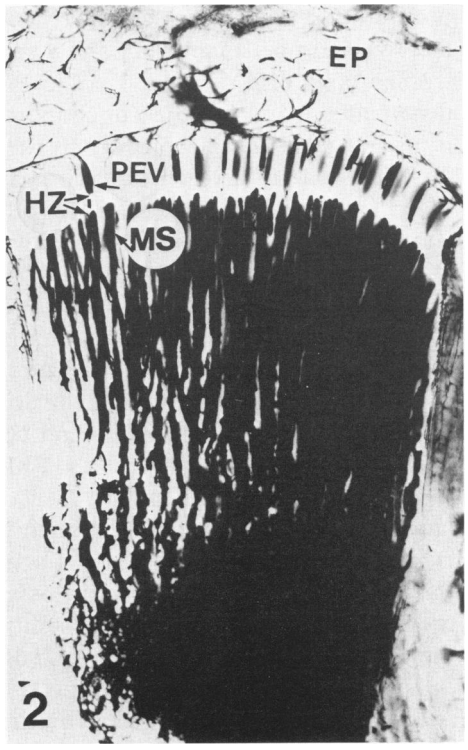
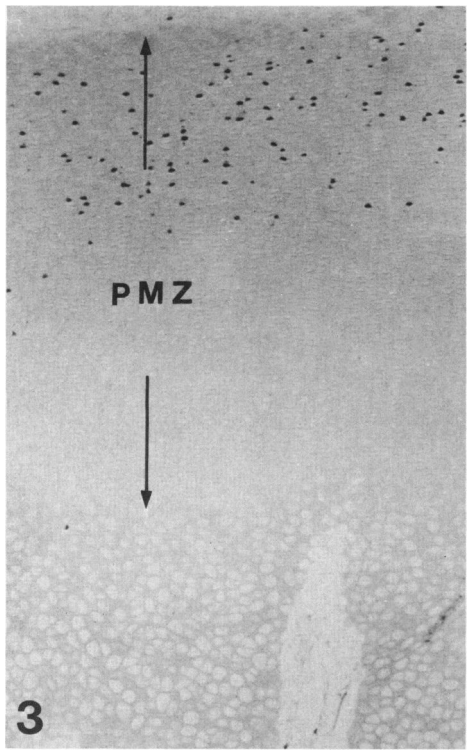


Figure 1 — Proximal tibia of a normal 3-week-old chick. *EP*, epiphysis; *OC*, ossification center; *PMZ*, proliferating and maturing zone of growth plate; *HZ*, hypertrophic zone; *CZ*, calcified hypertrophic zone; *PEV*, perforating epiphyseal vessel; *MS*, marrow space. (Von Kossa stain with H&E counterstain, ×7.1)

Figure 2 — Vascular supply of the proximal tibia of a normal 3-week-old chick. The hypertrophic zone is an avascular region between terminal portions of the PEVs and vessels within MSs. (India ink preparation, ×7.1)

side by vascular loops taking origin in the epiphysis, the perforating epiphyseal vessels (PEVs), and from its metaphyseal side by terminal portions of the nutrient and metaphyseal vascular systems, referred to collectively as metaphyseal vessels (MVs). Both the PEVs and the MVs occupy tunnels or canals within the cartilage of the growth plate and are accompanied within these tunnels by other cell types as described below.

The cartilage of the growth plate is divided into a



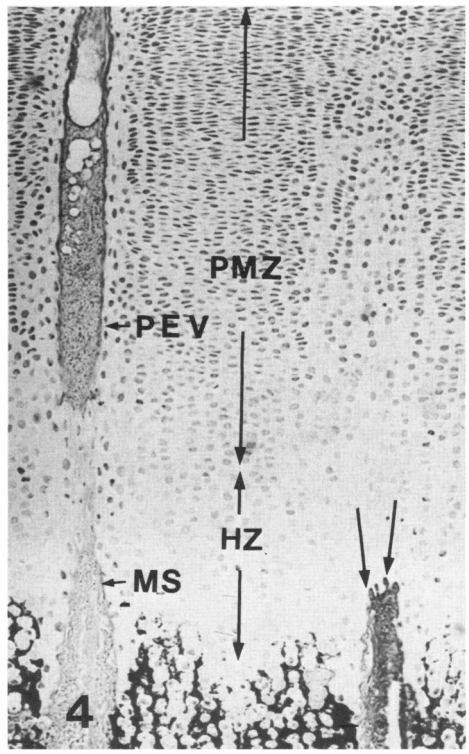


Figure 3 — Tritiated thymidine uptake in the proximal tibial growth plate of a normal 3-week-old chick. Labeled chondrocytes form a band in the proximal half of the PMZ. (Prepared as described in text; light green counterstain, ×100 **Figure 4** — Proximal tibial growth plate of a normal 3-week-old chick. Terminal PEVs and MVs are joined by scars passing through the avascular HZ. Vascular buds extending into partially opened chondrocyte lacunae are indicated by *arrows*. (Von Kossa stain with H&E counterstain, ×100)

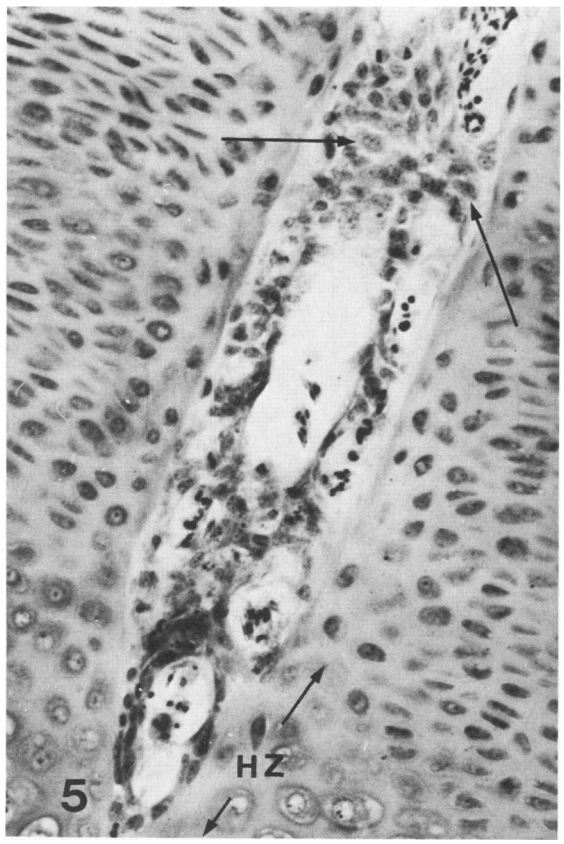
proliferating and maturing zone (PMZ), an uncalcified hypertrophic zone (HZ), and a calcifying hypertrophic zone (CZ) (Figure 1). The PMZ is sharply demarcated from the adjacent chondroepiphysis and is penetrated at regular intervals by the PEVs (Figure 2). There is no reserve cell zone comparable to mammalian growth plates. The PMZ adjacent to the chondroepiphysis is a region of active chondrocyte DNA synthesis (Figure 3). DNA synthesis declines toward the metaphyseal site of the PMZ. In this region cells enlarge slightly but retain their darkly staining nuclei and cytoplasm. (Figure 4).

