Pathology of vitamin D deficiency in growing turkeys

by

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A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

Signatures have been redacted for privacy

For the Graduate College

Iowa State University
Ames, Iowa
1986
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GENERAL INTRODUCTION

Leg weakness in turkeys results in significant economic losses for the turkey industry. Approximately 4-6% of male turkeys are culled or die from skeletal deformities and the losses are even more significant when depressed weight gain and removal of deformed limbs at processing are considered.\(^1\) \(^2\) \(^7\) Although part of the losses are due to infectious disease, noninfectious causes such as vitamin D-deficiency are major contributors to skeletal diseases.\(^1\) \(^5\) In conventional, light-restricted confinement housing, turkeys receive minimal quantities of vitamin D\(_3\) via photolysis and must rely upon dietary supplementation. Avian species discriminate against vitamin D\(_2\) and must receive adequate amounts of vitamin D\(_3\).\(^8\) \(^7\) Therefore, turkeys appear to be more vulnerable to low biopotent vitamin D\(_3\) in their ration or interference with absorption of vitamin D\(_3\) from the diet than other food producing animals.\(^1\) \(^4\) \(^7\) \(^8\) \(^9\) \(^7\) \(^2\) \(^7\) \(^2\) \(^7\)

Experimental and natural vitamin D-deficient rickets are well documented in chickens,\(^1\) \(^7\) \(^8\) \(^2\) \(^4\) \(^8\) \(^1\) \(^7\) \(^8\) \(^2\) \(^4\) \(^8\) but there are only a few reports in turkeys.\(^1\) \(^4\) \(^7\) \(^8\) \(^2\) \(^4\) \(^8\) \(^1\) \(^7\) \(^8\) \(^2\) \(^4\) \(^8\) Avian rickets primarily occurs during the time of most rapid skeletal growth (the first month of life) and is grossly apparent in areas that cartilage contributes most significantly to during skeletal growth.\(^11\) \(^0\) \(^2\) \(^1\) \(^0\) \(^2\) Experimental avian rickets is characterized by: lameness, retarded body growth, hypocalcemia, hypophosphatemia, elevated alkaline phosphataseemia, parathyroid hyperplasia, and severe bone distortion. Bone lesions are
characterized by distorted growth plates, failure of mineralization of cartilage and osteoid, decreased bone ash, fibrous osteodystrophy, and increased numbers of osteoclasts.

Growing turkeys in comparison to growing chickens have slightly higher levels of serum calcium and phosphorus,\textsuperscript{14} higher rates of gastrointestinal absorption of calcium and phosphorus per unit of body weight,\textsuperscript{104,105} a higher percentage of bone ash,\textsuperscript{14,39} greater intestinal absorption of dietary vitamin D,\textsuperscript{17} and higher amounts of intestinal calcium binding protein.\textsuperscript{14} These data point out important differences between turkeys and chickens in terms of vitamin D and mineral metabolism. When chicken breeds with faster growth rates are compared to slower growing chickens, the faster growing birds have elevated renal 1-hydroxylase activity and higher levels of intestinal calcium binding protein.\textsuperscript{16} In different avian bones, growth plates grow at different rates.\textsuperscript{168} When the above differences are considered, it may not be correct to assume that the clinical, gross and microscopic descriptions of vitamin D-deficiency in chickens are applicable to turkeys.

In turkeys, only one experimental vitamin D-deficiency study describing bone changes has been documented.\textsuperscript{34} In this study there were no clear statements concerning the lighting or possible exposure to sunlight, there were only two sampling times at 10 and 24 days, the bones were demineralized, estimates but no quantitation of osteoblasts and osteoclasts were made and the parathyroid glands were not examined. In this experiment, plasma phosphorus was depressed and calcium values were
normal which is inconsistent with experimental vitamin D-deficiency in chickens.

The objectives of this research were to i) record the clinical, gross, histological, histomorphometric, and ultrastructural temporal changes in vitamin D-deficient growing turkeys, ii) characterize the effect of vitamin D-deficiency among bones with different growth rates, and iii) compare vitamin D₃, 1,25(OH)₂D₃ and 1,25(OH)₂D₃ combined with 24,25(OH)₂D₃ for their capability to prevent qualitative changes of vitamin D-deficiency in growing turkey poults.

This dissertation uses the alternate format. The reference format is that used in the journal, Veterinary Pathology. Three manuscripts will be submitted for publication in refereed scientific journals. The first manuscript has been submitted to Veterinary Pathology, the second manuscript will be submitted to Veterinary Pathology, and the third manuscript will be submitted to the journal, Bone. A general introduction and literature review precedes the first manuscript and literature cited for this portion of the dissertation is listed in alphabetical order following the general summary and discussion. Literature cited for each manuscript follows the discussion and is listed in alphabetical order. A general summary and discussion follows manuscript three.

Olaf R. Hedstrom was the principal investigator for this study.
LITERATURE REVIEW

Historical background. No agreement has been reached as to when the first effects of vitamin D on man were discovered. Hints and clinical descriptions of rickets were described 100-200 AD but it was not until the eighteenth century that rickets was recognized as a disease state and not until the nineteenth century that nutritional (vitamin factor) or ultraviolet sun rays were associated with rickets.

DeLuca points out that Mellanby discovered and proved through scientific experimentation that rickets was caused by a dietary deficiency and that cod-liver oil had strong anti-rachitic effects. Norman credits Huldschinsky as the first to cure rickets with ultraviolet light and recognized that a deficiency of sunlight was an additional etiology for rickets. The next significant contribution came when McCollum et al. showed that the anti-rachitic effect of cod-liver oil was due to a new vitamin and not the fat soluble vitamin A portion. He named this new factor, vitamin D. The anti-rachitic efficacy of sunlight and nutritional factor of cod-liver oil were recognized by most researcher workers during the early portion of the nineteenth century. In 1924, Steenbock and Black found that food irradiated with UV light in the absence of the animal had strong anti-rachitic properties when fed back to ricketic rats. Subsequently, this anti-rachitic factor was associated with nonsaponifiable lipids, particularly sterols. The chemical structure of vitamin D was identified in 1932 and vitamin D in 1936. Vitamin D, was shown to
be a secosteroid and later a prohormone rather than a vitamin. From this point on the major focus of vitamin D research has been on the role that vitamin D partakes in calcium and phosphorus metabolism and homeostasis: namely bone formation, bone mobilization, gastrointestinal absorption, and renal excretion and reabsorption of calcium and phosphorous. Vitamin D metabolism involves a wide variety of cell types in complex physiologic processes and consequently is required for life in higher animals.