The HZ is a narrow avascular region between the terminal portions of the PEVs and MVs (Figures 2 and 4). The boundary between the PMZ and the HZ is very sharp (Figures 3 and 4). Cells in the HZ are larger and rounder and have less intensely stained nuclei and cytoplasm (Figures 4–6). Cartilage matrix in the HZ is not stained by the von Kossa method (Figures 4–6). In the CZ the matrix stains positively by the von Kossa method (Figures 4 and 6) but the chondrocytes are similar to those in the HZ (Figure 6).

The distal portions of the marrow are composed of thin-walled sinusoids and clusters of cells arranged around a central arteriole (Figure 6). The cells include elongated cells resembling fibroblasts, which are mitotically active and undifferentiated, large mononucleated and multinucleated cells with diffuse cytoplasmic acid phosphatase activity of varying intensity (Figure 8), and smaller granulocytes in which the granules also are acid-phosphatase-positive. The sinusoidal walls are composed of endothelial cells difficult to distinguish from the fibroblastlike cells, exhibiting weak diffuse cytoplasmic acid phosphatase activity.

PEV canals are occupied by capillaries, veins, arteries, and undifferentiated perithelial cells (Figs 5, 7).

In animals that were fed a vitamin-D–deficient diet (Group B) severe hypocalcemia and hyperalkaline phosphatemia developed (Table 3). In contrast, vitamin-D–deficient animals with diets supplemented by extra calcium were normocalcemic, hypophosphatemic, and had high alkaline phosphatase levels (Table 3). Their serum chemistries were similar to those of the vitamin-D_3–replete animals receiving $Al(OH)_3$ to bind phosphate (Table 3).



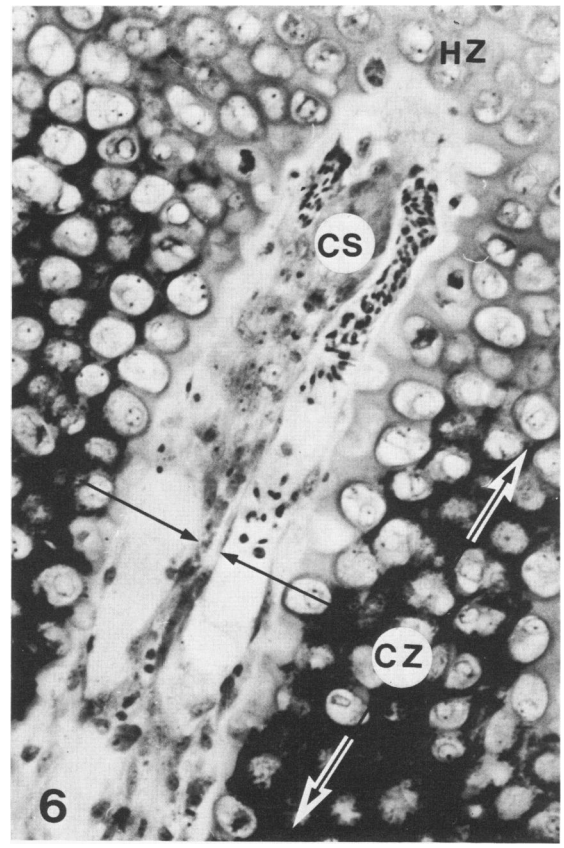
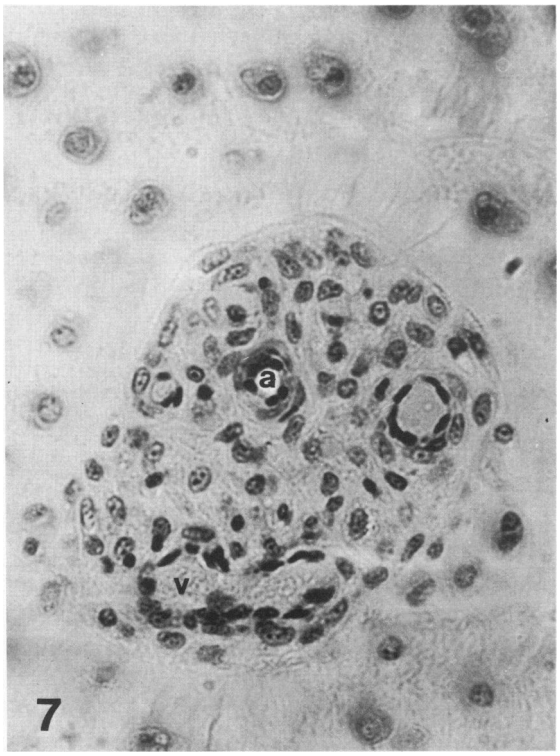


Figure 5 — Longitudinal section of the terminal portion of a PEV tunnel ending in HZ of normal 3-week-old chicks. Thin-walled vessels and oval perithelial cells (*arrows*) occupy the space. (Von Kossa stain with H&E counterstain, × 400) **Figure 6** — Terminal portion of an MS in the proximal tibial growth plate of a normal 3-week-old chick. The central stalk (*CS*) is surrounded by thin-walled vessels. A narrow central arteriole (*arrow*) enters the central stalk. (Von Kossa stain with H&E counterstain, × 400)



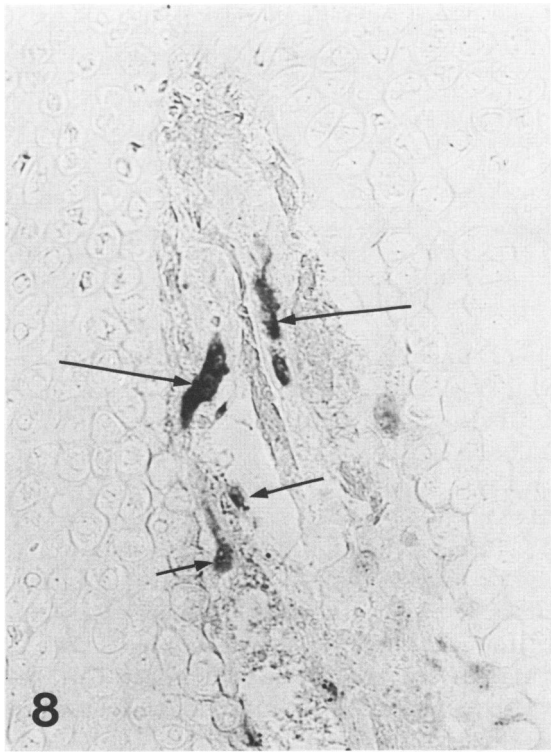


Figure 7 — Cross-section of a PEV tunnel in the epiphysis of a normal 3-week-old chick. Vessels at this level have differentiated to form small arteries (*a*) and veins (*v*). Perithelial cells show no differentiation. (Von Kossa stain with H&E counterstain, × 400) **Figure 8** — Tartrate-resistant acid-phosphatase-positive multinucleated (*long arrows*) and mononuclear cells (*short arrows*) in the MS of a normal 3-week-old chick. Similar cells with less intense acid phosphatase activity are also present. In addition, many of the smaller cells in the lower portion of the marrow space contain acid-phosphatase-positive cytoplasmic granules not well visualized in this photomicrograph. (Napthol AS-B1 phosphate substrate with fast garnet GBC capture, × 400)

The hypocalcemic vitamin-D-deficient animals had a markedly expanded PMZ region in the growth plate, as expected (Table 4, Figure 9), and an increased hypertrophic zone. The latter was expanded at the expense of calcified hypertrophic cartilage, since the total hypertrophic cartilage was normal (Table 4). Both groups of hypophosphatemic animals, in contrast, had normal PMZs, increased HZs, decreased CZs, and increased total hypertrophic cartilage (the sum of HZ and CZ) (Table 4, Figure 10).

PEVs in the hypocalcemic animals were markedly elongated and displayed lateral branching (Figure 11). The total number of PEVs in midlongitudinal section did not differ from normal, but the minimal and maximum length and the variation in length of PEVs was much greater than normal (Table 5). The terminal portions of the marrow tunnels in hypocalcemic animals were short, broad, and branched laterally (Figure 11). The number of marrow tunnels in midlongitudinal section was reduced, and the ratio of the number of PEVs to the number of MVs was increased (Table 5). The distance between the terminal capillaries of the marrow vessels and the metaphysis did not differ from normal (Table 5).

PEVs in hypophosphatemic animals did not vary significantly from normal with respect to length, total number, or variation in length (Figure 12; Table 5). The marrow vessels were longer than normal (Table 5) but the total number of marrow spaces and the ratio of number of PEVs to MVs did not differ from normal (Table 5). No significant differences between hypophosphatemic animals caused by $Al(OH)_3$ feeding or feeding vitamin-D_3-deficient, high-calcium diets were noted (Table 5).

Table 3 — Serum Calcium, Phosphate, and Alkaline Phosphatase Levels in Chicks Fed Normal and Deficient Diets

Group	Calcium (mg/dl)	Phosphate (mg/dl)	Alkaline phosphatase (kU/l)
A Normal	11.5 ± 1.3	9.4 ± 0.9	3.6 ± 0.8
B Vitamin-D-deficient	6.8 ± 0.9*	7.5 ± 2.1*	13.1 ± 5.8*
C Vitamin-D-deficient High calcium	10.6 ± 0.6	4.4 ± 0.6*	9.6 ± 3.6*
D Phosphate-deficient	11.3 ± 1.3	4.8 ± 1.4*	6.3 ± 2.6*

* Numerical values are group means ± 1 standard deviation. The series of values generating the numbers with asterisks are statistically different from the series generating the normal group mean values at a level of $P = 0.005$.

Table 4—Relative Size of Different Growth Plate Zones in Chicks on Normal and Deficient Diets

Group	Number of animals	PMZ	HZ	CZ	CZ + HZ
A Normal	9	76 ± 6	25 ± 7	166 ± 26	191 ± 34
B Vitamin-D-deficient	9	196 ± 74*	109 ± 36*	80 ± 33*	187 ± 43
C Vitamin-D-deficient High calcium	10	79 ± 17	216 ± 88*	59 ± 17*	275 ± 79*
D Phosphate-deficient	11	55 ± 11	165 ± 87*	88 ± 34*	253 ± 72*

* Numbers represent the mean ± 1 standard deviation for the total counts falling over the proliferating and maturing zone (PMZ), hypertrophic zone (HZ), and calcifying zone (CZ) in the growth plates of chickens in different dietary groups with the use of point-counting techniques described in the text. The series of values generating the numbers with asterisks are statistically different from the series generating the normal group means at a level of $P = 0.005$.

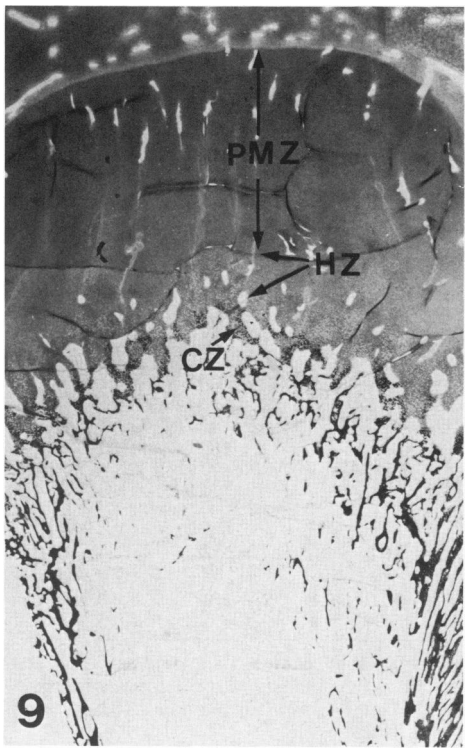
The total number of DNA-synthetized cells in longitudinally oriented 500-μ-wide strips of the PMZ of vitamin-D_3-deficient hypocalcemic animals was not statistically different from normal, but the density of these cells was significantly reduced (Table 6). This reduction in density was due to a change in their distribution. In normal animals, all DNA synthesizing cells were localized to the first 500 × 500 sq μ square adjacent to the epiphysis (Figure 3). In hypocalcemic animals, the labeled cells were distributed longitudinally throughout the increased thickness of the PMZ with a preferential distribution along the course of PEVs (Figure 13).

In hypophosphatemic animals, the total number and density of DNA-synthesizing cells per 500-μ-wide strip of PMZ was significantly reduced (Table 6). The labeled cells, however, were normally distributed within the first 500 × 500 sq μ square adjacent to the epiphysis (Figure 14).

Orientation of PMZ chondrocytes was abnormal in hypocalcemic animals. In normal animals, the long axis of proliferating chondrocytes was parallel to the transverse axis of the growth plate and perpendicular to the PEVs (Figure 4). In hypocalcemic animals, the orientation of the long axis of these cells was random or parallel to PEVs (Figure 15).

The transition from proliferating and maturing to hypertrophic chondrocytes occurred in the avascular zone between terminal portions of PEVs and MVs in the hypocalcemic and hypophosphatemic animals as it does in normals (Figures 4 and 16). The boundary beween PMZ and HZ in hypocalcemic animals was much more irregular than normal because of variation in the length of PEVs and interdigitation of PEVs and MVs.

The histologic composition of the marrow tunnels was abnormal in hypocalcemic and hypophosphatemic animals. Hypocalcemic animals had an abnor-



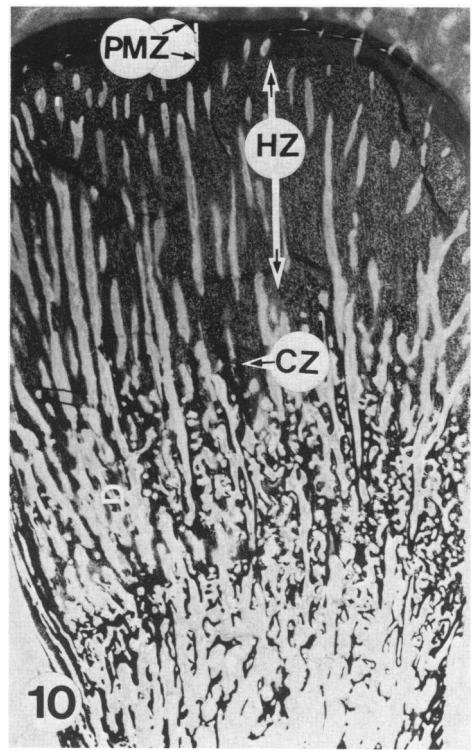


Figure 9—Proximal tibia of a hypocalcemic vitamin-D-deficient 3-week-old chick. The PMZ is markedly enlarged. The HZ is expanded at the expense of a reduced CZ. (Von Kossa stain with H&E and counterstain, ×7.1)
Figure 10—Proximal tibia of a hypophosphatemic vitamin-D-deficient 3-week-old chick. The PMZ is normal. The HZ is markedly expanded and the CZ reduced. (Von Kossa stain with H&E counterstain, ×7.1)