**Vitamin D metabolism.** The major biologic function of vitamin D, in concert with parathyroid and calcitonin hormones, is to promote normal skeletal development and integrity through calcium and phosphorus homeostasis. Mineral homeostasis is manifested by the action of these three hormones on the intestine, bone and kidney.\(^{142}\)

Ultraviolet light exposure to the stratum germinativum layer of the skin initiates a nonenzymatic photolysis reaction (acting on 7-dehydrocholesterol), followed by synthesis of vitamin D\(_3\)^{94,141} A transport protein transfers the newly synthesized vitamin D\(_3\) from the skin to the liver.\(^{94}\)

Because vitamin D is a fat soluble vitamin, dietary exposure requires chylomicron formation, followed by absorption into small intestinal lacteals and transport by an alpha-2 globulin to the liver.\(^{68}\)

Vitamin D requires hepatic hydroxylation at carbon 25 before it can begin to exert its physiological activity or undergo any further metabolism.\(^{58,84,141,142}\) Both vitamin D\(_2\) (ergocalciferol) and D\(_3\) (cholecalciferol) undergo this hydroxylation. Only vitamin D\(_3\) metabolism will be discussed from this point forward because vitamin D\(_2\) can
undergo most metabolic transformations that vitamin D₃ does, avian species discriminate against vitamin D₂,¹⁹ and I used only vitamin D₃ in my experiments.

Hydroxylation of vitamin D₃ at carbon 25 occurs primarily within hepatocyte endoplasmic reticulum by a NADPH-dependent cytochrome P-450 enzyme system (25-hydroxylase).²¹⁸ A mixed function mitochondrial enzyme system can also hydroxylate vitamin D₃ at carbon 25.²⁵ The intestine and kidney can carry out this hydroxylation, but to a much lesser degree than found in the liver.²⁰¹ This metabolite leaves the liver and is transported to other organs by a plasma protein for further metabolism.³²

The major site of 25OHD₃ metabolism and further hydroxylation occurs in the kidney.⁵⁸,⁸⁴,¹⁴¹,¹⁴² ²⁵ hydroxyvitamin D₃ is hydroxylated by proximal-tubule, mitochondrial, mixed-function-monooxygenase (25 OH D₃-1-hydroxylase) to 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃).⁸⁵,²¹⁷ Extrarenal hydroxylation of 25OHD₃ to 1,25(OH)₂D₃ can occur in bone⁷⁰,⁹⁹ and placenta²⁰⁹ but to a much lesser amount than occurs in the kidney.⁵⁸ Oxidative cleavage of 1,25-(OH)₂D₃ forms calcitroic acid, a bile excretory product.¹⁵¹ Hydroxylation of 25OHD₃ at carbon 24 by a proximal-tubule, mitochondrial, cytochrome P-450 enzyme system (25-OH-D-24-hydroxylase) forms 24,25 dihydroxyvitamin D₃ (24,25(OH)₂D₃).⁵⁸,¹⁴² 24,25 dihydroxy-vitamin D₃ may be excreted into the biliary system after further metabolism to cholecalcific acid.⁵⁸ Much higher plasma concentrations of 24,25(OH)₂D₃ than 1,25(OH)₂D₃ are found in most animal
species. Intestine, cartilage and bone have minor 24-hydroxylase activity.

Additional metabolic pathways exist for 25OHD₃ or 1,25(OH)₂D₃. These metabolites are generally not physiologically significant or at least their significance is not yet known. Hydroxylation of 25OHD₃ at carbon 26 forms 25,26 dihydroxyvitamin D₃. 25 hydroxyvitamin D₃ can be functionalized at carbon 23, then oxidized at carbon 26, and finally lactonized at carbon 26, yielding 25-OH-D₃ 26,23-lactone. Another metabolite, 23,25 dihydroxyvitamin D₃ may represent an intermediate metabolite in the synthesis of this latter metabolite. Lactonization of 23,25(OH)₂D₃ may represent an excretory route. Oxidation of 1,25(OH)₂D₃ at carbon 23 forms 23-oxo-1,25(OH)₂D₃ and 23-oxy-1,25,26(OH)₃D₃ has been identified. Many additional metabolites have been identified but these metabolites are thought to be nonsignificant because their synthesis requires laboratory manipulation through administration of massive doses of vitamin D₃.

The major vitamin D₃ metabolite responsible for the physiologic actions of vitamin D is currently believed to be 1,25(OH)₂D₃. Following renal synthesis, 1,25(OH)₂D₃ is released and transferred in the blood stream to multiple tissues that contain specific receptor sites for 1,25(OH)₂D₃. 1,25 dihydroxyvitamin D₃ is thought to attach to a cytosol receptor protein and then somehow is transferred to the nucleus where its effects are mediated. Cells shown to have nuclear localization of 1,25(OH)₂D₃ include:
intestinal villi and crypt cells, kidney, bone osteoblasts and osteoclasts, stomach, skin, pituitary gland, parathyroid glands, mammary gland, and egg chorioallantoic membrane. After nuclear stimulation by 1,25(OH)\textsubscript{2}D\textsubscript{3}, transcription, translation and m-RNA induced protein synthesis then occurs. Consequently, 1,25(OH)\textsubscript{2}D\textsubscript{3} produces its effect similar to other steroid hormones. One such gene product is cytoplasmic calcium binding protein. 1,25-dihydroxyvitamin D\textsubscript{3} dependent calcium binding proteins have been detected in intestine, kidney, bone, skin, pancreatic islet cells and parathyroid glands. Intestinal synthesis of alkaline phosphatase and calcium activated ATPase can also be induced by 1,25(OH)\textsubscript{2}D\textsubscript{3}.