Table 5—Measurements of PEVs and MVs in Chicks Fed Normal and Deficient Diets

Group	PEVs (number)	Length (mm) Maximum	Length (mm) Minimum	MVs (number)	Length (mm) Maximum	Length (mm) Minimum	Number PEVs Number MVs
A Normal	11 ± 3	0.8 ± 0.1	0.8 ± 0.1	26 ± 5	2.6 ± 0.4	2.6 ± 0.4	0.40 ± 0.07
B Vitamin-D_3-deficient	11 ± 2	2.5 ± 0.9*	1.3 ± 0.5*	15 ± 2*	2.5 ± 1.4	2.2 ± 1.2	0.79 ± 0.18*
C Vitamin-D_3-deficient High calcium	9 ± 3	0.9 ± 0.3	0.8 ± 0.2	21 ± 7	3.8 ± 1.3*	3.8 ± 1.4*	0.40 ± 0.06
D $Al(OH)_3$	9 ± 4	0.6 ± 0.1	0.6 ± 0.1	22 ± 4	3.9 ± 1.9*	3.8 ± 2.0*	0.42 ± 0.14

* The series of values generating the values marked with asterisks differ from the series generating normal values at a level of $P = 0.01$.

mally large proportion of the terminal marrow spaces occupied by cells (Figure 17, Table 7). Many of the cells were large cells with intense diffuse cytoplasmic acid phosphatase activity (Figure 18). Hypophosphatemic animals had an abnormally low proportion of the terminal marrow occupied by cells (Figure 16, Table 7). Large cells with strong acid phosphatase activity were not prominent in these animals (Figure 19).

Discussion

The observations that growth plate lesions in vitamin-D-deficient chicks differ depending on whether the animals become hypocalcemic or hypophosphatemic[1-3] and that the corresponding lesions can be produced by vitamin-D-replete diets producing hypocalcemia or hypophosphatemia[23] are not new, but they have not been widely noted in the literature. It is obviously important to be aware of such a distinction if the results of studies in chicks are to be applied to studies of mammalian rickets.[22]

Vitamin-D-deficient growth plate abnormalities in mammals, including man, are morphologically and etiologically comparable to hypophosphatemic lesions in chicks. Vitamin D deficiency in mammals is characterized by an increase in hypertrophic chondrocytes[23-31] and hypophosphatemia, rather than hypocalcemia,[24,32] and is generally produced by diets low in phosphate and high in calcium, in addition to being D-deficient.[24,26] As is the case with birds, similar lesions can be produced by vitamin-D-replete diets low in phosphates.[28] Rachitic lesions in D-deficient rats can be healed by feeding them D-deficient diets with high phosphate levels.[33] These data indicate that studies of hypophosphatemic or vitamin-

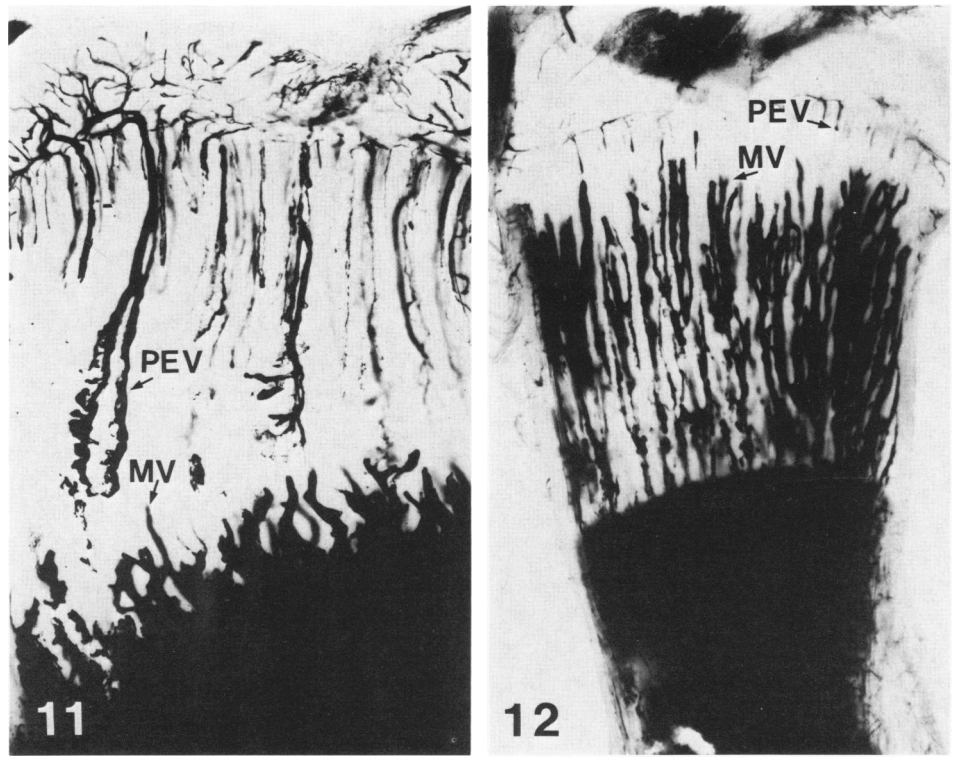


Figure 11—Vascular supply of the proximal tibia of a hypocalcemic vitamin-D-deficient 3-week-old chick. PEVs are markedly elongated and display lateral branching. MVs are short and blunt and display lateral branching. (India ink preparation, × 10)
Figure 12—Vascular supply of the proximal tibia of a hypophosphatemic vitamin-D-deficient 3-week-old chick. PEVs are of normal length but reduced in number. MVs have a normal distribution and appearance. (India ink preparation, × 7.1)

Table 6—Tritiated-Thymidine-Labeled Cells in Growth Plates from Chicks Fed Normal and Deficient Diets

Group	Cells/unit width*	Cells/unit area†
A Normal (n = 8) (Ca 11.5 ± 1.3, P 9.4 ± 0.9)	135 ± 27	135 ± 27
B Vitamin-D_3-deficient (n = 8) (Ca 6.8 ± 0.9; P 7.5 ± 2.1)	120 ± 28	39 ± 14‡
D Phosphate-deficient (n = 9) (Ca 11.3 ± 1.3; P 4.8 ± 1.4)	55 ± 17‡	55 ± 17‡

* Mean ± 1 standard deviation of the total number of labeled cells per 500 μ wide strip between epiphysis and hypertrophic zone.

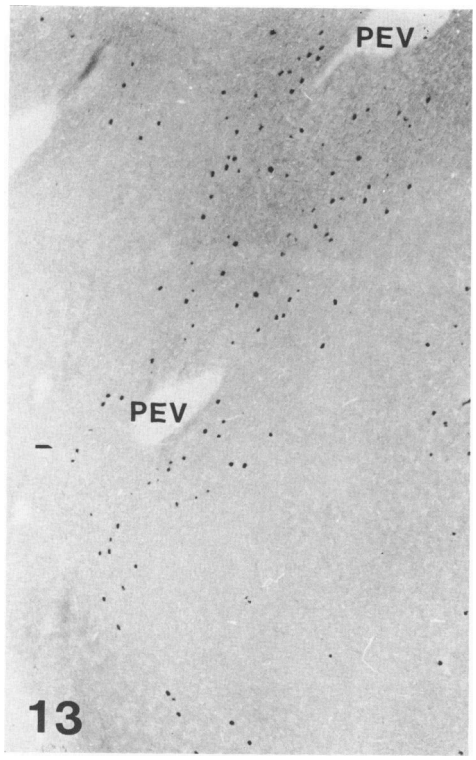
† Mean ± 1 standard deviation of the total number of labeled cells per 500 μ wide strip divided by number of 500 × 500 μ^2 fields containing labeled cells in the strip.

‡ These series of values differ from the series generating normal group mean at a level of $P = 0.01$.

D-deficient hypophosphatemic chicks are likely to be applicable to mammals.

On the other hand, it is not clear whether data on hypocalcemic or vitamin-D-deficient hypocalcemic chicks are applicable to mammals. Two types of rickets have been described in men[32] and rats,[34] based on differences in diet and serum calcium and phosphate levels. Human rickets resulting from a calcium-deficient diet and healed by calcium therapy has been documented radiologically.[35] The patient was hypocalcemic and hypophosphatemic. Rachitic lesions were attributed to the latter condition which were thought to be the result of secondary hyperparathyroidism.

Histologic abnormalities in mammals with rickets differing etiologically from typical hypophosphatemic vitamin-D-deficient or pure hypophosphatemic types fall into several categories. Vitamin-D-deficient hypocalcemic normoposphatemic rats[36] and normocalcemic hyperphosphatemic uremic rats[37,38] have lesions typical of hypophosphatemic rickets. The latter heal by vitamin D therapy.[38] Hypocalcemic hyperphosphatemic uremic rats do not have typical hypophosphatemic growth plate lesions and have marrow changes similar to those in hypocalcemic chicks.[37,38] Most uremic children have narrow growth plates due to growth arrest or excessive resorption by marrow,[40-42] but some growth plates are thickened (Figure 1b in Krempien et al,[40] for example) and the growth plate changes in uremic children include marked disturbances in the proliferation zone with loss of chondrocyte orientation, a normal or reduced hypertrophic zone, and irregular invasion by marrow,[40,41] all changes similar to those in hypocalcemic chicks. Humans and other mammals with long-standing nutritional rickets also develop invasion of the growth plate by epiphyseal vessels[26,29] and "bizarre" cartilage similar to the excessive proliferative cartilage in hypercalcemic chicks.[29] It appears, therefore, that mammals develop growth plate abnormalities similar to those typical of hypocalcemic avian rickets, but the lesions have not yet been specifically ascribed to hypocalcemia in mammals.



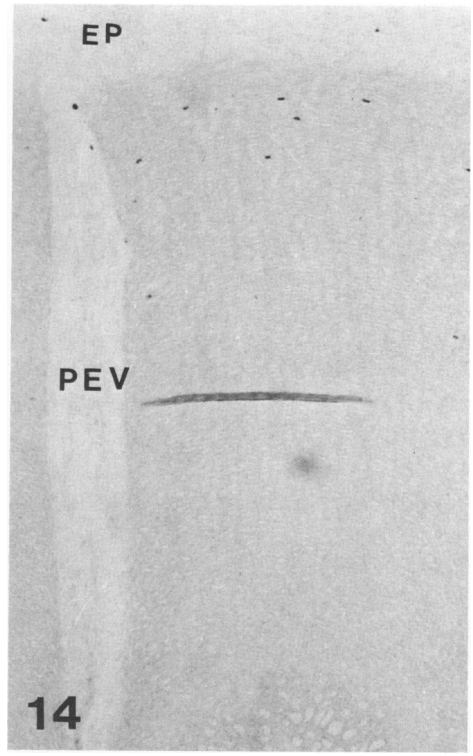


Figure 13—Distribution of DNA synthesizing chondrocytes in the proximal tibial growth plate of a hypocalcemic vitamin-D-deficient 3-week-old chick. Tritiated thymidine uptake is demonstrated throughout the PMZ, especially along the course of elongated PEVs. (Preparation as described in text; light green counterstain, × 100) **Figure 14**—Distribution of DNA-synthesizing chondrocytes in the proximal tibial growth plate of a hypophosphatemic vitamin-D-deficient chick. Labeled cells are normally distributed but reduced in number. (Light green counterstain, × 100)

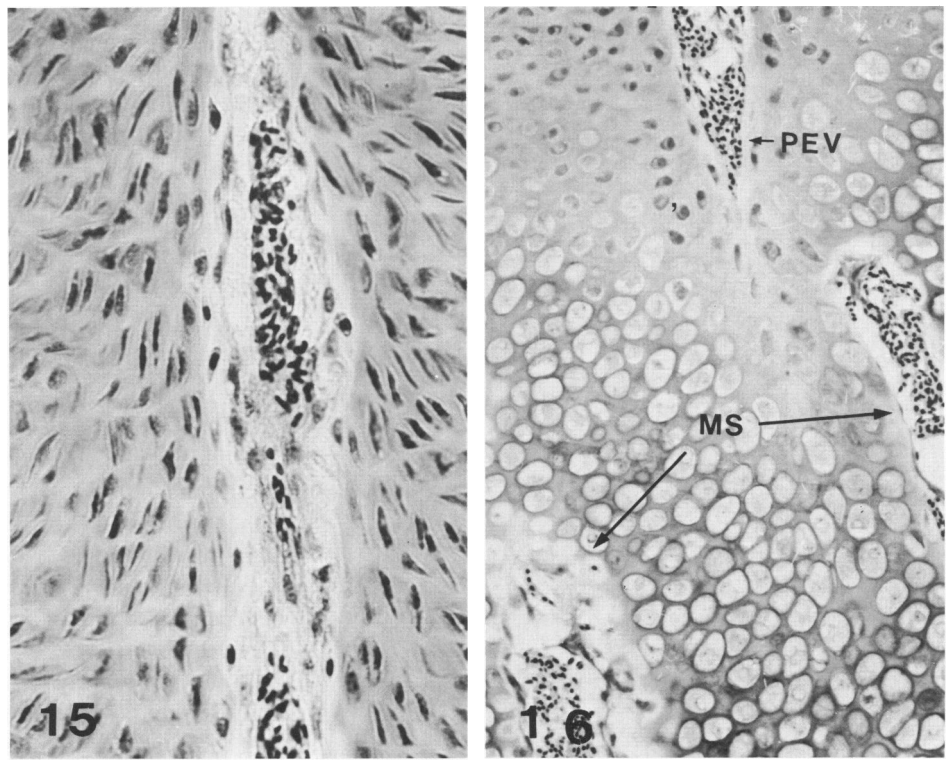


Figure 15—PEV in the PMZ of a hypocalcemic vitamin-D-deficient 3-week-old chick. Vessels appear normal but surrounding chondrocytes are abnormally oriented. (Von Kossa stain with H&E counterstain, ×400) **Figure 16**—PEV and terminal MSs in a hypophosphatemic vitamin-D-deficient 3-week-old chick. PEVs and chondrocytes are normal, but cells are reduced and vessels correspondingly more prominent than normal in the terminal MS. (Von Kossa stain with H&E counterstain, ×100)