The primary function of 1,25(OH)\textsubscript{2}D\textsubscript{3} is to maintain and regulate plasma calcium and phosphorus levels, usually in concert with parathyroid or calcitonin hormones. Tightly controlled levels of calcium are absolutely essential to sustain life because of the diverse role calcium plays in body functions including: bone formation, neuronal excitation, muscle contraction, membrane structural integrity and permeability, cell adhesion, cell proliferation, enzyme activity, blood coagulation, intercellular communication and hormone release. Phosphorus is important for bone structure, intermediary metabolism (phosphorylated intermediates), genetic material (DNA, RNA), phospholipids, enzyme function, and membrane structure.

The major effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} are mediated through its interaction with the intestine, bone and kidney. Under normal circum-
stances, plasma levels of calcium and phosphorus are primarily increased by 1,25(OH)$_2$D$_3$ induced intestinal transport versus the important but often lesser effects of the other two organ systems. In the intestine, 1,25(OH)$_2$D$_3$ stimulates active transport of these minerals through separate transport systems.

Unfortunately, the exact mechanism of intestinal calcium and phosphorus transport are unknown. In rats, 1,25(OH)$_2$D$_3$ induced calcium transport has a biphasic response. The first response is rapid and probably nuclear mediated following cytosol receptor stimulation. After a precipitous drop, a second elevated and sustained response is found, which may be a programmed function of crypt cells. Nuclear stimulation results in synthesis of calcium transport proteins via gene transcription. Transport proteins may function at enterocyte brush border transferring calcium to the cytoplasm where it is sequestered by vesicles or mitochondria and later extruded into interstitial fluid at baso-lateral membranes. Calcium binding protein, a 1,25-(OH)$_2$D$_3$ induced gene product, at one time was thought to play a physiological significant role as a carrier protein for calcium transport; but recently, after much conflicting data in the literature, it is now thought to protect cells from large calcium fluxes induced by 1,25(OH)$_2$D$_3$. Calcium binding protein and other 1,25(OH)$_2$D$_3$ induced transport proteins have lost favor primarily because of the delay required for their synthesis (6-7 hours) versus the immediate response (1-2 hours) of increased intestinal calcium transport following 1,25(OH)$_2$D$_3$ stimulation. Calmodulin may also act as a
calcium carrier protein or act as a calcium sink when other calcium sinks such as mitochondria are not available. Brush border sites of chick enterocytes can be induced by 1,25(OH)$_2$D$_3$ to produce membrane lined vesicles capable of active transport of calcium or possible phosphorus. Others suggest that 1,25(OH)$_2$D$_3$ induces cell membrane lipid changes (ratio of phosphatidylcholine to phosphatidylethanolamine) which result in elevated calcium transport. Basal-lateral membrane transport of calcium may also be a 1,25(OH)$_2$D$_3$ inducible transport system.

Another mechanism by which 1,25(OH)$_2$D$_3$ increases serum calcium and phosphorus is through bone resorption. How this occurs is not known; in vivo, parathyroid hormone and 1,25(OH)$_2$D$_3$ probably act in concert to mobilize bone mineral. Both osteoclastic and osteocytic osteolysis may occur in direct response to these hormones. In vitro, 1,25(OH)$_2$D$_3$ and parathyroid hormones can inhibit bone collagen synthesis, alkaline phosphatase activity and other osteoblastic functions which may be necessary for bone resorption. Bone mobilization may be due to the steroid effect that 1,25(OH)$_2$D$_3$ has on osteoblasts and is initiated by specific osteoblast receptors for this hormone. These receptors appear to be regulated by glucocorticoids and cell replication. Furthermore, gene controlled protein synthesis of calcium binding protein following receptor stimulation by 1,25(OH)$_2$D$_3$ may somehow be involved with bone resorption.

Even less is known about the effects of 1,25(OH)$_2$D$_3$ on the kidney. Renal tubular reabsorption of calcium and phosphate secretion is
primarily controlled by parathyroid hormone.\textsuperscript{43} However, 1,25-(OH)\textsubscript{2}D\textsubscript{3} has been reported to stimulate renal calcium and phosphorus reabsorption in parathyroidectomized animals.\textsuperscript{84} Moreover, 1,25-(OH)\textsubscript{2}D\textsubscript{3} can rapidly stimulate synthesis of calcium binding protein in chick kidney.\textsuperscript{84}

Considerable controversy surrounds the significance of 24,25-(OH)\textsubscript{2}D\textsubscript{3} as an important metabolite required for the physiologic actions of vitamin D. 24,25 dihydroxyvitamin D\textsubscript{3} has shown significant vitamin D effects, usually when combined with 1,25(OH)\textsubscript{2}D\textsubscript{3}. This combination has been reported to be important in bone metabolism,\textsuperscript{92,125} cartilage growth and differentiation,\textsuperscript{52,64} calcium homeostasis,\textsuperscript{144} healing of rickets in chicks,\textsuperscript{29} or rachitic chick fracture repair,\textsuperscript{55,140} healing of rickets in rats,\textsuperscript{10,83} embryonic chick development,\textsuperscript{87} and maintenance of chick parathyroid gland size.\textsuperscript{88} Yet, these results are not supported by studies using the synthetic sterol 24,24-difluoro-25 hydroxyvitamin D\textsubscript{3} which cannot be hydroxylated at carbon 24, hence inhibiting conversion to 24,25(OH)\textsubscript{2}D\textsubscript{3}.\textsuperscript{79,147} The biologic activity of this synthetic sterol has been reported to be equivalent to 25OH\textsubscript{D}3 in reversing ricketic bone lesions in growing rats\textsuperscript{79,147} and this sterol maintained rats for two generations.\textsuperscript{37,109} Embryonic chicks grow and develop normally with this sterol.\textsuperscript{3} Consequently, these studies do not support a role for 24,25(OH)\textsubscript{2}D\textsubscript{3} as an active vitamin D metabolite. Some evidence suggests that 24-hydroxylation represents the first step in a degradative pathway.\textsuperscript{37,93}
Regulation of vitamin D metabolism centers around the control of renal 1-hydroxylase which is modulated according to the calcium and phosphorus needs of the animal. This enzyme system is responsive to 1,25(OH)₂D₃ itself as a negative feedback regulator so that elevated levels of 1,25(OH)₂D₃ inhibit 1-hydroxylase activity but in vitamin D deficient states this enzyme system is hyperactive. Furthermore, elevated levels of 1,25(OH)₂D₃ activate the 24-hydroxylase enzyme system so that 24,25(OH)₂D₃ is synthesized in times of excess production of 1,25(OH)₂D₃. Hypocalcemia and hypophosphatemia are strong stimulators of 1-hydroxylase activity. In hypocalcemic conditions, parathyroid hormone synthesis is increased; parathyroid hormone in turn is a strong activator of the 1-hydroxylase enzyme system. In chicks, insulin may be required to potentiate parathyroid hormone stimulatory effect on renal 1-hydroxylase activity. When serum calcium levels become normalized, synthesis can shift from 1,25(OH)₂D₃ towards 24,25(OH)₂D₃. In vivo, production of 1,25(OH)₂D₃ is, therefore, tightly monitored by calcium demand through bimodal regulation of renal 1-hydroxylase. Renal 1-hydroxylase immediately responds to mitochondrial calcium and phosphorus ionic changes versus a delayed response to parathyroid hormone. For example, in vitamin D-deficiency, low plasma levels of 1,25(OH)₂D₃ and hypocalcemia with subsequent hyperparathyroidism will markedly stimulate renal 1-hydroxylase activity. Estrogen, progesterone and testosterone can synergistically stimulate 1-hydroxylase activity in egg laying hens, which indirectly affects serum calcium.
Growth hormone and prolactin are additional hormones that can stimulate 1-hydroxylase activity. 25-hydroxylase activity is not tightly controlled since it is not affected by calcium or phosphorus levels and the concentration of 250HD_3 largely depends upon the dietary intake of vitamin D or exposure to sunlight.

Besides regulating skeletal homeostasis, vitamin D has been shown to be required for maximum fertility in the rat, and vitamin D-deficient chickens lay progressively fewer numbers of thinner eggs until laying stops. In addition, chick embryos do not develop properly when treated by only 1,25(OH)_2D_3.

Recently, additional important functions have been attributed to 1,25(OH)_2D_3: induction of further differentiation of leukemia cells, induction of monocyte differentiation (which then could be associated with osteoclastogenesis), inhibition of T lymphocyte interleukin-2 production, inhibition of antigen induced T cell function and interaction with receptors on circulating monocytes. Therefore, 1,25(OH)_2D_3 may have some chemotherapy potential for certain kinds of neoplasms and it might be an immunoregulatory hormone.

Bone development. Osteogenesis commences by two major processes, intramembranous and endochondral ossification. Intramembranous ossification begins in flat sheets of primitive mesenchymal connective tissue whereas endochondral ossification begins in preexisting hyaline cartilage. Both processes are essentially the same, differing only by the initial material from which they form primary
trabeculae made of immature bone. Immature bone is initially laid down as woven bone in primary spongiosa.

Intramembranous ossification originates in well-vascularized mesenchymal tissue that usually contacts an epithelial border. Mesenchymal cells differentiate directly into osteoid secreting osteoblasts. Osteoid becomes mineralized and entraps the secreting osteoblasts, so that eventually, thin trabecular spicules of immature or woven bone forms. Intramembranous ossification regulates bone growth in width and consequently generates cortical and cancellous bone. In endochondrial ossification, hyaline cartilage chondrocytes undergo hypertrophy, followed by mineralization of the interterritorial matrix. This mineralized matrix provides a scaffold for osteoblasts which surround the cartilagenous core with osteoid. Osteoid becomes mineralized and primary trabeculae composed of woven bone are thus formed.

Microscopically, woven bone displays randomly oriented collagen fibers and a few scattered osteocytes embedded within mineralized matrix. Woven bone also shows up in areas of fracture healing, ectopic ossification and bone tumor formation. In lamellar bone (mature or secondary bone), regularly arranged sheets of collagen fibers form individual lamellae. Lamellae are further organized into parallel sheets or concentrically around blood vessels forming osteons. In lamellar bone, collagen fibers of one lamellae align perpendicular to collagen fibers of adjacent lamellae. This arrangement displays alternating light and dark bands under polarized light and is the primary
method used to separate woven and lamellar bone. Lamellar bone also contains a higher population of uniformly distributed osteocytes than found in woven bone.

Bone organogenesis must incorporate the function of bone (structural support, protection, mechanical strength, reservoir of calcium and phosphorus and other metabolic and hematopoietic activities) into its construction. Consequently, it is an immensely complex process requiring close integration of cell-cell, cell-matrix, intramembranous ossification, endochondral ossification, growth, molding of bone into its final shape and constant remodeling to maintain its integrity.

Organogenesis begins when mesenchymal tissue, which forms a rough outline of the developing bone, grows and forms hyaline cartilage. Perichondrium is invaded by capillaries and intramembranous ossification forms a periosteal bone collar. Concurrently, endochondral ossification transforms the central portion of the hyaline cartilage model into a primary ossification center with a central marrow cavity. Most bones contain secondary ossification centers which are located in the epiphyseal regions and through endochondral ossification form the epiphyseal regions of long bones. The continued growth and expansion of these ossification centers results in the formation of growth plates, usually located at both ends of long bones.

The growth plate is a transverse disc that separates epiphyseal from diaphyseal bone and its major function is to provide elongation of long bones. The majority of all metaphyseal trabecular bone (spon-
gosia is initially generated at a growth plate because the growth plate provides a mineralized cartilagenous trabecular scaffold for the construction of primary trabeculae. The growth plate maintains a fairly constant thickness throughout longitudinal growth but different growth plates have characteristic rates of growth, usually dependent upon the size of the proliferative zone.

The growth plate can be divided into zones. The avian growth plate sequentially consists of a cartilagenous epiphysis, proliferating-prehypertrophied zone, hypertrophied zone, degenerating hypertrophied zone, and metaphyseal primary and secondary trabeculae (spongiosa). Avian and mammalian growth plates differ considerably. In avian species, the zone of proliferation has an overall increase in the number of chondrocytes per unit area, and more columns of chondrocytes with longer lengths when compared to equivalent areas in mammals. Epiphyseal blood vessels do not penetrate the mammalian proliferative zone as found in avian proliferating-prehypertrophied zone. In mammals, one metaphyseal blood-vessel-tunnel ascends per cartilage column whereas one metaphyseal blood-vessel ascends per several cartilage columns in avian growth plates. As a result, avian metaphyseal cartilage trabeculae are much wider than in mammals. Finally, avian metaphyseal and diaphyseal regions contain a cartilage cone that can persist up to 14 days; similar structures are not found in mammals.