We undertook this study to ascertain what changes occur in growth plate vessels in hypocalcemic and hypophosphatemic avian rickets. Truetta's observation that cutting[4] or compressing[5] metaphyseal vessels resulted in accumulation of uncalcified hypertropic cartilage in the growth plate in a manner similar to that of rickets suggested that in hypophosphatemic avian rickets, there would be decreased growth of metaphyseal vessels. Studies on the vascular changes in the growth plate of rachitic rats fed a Steenbock Black (hypophosphatemic) diet showing early dilatation and lack of penetration of cartilage by terminal metaphyseal capillaries as the cause of accumulation of hypertrophic chondrocytes[25,26] supported that hypothesis. On the other hand, during later stages of mammalian rickets, renewed penetration of cartilage occurs in the form of irregular bushlike tufts.[25,26,29] This occurs at the time when proliferative activity by growth plate chondrocytes has ceased and is not associated with further accumulation of hypertrophic cartilage.[25] In some cases, the abnormal vascular tufts grow all the way through the growth plate and form anastomoses with epiphyseal vessels.[25]

Our study showed that in hypophosphatemic avian rickets, longitudinal growth of metaphyseal vessels into cartilage was not impaired, and in fact the marrow spaces were longer than normal. Despite this, the amount of hypertrophic cartilage in the growth plate was greater than normal. The most likely explanation we could find to explain these findings was a decrease in the ratio of cells to blood vessels in the marrow spaces. Ultrastructural studies of normal avian growth plates have been interpreted as showing that longitudinal penetration of avian growth plate cartilage is carried out by inconspicuous mononuclear cells opening chondrocyte lacunae for subsequent ingrowth of vessels. In contrast, lateral resorption of cartilage around the sides of the marrow spaces is carried out by large numbers of multinuclear and mononuclear chondroclasts.[43] If that interpretation is correct, the decrease in the ratio of cells to blood vessels found in our study would explain how the length of marrow spaces and the total amount of hypertrophic cartilage could both be increased in hypophosphatemic rickets. Other investigators have shown that the decrease in marrow cells includes a decrease in the number of chondroclasts thought to be due to suppression of the parathyroid glands by hypophosphatemia.[2,3]

It seems unlikely from our results that the accumulation of hypertrophic cartilage is due to increased proliferation of chondrocytes, because the total number of DNA-synthesizing cells per unit width of growth plate is reduced. This is in keeping with studies showing normal[44] or decreased[45] DNA synthesis in hypophosphatemic rats. It is also unlikely that increased synthesis of matrix is the cause of the increased hypertrophic zone, since in hypophosphatemic rickets

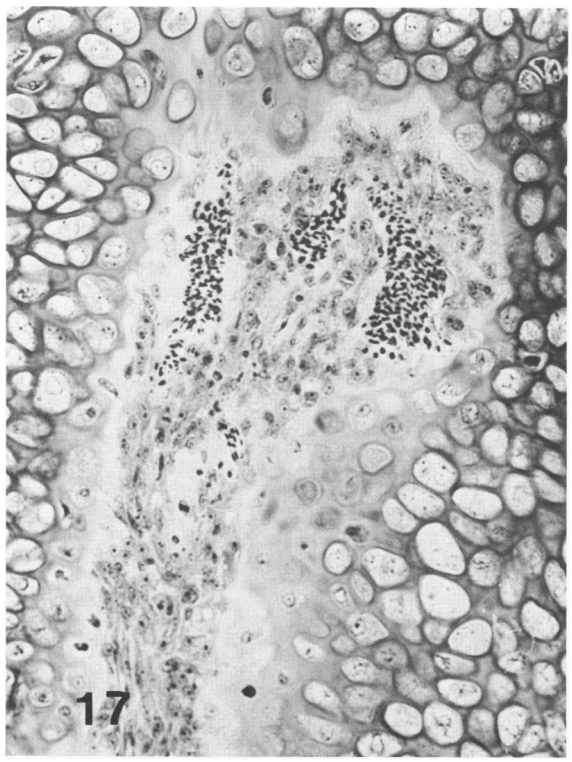


Figure 17—Terminal MS in a hypocalcemic vitamin-D-deficient 3-week-old chick. The number of cells in the stalk and adjacent to cartilage is increased, and the vascular space is reduced in comparison with normal. (Von Kossa stain with H&E counterstain, ×400)

in mammals matrix synthesis stops in the hypertrophic zone.[44,46,47] As we have shown above, hypophosphatemic rickets in birds and mammals are similar in most other aspects; so it is likely that they are also similar with respect to cartilage matrix synthesis. From these considerations, we agree with the conclusions of other investigations[22,23,25,26,28,29] that the accumulation of hypertrophic cartilage in hypophosphatemic rickets is probably due to decreased resorption of cartilage by marrow. Our studies indicate that in birds the major deficit is decreased lateral resorption of cartilage because of decreased marrow cells, as opposed to decreased longitudinal penetration of cartilage, which is carried out by small mononuclear cells and vessels.

Table 7—Proportion of Marrow Occupied by Cells and Blood Vessels in Chicks Fed Normal and Deficient Diets

Group	Cell counts/ vessel counts
A Normal (n = 7)	1.8 ± 0.4
B Vitamin-D_3-deficient (n = 7)	3.9 ± 0.8*
C Vitamin-D_3-deficient, high calcium (n = 8)	0.30 ± 0.13*
D Normal + $Al(OH)_3$ (n = 9)	0.34 ± 0.12*

The series of values generating the values with asterisks differs from the series generating the normal group mean at a level of $P = 0.01$.

In contrast to the findings on hypophosphatemic rickets, hypocalcemic rickets is characterized by an increased ratio of cells to vessels in the marrow and reduced numbers of broad blunt marrow spaces of normal length. The total amount of hypertrophic cartilage in these animals is normal. These findings indicate that in this form of rickets the relative rates of chondrocyte hypertrophy and resorption of cartilage by marrow are equal, as they are in normal animals. As discussed further below, the rate of chondrocyte hypertrophy may be reduced, implying that the rate of cartilage resorption must be correspondingly reduced, or the hypertrophic cartilage of the growth plate would disappear entirely. This phenomenon has been described in uremic mammals[39,40] and occurs in some of our hypocalcemic chicks, although it is infrequent enough that it does not show up in the statistics of a large group.

Nutrition and viability of mammalian growth plates is dependent on an intact epiphyseal vascular system.[4,5] The vessels directly responsible are a capillary plexus between the epiphyseal boney plate and the reserve cell zone of the growth plate.[7] The avian counterparts of these vessels are the perforating epiphyseal vessels.[48–50] Because they are thought to play a nutritive