The zone of proliferation is characterized by many mitotic figures, flattened chondrocytes and the synthesis and secretion of extracellular matrix. This zone is the driving force primarily respon-
sible for bone elongation. Hypertrophied chondrocytes are large vacuolated cells with pyknotic nuclei and abundant mitochondria and rough endoplasmic reticulum. In this zone, hyaline cartilage interterritorial matrix contains abundant protoglycan granules bound to delicate collagen fibers and matrix vesicles which are associated with mineralization.

Degenerating hypertrophied chondrocytes display reduced amounts of cytoplasmic organelles, large cytoplasmic clear spaces and mineralized interterritorial matrix. Mineralized interterritorial matrix provides scaffolding for osteoblasts, which differentiate from advancing perivascular metaphyseal tissue to lay down osteoid. Woven bone (primary spongiosa) is formed when this osteoid is mineralized. Eventually, primary spongiosa is converted into secondary spongiosa by the simultaneous removal of woven bone and the calcified cartilage core with concurrent addition of lamellar bone. Metaphyseal spongiosa consists of these newly formed primary and secondary trabeculae.

Concurrently, during long bone elongation, diaphyseal woven bone (cortical bone) is laid down by intramembranous bone formation. In concert with the addition of periosteal bone, endosteal bone is slowly resorbed so that the net result is an enlarged marrow cavity and increased outer cortical diameter and thickened shaft wall. Haversian systems or osteons are formed as woven bone is subsequently replaced by lamellar bone. Osteons represent the main structural unit of cortical bone. Osteons consist of central blood vessels and connective tissue components (Haversian canal) surrounded by concentric lamellae and a
prominent outer border of mineralized matrix lacking collagen fibers (cement line). Haversian canals communicate with the periosteum, bone marrow, and each other through transverse channels (Volkmans canals).

In growing bone, chondroblasts, chondrocytes, osteoprogenitor cells, osteoblasts, osteocytes, osteoclasts, bone-lining cells and marrow hematopoietic cells can be found. Osteoprogenitor cells have the capacity for mitosis and further differentiation. They are spindle shaped fibroblast-like cells containing few cytoplasmic organelles. Active osteoblasts are plump cuboidal or polygonal non-polarized cells that have abundant rough endoplasmic reticulum, well-developed Golgi apparatus with numerous saccules containing collagen precursors, many cytoplasmic projections that extend into developing bone matrix, and abundant amounts of alkaline phosphatase within their cell membranes. Osteocytes are housed in lacunae surrounded by mineralized matrix. Ultrastructurally, they have reduced amounts of cytoplasmic organelles and abundant interconnecting cytoplasmic projections linking them to other osteocytes. The cytoplasmic projections and contact between cells appear to be necessary for survival of these isolated osteocytes because these channels allow for passage of nutrients and waste removal. Osteocytic osteolysis may remove only a minute amount of perilacunar bone.

Osteoclasts are large multinucleate cells with abundant foamy light eosinophilic cytoplasm. They originate from circulating monocytes and are responsible for the majority of bone resorption and often are
found in resorption pits (Howships lacunae). Ultrastructurally, osteoclasts have a unique surface membrane structure, the ruffled border, which facilitates bone resorption because it increases cell membrane surface area. The ruffled border is often surrounded by clear zones that contain many actin filaments. The function of the clear zone may be to adhere osteoclasts to bone surfaces and form a seal, hence maintaining a microenvironment favorable to bone resorption. Active osteoclasts contain abundant mitochondria, Golgi complexes, and vacuoles and lysosomes containing acid phosphotase usually subadjacent the ruffled border. Osteoclasts rapidly respond to parathyroid hormone by immediate development of ruffled borders.

The mechanism of osteoclastic bone resorption is not known. Generally, during bone resorption, osteoclasts synthesize increased levels of lysosomal enzymes and other proteases followed with increased release of acid compounds such as lactic and citrate acid. Mineral dissolution requires chelating agents, lowered pH and the activation of ionic pumps to remove ions from the bone microenvironment. Following mineral dissolution, the matrix is probably resorbed by collagenase. Prostaglandin E\textsubscript{2}, 1,25(OH)\textsubscript{2}D\textsubscript{3}, thyroid hormone, and osteoclast activating factor (derived from lymphocytes) are other factors that can stimulate osteoclastic bone resorption. Peripheral-blood monocytes and tissue macrophages are also important cells in bone resorption and bone remodeling processes. In addition, bone resorption products and bone matrix components can be chemotactic for monocytes.
Bone lining cells are flat elongated cells containing few cytoplasmic organelles. Their exact function is not known. They may represent osteoblastic reserve cells and provide a continuous membrane barrier which regulates mineral homeostasis through control of calcium and phosphate fluxes in and out of bone fluids.

Hyaline cartilage matrix (i.e., interterritorial regions), contains type II collagen. Type II collagen fibers are finer than those found in type I collagen fibers of bone and they have variable periodicity so that they fail to show the characteristic banding seen in type II bone collagen. Collagen represents the largest proportion of the dry weight of cartilage matrix.

Proteoglycan aggregates represent the major macromolecule responsible for the structure and function of hyaline cartilage matrix. Proteoglycan subunits are polyanions containing a protein core with side chains of covalently bound carbohydrates (glycosaminoglycans). Glycosaminoglycans are polymers of repeating disaccaride units, primarily chondroitin sulfate and keratin sulfate. Proteoglycans are classified according to the composition of these polysaccharide side chains. Individual proteoglycan subunits can become noncovalently associated with a hyaluronic acid backbone to form proteoglycan macromolecular aggregates. Proteoglycan abundant acidic sidegroups can weakly bind cations which play an important role in transport and retention of water and electrolytes within matrix. In addition, hyaluronic acid (proteoglycan aggregate backbone) anchors
itself to collagen fibers and forms a network. These characteristics give cartilage matrix its gel-like qualities, stiffness and resilience.

Intact, matrix proteoglycans inhibit mineralization (hydroxyapatite nucleation); mineralization is thought to occur only after a decline, removal or alteration of proteoglycan molecules. Apparently, the large size and capability to fill large volumes with dense networks of chondroitin sulfate side chains gives proteoglycans their potent inhibitory properties. In this light, proteoglycans may control the extent or progression of provisional mineralization. Others argue that the amount of proteoglycans does not decrease before the onset of mineralization and the amount present is similar to that found in mineralized tissue. Furthermore, these same authors suggest that the degradation of proteoglycans may occur naturally during the formation of the marrow cavity and excavation of cartilage. Therefore, the degradation of proteoglycans would have no significant affect on calcification as other authors suggest.

Biological calcification depends upon the interaction of cells, extracellular macromolecules (collagen, noncollagenous proteins, proteoglycans, proteolipids and glycoproteins) and other matrix components such as matrix vesicles. The mechanisms of provisional mineralization are not entirely understood. Yet, a characteristic feature of provisional calcification of cartilage is that it occurs in proteoglycan rich type II collagen-containing matrix usually containing matrix vesicles. Matrix vesicles are extracellular membrane bound electron dense particles which are 100-200 nm in
Matrix vesicles contain membrane bound phosphatases (primarily alkaline phosphatase) and phospholipids (primarily phosphatidylserine) which promote calcification. In general, matrix vesicles are thought to promote calcification by concentrating or transporting calcium and phosphate while removing or excluding inhibitors of calcification. Mitochondria from normal ossifying tissues or in vitamin D deficient studies have been shown to contain deposits of calcium and phosphate. Matrix vesicle formation during or prior to budding from chondrocyte plasma membranes appears to be coupled with mitochondrial release of these minerals. Consequently, matrix vesicles then contain the necessary lipid-calcium-phosphorus complex needed to initiate mineralization. In addition, alkaline phosphatase appears to be vital to matrix vesicle mediated mineralization because it probably functions as a phosphorus binding and transport protein; the role of this enzyme in the hydrolysis of pyrophosphate (a strong inhibitor of hydroxyapatite formation) cannot be overlooked. Further accumulation of calcium within matrix vesicles probably occurs because of the high affinity of its membrane acidic phospholipids for calcium. Once provisional mineralization is initiated, proliferation of mineral crystals is dependent upon regulatory factors such as the amount of extracellular calcium, phosphate, and other matrix promoters or inhibitors such as pyrophosphate.

Woven or lamellar bone formation begins with synthesis of matrix followed by mineralization of this matrix. Both substances comprise the
major components of bone. Because mineralization lags behind matrix production, a thin layer of unmineralized osteoid remains behind (calcification front).

The organic matrix of bone is secreted by active osteoblasts which eventually become trapped by matrix and then become less active osteocytes. Organic matrix is composed of collagen fibers embedded in amorphous ground substance. Bone collagen is type I collagen. Collagen cross striations are due to the staggered arrangement of collagen molecules in the process of forming fibrils. The staggered arrangement produces gaps or hole zones between successive collagen molecules and these sites represent areas where 50% of hydroxyapatite mineral crystals are deposited. In addition, bone collagen contains higher numbers of crosslinks formed by hydroxyllysine residues than found in other types of collagen molecules. Bone collagen synthesis can be inhibited by parathyroid hormone or 1,25(OH)\textsubscript{2}D\textsubscript{3}. Control of collagen synthesis by 1,25(OH)\textsubscript{2}D\textsubscript{3} occurs at transcription of procollagen mRNA.

Amorphous ground substance is a noncollagenous cementing substance that embeds mineral crystals and collagen fibers and at times forms complexes with collagen fibers. Ground substance contains sialoproteins, phosphoproteins (osteonectin), gamma carboxyglutamic acid proteins (osteocalcin), glycoproteins and small amounts of proteoglycans, lipids, and peptides. These substances may act as carriers of calcium and phosphorus, mineral crystal nucleators, or they may affect collagen spacing and cross-linking. Osteonectin and osteocalcin can
limit hydroxyapatite formation, and therefore, they may be regulators of matrix mineralization.\textsuperscript{129} 

Mineral constitutes the major portion of bone weight.\textsuperscript{13,65} Hydroxyapatite (\(\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2\)) crystals compose the major portion of bone mineral, yet other forms of mineral (bushite or amorphous hydroxyapatite) are thought to be present in early solid deposits of mineral.\textsuperscript{30,65,175} Furthermore, bone crystals may contain other ions and minerals.\textsuperscript{65} 

The mechanism of \textit{de novo} mineral deposition of bone matrix is not clear. Bone mineralization mediated by osteoblasts needs to be clearly distinguished from those processes occurring in provisional mineralization of cartilage, which are mediated by hypertrophic chondrocytes and different matrix materials. Nevertheless, in both processes, there are similar features occurring during the overall process of calcification.\textsuperscript{215} 

Heterogenous nucleation of collagen alone or possibly combined with several closely associated matrix proteins was originally thought to represent a means of \textit{de novo} calcification of bone matrix.\textsuperscript{30,118,129,149,159,215} The presence of holes created by the packing arrangement of collagen molecules or the periodicity of collagen, even though early deposits of mineral are associated with these regions, probably does not initiate mineralization.\textsuperscript{41,80,215} Recent studies suggest that native collagen does not possess the nucleation ability required of bone undergoing rapid mineralization but rather collagen serves as a secondary nucleation receptor and propagator of mineralization.\textsuperscript{30,215}
Currently, cells within calcifying tissues are thought to be the central factor controlling deposition of mineral crystals in extracellular matrix. De novo calcification in bone may be mediated by cell processes through involvement of mitochondria, or matrix vesicles as previously described for provisional mineralization of cartilage. Some authors do not support matrix vesicles as initiators of mineralization in bone. In addition, bone matrix vesicles have been found only in woven bone where large amounts of osteoid exist and in sites of rapid calcification (i.e., embryonic bone) and have not been observed in mature bone. Osteoblasts and osteocytes can possibly regulate local concentrations of calcium and phosphorus; therefore, they may be able to promote or regulate a phase of mineralization. The presence of high levels of alkaline phosphatase within osteoblasts may also be involved with mineralization because this enzyme degrades pyrophosphate, a strong inhibitor of mineralization.