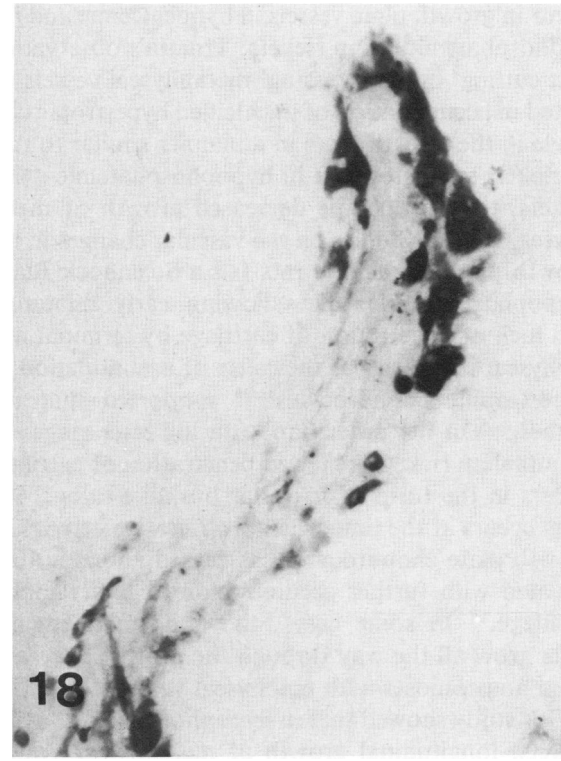


Figure 18—Tartrate-resistant acid-phosphatase-positive cells in the marrow of a hypocalcemic vitamin-D-deficient 3-week-old chick. The number of positive cells and intensity of staining is increased in comparison with normal and phosphate-deficient chicks. (Napthol AS-B1 substrate with fast garnet GBC capture, ×400)

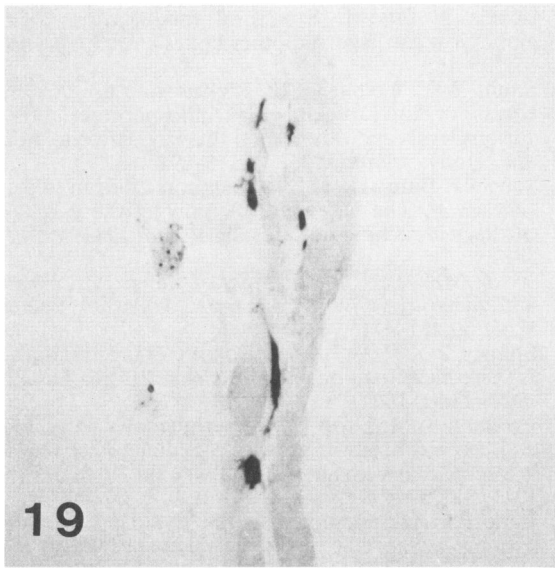


Figure 19 — Tartrate-resistant acid-phosphatase-positive cells in the marrow of a hypophosphatemic vitamin-D-deficient 3-week-old chick. The number of positive cells and intensity of staining is decreased in comparison with hypocalcemic animals. (Napthol AS-B1 substrate with fast garnet GBC capture, × 400)

role for proliferating and maturing chondrocytes,[48,50,51] we hypothesized that they would show increased growth in a situation, such as hypocalcemic rickets, in which the PMZ of the growth plate is increased. This study showed that this hypothesis is correct.

The most likely explanation for the increase in size of the PMZ is a delay in chondrocyte hypertrophy, as suggested by Bisaz et al.[22] It is unlikely that increased chondrocyte replication is the mechanism, because the total number of DNA-synthesizing chondrocytes per unit width of growth plate is not increased. In studies still in progress we used point-counting techniques to measure parameters of chondrocyte maturation. Preliminary results indicate that the total number of cells per unit width in the full thickness of the growth plate (from epipysis to metaphysis) is essentially the same in normal, hypocalcemic, and hypophosphatemic growth plates. In hypocalcemic growth plates, the major factors responsible for increased growth plate thickness are an increase in the relative cell size and in the relative amount of intercellular matrix in the PMZ. This is accompanied by a decrease in cell density. These data support the concept that prolonged chondrocyte maturation and matrix synthesis, rather than increased chondrocyte proliferation, is the major cause of growth plate thickening in hypocalcemic rickets.

We do not know the cause of chondrocyte hypertrophy, nor precisely how it is related to growth of perforating epiphyseal vessels and marrow. As shown in this study, the zone of chondrocyte hypertrophy in birds always corresponds to the level at which perforating epiphyseal and metaphyseal vessels end as they penetrate the growth plate from opposite sides. Based on their studies on the vascular supply of mammalian growth plates, Trueta and colleagues have proposed that chondrocyte hypertrophy is the result of displacement of cells from their nutritional supply adjacent to the reserve cell zone.[6] Wise and Jennings have suggested that in birds chondrocyte hypertrophy causes obliteration of perforating epiphyseal vessels by increasing the external pressure on them, and that the resulting scars act as preferred pathways for growth of marrow into the growth plate.

In the following paper on recovery from hypocalcemic rickets,[52] we present further data relevant to the theories above. That study shows that obliteration of perforating epiphyseal vessels accompanies an elevation of serum calcium. Obliteration is followed by necrosis of chondrocytes in newly devascularized areas and by chondrocyte hypertrophy in areas in which perforating epiphyseal vessels survive. These findings indicate that PEVs are necessary for viability of chondrocytes and also pay a positive role in determining where hypertrophy will occur. In that sense, their length is causally related to the thickness of the PMZ as the growth plate reverts to normal after hypercalcemia. As shown in this study, their length is also related to the position of the hypertrophic zone (and hence the thickness of the PMZ) under hypocalcemic conditions, although here cause-and-effect relationships are less clear. We have attempted to explain the observations in this and the following paper by hypothesizing that chondrocyte hypertrophy, longitudinal growth of marrow and epiphyseal vessels, and proliferation and differentiation of cartilage-resorbing marrow cells are controlled directly or indirectly by calcium. Without our specifying exact mechanisms, it appears that calcium promotes chondrocyte hypertrophy and longitudinal growth of marrow and inhibits growth of perforating epiphyseal vessels and lateral resorption of cartilage by marrow. It is also possible that some of these are direct effects of phosphate and/or secondary effects of parathormone. Further data and discussion relevant to these hypotheses are presented in the following paper.

References

1. Jande SS, Dickson IR: Comparative histologic study of the effects of high calcium diet and vitamin D supplements on epiphyseal plates of vitamin D deficient chicks. Acta Anat 1980; 108:463-468

2. Groth W: Experimentelle Untersuchingen über Calcium und Phosphat Mangel osteopathien und ihre Beziehung zur Epithelkörperchenfunktion und zum Mineralstoffgehalt des Blutes bei Wachsenden hühnern. Zentralbl Veterinaermed 1962; 9:1009-1040
3. Groth W, Frey H: Zur Differenzierung der Wirkung eines Mangels an Calcium Phosphor oder Vitamin D auf Knochen, Blut und inersekretorische Drüsen des hühnern. Zentralbl Veterinaermed 1966; 13:202-319
4. Trueta J, Amato VP: The vascular contribution to osteogenesis: III. Changes in the growth cartilage. J Bone Jt Surg 1960; 42B:571-587
5. Trueta J, Trias A. The vascular contribution to osteogenesis: IV. The effect of pressure on the epiphyseal cartilage of the rabbit. J Bone Jt Surg 1961; 43B:800-813
6. Trueta J, Little K: The vascular contribution to osteogenesis: II. Studies with the electron microscope. J Bone Jt Surg 1960; 42B:367-376
7. Trueta J, Morgan JD: The vascular contribution to osteogenesis: I. Studies by the injection method. J Bone Jt Surg 1960; 42B:97-109
8. Zettner A, Seligson D: Application of atomic absorption spectrophotometry in the determination of calcium in serum. Clin Chem 1964; 10:869-890
9. Henry RJ: Inorganic ions: Determination of inorganic phosphorus: Clinical Chemistry: Principles and Techniques. New York, Hoeber Medical Division, Harper and Row, 1964; pp. 411-416
10. Hurst RO: The determination of nucleotide phosphorus with a stannous chloride-hydrazine sulphate reagent. Can J Biochem 1964; 42:287-292
11. Phosphorus Rapid Stat Kit 1976; Pierce Chemical Co.
12. Stefanini M, DeMartino C, Zamboni L: Fixation of ejaculated spermatozoa for electron microscopy. Nature 1967; 216:173-174
13. JB-4 Embedding Kit. Data Sheet 123; 1976. Polysciences Inc, Warrington, Pennsylvania
14. Graham RC, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 1966; 14:291-302
15. Pearse AGE: Histochemistry Theoretical and Applied. 2nd edition. Boston, Little, Brown, 1961; p. 932
16. Pearse AGE:[15]
17. Sigma Technical Bulletin 386, January 1980.
18. Garner A, Ball J: Quantitative observations on mineralized and unmineralized bone in chronic renal azotemia and intestinal malabsorption syndrome. J Pathol Bacteriol 1966; 91:545-561
19. Remington RD, Schort MA: Distribution free and nonparametric methods: The Wilcoxon Rank Sum Test, Statistics with Applications to the Biological and Health Sciences. Englewood Cliffs, NJ, Prentice-Hall, Inc, 1970; pp 313-315
20. Hauseman TU, Helger R, Rich W, Gross W: Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. Clin Chem Acta 1967; 15:241-245
21. BMC Reagent Set, Alkaline Phosphatase, Catalog No. 1238454. Bio Dynamics/BMC, Indianapolis, Ind
22. Bisaz SY, Schenk R, Kunin AS, Russell RGG, Muhlbauer R, Fleisch H: The comparative effects of vitamin D deficiency and ethane-1-hydroxy-1,1-diphosphonate administration on the histology and glycolysis of chick epiphyseal and articular cartilage. Calc Tiss Res 1975; 19:139-152
23. Dodds GS, Cameron HC: Studies on experimental rickets in rats: I. Structural modifications of the epiphyseal cartilage and other bones. Am J Anat 1934; 55:135-165
24. Shohl AT, Wolbach SB: Rickets in rats: XV. The effects of low calcium high phosphorous diets at various levels and ratios upon the production of rickets and tetany. J Nutr 1935, 11:275-291
25. Trueta J, Buhr AJ: The vascular contribution to osteogenesis: V. The vasculature supplying the epiphyseal cartilage in rachitic rats. J Bone Jt Surg 1963; 45B:572-581
26. Irving MH: The blood supply of the growth cartilage and metaphysis in rachitic rats. J Pathol Bacteriol 1965, 89:461-471
27. Shipley PG, Park EA, McCollum EU, Simmonds N: Is there more than one kind of rickets? Am J Dis Child 1922, 23:91-106
28. Freeman S, McClean FC: Experimental rickets: Blood and tissue changes in puppies receiving a diet very low in phosphorus with and without vitamin D. Arch Pathol 1941, 32:387-408
29. Park EA: Observations on the pathology of rickets with particular reference to the changes at the cartilage-shaft junctions of the growing bones. Harvey Lectures 1939, 34:157-213
30. Follis RH: Renal rickets and osteitis fibrosa in children and adolescents. Bull Johns Hopkins Hosp 1950, 87: 393-614
31. Follis RH, Park EA, Jackson D: The prevalence of rickets during the first two years of life. Bull Johns Hopkins Hosp 1952, 91:480-497
32. Lapatsanis P, Makaronis G, Uretos C, Doxiadis S: Two types of nutritional rickets in children. Am J Clin Nutr 1976, 29:1222-1226
33. Jones JH, Cohn BNE: The healing of rickets in rats on a diet containing negligible amounts of calcium and vitamin D. J Nutr 1935, 11:293-301
34. Harrison JE, Hitchman AJW, Hitchman A, Hasany SA, McNeil KG, Tam LS: Differences between the effects of phosphate deficiency and vitamin D deficiency on bone metabolism. Metabolism 1980, 29:1225-1233
35. Koon SW, Fraser D, Reilley BJ, Hamilton JR, Gall DG, Bell L: Rickets due to calcium deficiency. N Engl J Med 1979, 297:1264-1266
36. Rasmussen P: The action of vitamin D deficiency on bone tissue and the epiphyseal plate in rats given adequate amounts of calcium and phosphorus in the diet. Arch Oral Biol 1969, 14:1293-1304
37. Mehls O, Ritz E, Gilli G, Schmidt-Gay KH, Krempien B, Wesch H, Pryer P: Skeletal Changes in growth in experimental uremia. Nephron 1977, 18:288-300
38. Mehls O, Ritz E, Gilli G, Krampien B, Schmidt-Gay KH, Udes H: Growth and bone lesions in experimental uremia, Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism. Edited by SW Norman. Berlin, deGruyter, 1977, pp 685-688
39. Mehls O, Ritz E, Gilli G, Wangdaz T, Krempien B: Effect of vitamin D on growth in experimental uremia. Am J Clin Nutr 1978, 31:1927-1937
40. Krempien B, Mehls O, Ritz E: Morphological studies on the pathogenesis of epiphyseal slipping in uremic children. Virchows Archiv [Pathol Anat] 1974, 362: 129-143
41. Mehls O, Ritz E, Krensser W, Krempien B: Renal osteodystrophy in uremic children, Clinics in Endocrinology and Metabolism. Vol 9, Metabolic Bone Disease. Edited by LV Alvioli, LG Raizz. Philadelphia, W. B. Saunders Co., 1980, pp 151-176
42. Mehls O, Ritz E, Krempien B, Gilli G, Link K, Willich E, Scharer K: Slipped epiphyses in renal osteodystro-

phy. Arch Dis Child 1975, 50:545–554
43. Howlett CR: The fine structure of the proximal growth plate and metaphysis of the avian tibia: Endochondral osteogenesis. J Anat 1980, 130:745–768
44. Mankin HJ, Lippiello L: Nucleic acid and protein synthesis in epiphyseal plates of rachitic rats: An autoradiographic study. J Bone Jt Surg 1969, 51A:862–879
45. Simmons DV, Kunin AS: Development and healing of rickets in rats: I. Studies with tritiated thymidine and nutritional considerations. Clin Orthop 1970, 68:251–200
46. Simmons DJ, Kunin AS: Development and healing of rickets in rats: II. Studies with tritiated proline. Clin Orthop 1970, 68:216–272
47. Hjertquist SO: Autoradiographic study of the proximal tibial growth plate in normal and rachitic rats after administration of radiosulphate. Acta Pathol Microbiol Scand Suppl 1962, 54:99–100
48. Beaumont GD: The intraosseous vasculature of the ulna of Gallus domesticos. J Anat 1967, 101:543–554
49. Lutfi AM: Mode of growth, fate and function of cartilage canals. J Anat 1970, 106:135–145
50. Wise DR, Jennings AR: The development and morphology of the growth plates of long bones of the turkey. Res Vet Sci 1973, 14:161–166
51. Hunt CD, Ollerich DA, Nielsen FH: Morphology of the perforating cartilage canals in the proximal tibial growth plate of the chick. Anat Rec 1979, 194:143–147
52. Huffer WE, Lacey DL: Studies on the pathogenesis of avian rickets: II. Necrosis of perforating epiphyseal blood vessels during recovery from hypocalcemic vitamin-D_3-deficient rickets. Am J Pathol 1982, 109:304–311