Phosphoproteins and gamma-carboxyglutamate containing proteins (osteocalcin) are the two major types of noncollagenous bone proteins. Synthesis of osteocalcin, a vitamin-K-dependent protein, apparently depends upon the presence of vitamin D; in vitamin D deficiency, synthesis is decreased, but synthesis is stimulated by \(1,25(OH)_2D_3\). Osteocalcin has been shown to inhibit hydroxyapatite formation but is able to bind calcium with its gamma-carboxyglutamic acid residues. Osteonectin, a phosphoprotein which is analogous to fibronectin is thought to bind collagen to bone.
Both osteonectin and osteocalcin probably function more as regulators controlling the orientation, size and distribution of apatite crystals in matrix mineralization instead of functioning as initiators of mineralization. The continuous discovery of additional compounds such as bone morphogenic protein, which irreversibly induces differentiation of perivascular mesenchymal cells into osteoprogenitor cells or bone derived growth factors which stimulate DNA synthesis of differentiated osteoblasts will continue to contribute knowledge to the immensely complex organ system of bone.

**Parathyroid glands and ultimobranchial bodies.** Most animal species have two paired parathyroid glands occupying the anterior cervical region. The parathyroid glands are derived from third and fourth pharyngeal pouch entoderm. Both avian parathyroid glands are closely apposed and form a small white round gland usually located at the bifurcation of the carotid and anterior subclavian arteries. Anterior to this, the thymus and thyroid glands can be found, which may contain accessory parathyroid tissue. Histologically, avian parathyroid glands are characterized by solid islands and cords of densely packed polygonal chief cells surrounded by delicate fibrovascular stroma. Chief cells contain a small amount of basophilic cytoplasm and round hyperchromatic nuclei.

Avian ultimobranchial bodies contain calcitonin secreting cells (C cells) originating from the neural crest and are closely associated with the parathyroid glands as independent organ structures or are incorporated into parenchyma of the parathyroid glands.
structures usually require histology for conformation because they cannot be identified grossly. Ultimobranchial bodies are organized into small acini or cords with interspersed variable amounts of fibrous connective tissue. Usually the fibrous connective tissue is more abundant in ultimobranchial bodies than in the parathyroid glands.

Morphologic evidence suggests that certain ultrastructural characteristics of chief cells can be associated with different stages of their synthetic and secretory cycle. In most species, chief cells are in the inactive stage of their secretory cycle. Yet, chief cells from normal growing chickens and normal laying hens show active secretion, most likely due to their high rate of calcium metabolism during rapid bone growth or egg laying. Chief cells which are interpreted to be in the inactive stage of their secretory cycle are small and cuboidal, and plasma membranes of contiguous cells are straight. Their cytoplasm is electron lucent because of infrequent secretory granules and poorly developed cytoplasmic organelles. Golgi complexes are small and composed of tightly layered saccules and associated with few vesicles or prosecretory granules. Individual profiles of granular endoplasmic reticulum and a few ribosomes can be found scattered throughout their cytoplasm.

In the early active phase (synthesis stage) of the secretory cycle of chief cells, abundant endoplasmic reticulum aggregates into large lamellar arrays and abundant ribosomes form distinct clusters. Proparathyroid hormone is probably synthesized at this stage. Following the synthesis stage, a packaging phase begins where secretory granules
develop by sequential accumulation and condensation within Golgi complexes. Consequently, chief cell Golgi apparatus enlarges, and becomes complex with numerous associated vacuoles, vesicles and prosecretory granules. Granular endoplasmic reticulum at this stage begins to involute and disperse. Mature secretory granules form and move to the periphery of the cell from where they are eventually secreted by exocytosis within cytoplasmic projections into perivascular spaces. Next, cellular cytoplasmic organelles begin to involute and the cell eventually returns to its inactive status.

Blood calcium levels regulate parathyroid hormone biosynthesis and secretion. Plasma calcium affects chief cells during the secreting phase; that is, low ambient calcium speeds up the rate of secretion and shortens the resting or inactive phase whereas high plasma levels of calcium have just the opposite effect. 43,172

Mature avian secretory granules range in size from 100-400 nm in their greatest diameter, 139,146,219 and are round and regular in shape. They are lined by a closely apposed membrane and contain fine central electron dense particles. Secretory granules contain parathyroid hormone which is a straight chain polypeptide containing 84 amino acid residues. 35 In addition, chief cell secretory granules contain parathyroid secretory protein, which may represent a binding protein for parathyroid hormone. 43 The number of mature secretory granules in chicks is low. 76,139,171 Furthermore, the number of secretory granules in chief cells can vary markedly between closely related species; they are reported to be numerous in mice but infrequent in rats. 43,171
Physiologically, parathyroid hormone is the most important regulator of minute-to-minute blood calcium levels. Parathyroid hormone functions through its effects on target cells, primarily in bone and kidney, and possibly the intestine. Therefore, the primary biologic effects of parathyroid hormone are to maintain blood calcium levels as follows: increase bone resorption and skeletal remodeling through activation of osteoclasts and osteolysis; increase 1,25(OH)$_2$D$_3$ synthesis through activation of renal 1-hydroxylase with subsequent increased intestinal transport of calcium; and increased renal distal tubule reabsorption of calcium which decreases calcium loss in urine; and increased renal excretion of phosphorus by decreasing proximal tubule reabsorption of phosphorus, hence phosphaturia. Bone or soft tissues could become remineralized if parathyroid hormone did not stimulate renal excretion of phosphorus.

Bony skeleton contains 99% of the total body calcium, therefore, bone resorption occurs to help meet calcium demands. The exact mechanisms by which parathyroid hormone mobilizes calcium and phosphorus from bone are not known. One immediate effect of parathyroid hormone usually in concert with 1,25(OH)$_2$D$_3$ might be due to stimulation of osteocytic osteolysis and/or activation of bone lining cells and osteocytes to pump calcium and phosphorus from the bone fluid compartments and bone surfaces to extracellular spaces. The greatest amount of bone resorption and remodeling is produced by parathyroid hormone activation of osteoclasts and recruitment of
mononuclear cells to form osteoclasts. The proposed mechanisms of osteoclastic bone resorption were discussed above. Parathyroid hormone induced bone resorption of calcium and excretion of phosphorus appears to be due to activation of membrane bound adenyl cyclase followed by synthesis of cyclic AMP from ATP. The extracellular concentration of calcium also effects the magnitude of response that cyclic AMP produces in response to parathyroid hormone stimulation, therefore, calcium and cyclic AMP may act in concert to mediate the actions of parathyroid hormone.  

Calcitonin can produce distinct hypocalcemic and hypophosphatemic effects but probably is not an important regulator of bone formation. A major function of calcitonin may be to protect against hypercalcemia during rapid postprandial absorption of calcium. The effects of calcitonin are primarily mediated by inhibiting osteocytic and osteoclastic bone resorption characterized by osteoclastic ruffled borders and clear zone atrophy and there is a decrease in number of osteoclasts following calcitonin administration. Calcitonin also reduces renal tubular reabsorption of phosphate, eventually leading to hypophosphatemia. Calcitonin can inhibit gastrointestinal release of gastrin and acid secretions but it has not been shown to have major effects on intestinal absorption of calcium. Calcitonin secretion by ultimobranchial bodies is regulated by the concentration of calcium; high calcium concentrations stimulate calcitonin release whereas low calcium levels inhibit it. Magnesium levels, gastrointestinal hormones, biogenic amines,
somatostatin and other factors can also regulate calcitonin secretion. 12

**Rickets.** Rickets is a disease of young growing animals caused by a deficiency of vitamin D, calcium or phosphorus and has been reported to affect many animal species as natural or experimental disease. 110 Failure to mineralize cartilaginous matrix and bone matrix (osteoid) are manifestations of these deficiencies. Osteomalacia occurs in adult animals due to similar deficiencies. 110 In this latter disease, only osteoid fails to become mineralized because endochondral ossification is usually completed in adult animals.

Avian rickets due to vitamin D-deficiency most often occurs within the first two to three weeks of life. 167,168,210 Because of bone pain, birds have clinical signs of lameness, ataxia, leg weakness (reluctance to move) or other forms of gait disturbances, and reduced food and water consumption. 168,169 As a result of reduced feed and water intake and other metabolic consequences of vitamin D deficiency such as metabolic acidosis, 27 birds fail to grow, become dehydrated, and often starve to death.

Clinically, vitamin D-deficient birds usually have reduced plasma calcium, inorganic phosphorus, and vitamin D metabolites, intestinal calcium binding protein, bone ash, and renal 24-hydroxylase activity. 15,48,107,169 Plasma alkaline phosphatase and renal 1-hydroxylase activity are increased. 15,107,169

The macroscopic bone lesions of rickets are prominent in areas that cartilage contributes significantly to during skeletal growth. 110 In
avian vitamin D-deficient rickets, costovertebral and costochondral junctions become nodular (rachitic rosary) and rib bones become crooked and malformed. \(^{48,107}\) Bones are soft, pliable and easy to cut because they lack mineral. Avian long bone diaphyseal regions are shorter and cortex is thinned; epiphysyal and metaphysyal regions are elongated but do not necessarily have flaring as often occurs in mammals. \(^{48,107,110,169}\) Parathyroid glands are enlarged and distinct. \(^{106}\)

Histologically, avian growth plates and metaphysyal regions become highly distorted. \(^{48,107,108,113,121,169}\) The proliferating-prehypertrophied zone has marked but variable lengthening and its distal margin is irregular. Failure to mineralize hypertrophic cartilage inhibits its further degeneration, and it accumulates as large nonmineralized irregular cartilagenous trabeculae. Mineralization of hypertropic cartilage that does occur is often irregular. Consequently, there is a loss of the orderly progression of hypertropic cartilage to primary spongiosa. Metaphysyal cartilage columns may collapse with subsequent areas of necrosis. Thick layers of osteoid line cartilagenous trabeculae, primary and secondary spongiosa, and periosteal and endosteal surfaces. Metaphysyal marrow spaces and epiphysyal capillaries may have abnormal branching. Metaphysyal regions of osteodystrophia fibrosa may develop.

Ultrastructurally, besides a lack of mineralization, osteoclast, osteoblast, osteocyte, chondrocyte and matrix changes in vitamin D-deficiency are minimal when compared with vitamin D treated controls. \(^{43,46,48,63}\) All zones of chicken ricketic chondrocytes contain increased
amounts of intracytoplasmic lipid and chondrocytes contain chains of intracytoplasmic vesicles. In ricketic rat chondrocytes, mitochondria contain abundant calcium and phosphate crystals whereas in another study rat chondrocytes contained increased numbers of mitochondrial profiles and rough endoplasmic reticulum. Some of these changes were thought to be due to interference with normal cartilage lipid metabolism or secondarily to inefficient oxidative energy production. Cartilage matrix vesicles from ricketic rats morphologically resemble matrix vesicles from vitamin D₃ treated rats, they retain alkaline phosphatase activity and represent initial sites of mineralization when the rats are treated with vitamin D₃. Histologically, parathyroid glands from vitamin D-deficient birds are hyperplastic. Chief cells may appear smaller and shorter than normal and have reduced nuclear volume, cytoplasmic mass and granularity. Chief cell swelling and increased numbers of mitotic figures may be seen.

Similar chief cell ultrastructural changes have been described for both mammals and avian species with vitamin D-deficiency or hypocalcemia. Chief cells may vary in size. Chief cell cytoplasmic volume may be increased and it usually contains abundant organelles concerned with protein synthesis and secretion: expanded Golgi complexes associated with increased vesicles and prosecretory granules; aggregates of rough endoplasmic reticulum that with time decrease in amount. Numbers of mature secretory granules are often reduced. Contiguous chief cells show complex intricately interdigitated
plasma membranes and some chief cells may show cytoplasmic vacuolar changes.

Rickets due to a dietary deficiency of calcium or phosphorus have been described.\(^ {108,113,119,120}\) In chicks hypocalcemic due to dietary deficiency of calcium, the degenerative hypertrophic zone becomes elongated whereas the proliferating-prehypertrophied zone is similar in length to that of vitamin D\(_3\) treated birds.\(^ {108}\) Recently, other authors\(^ {120}\) suggest that in early dietary calcium deficiency, broiler chicks produce similar histological bone lesions as found in vitamin D-deficient broiler chicks (primarily lengthening of the proliferating-prehypertrophied zone) except that fibrous osteodystrophy may be more prominent in calcium deficiency than found in vitamin D-deficiency.

Histological bone lesions in broiler chicks fed a phosphorus deficient diet have elongated metaphyseal regions because of a failure to mineralize matrix; hence, lengthened hypertrophic and degenerating hypertrophied zones and metaphyseal primary spongiosa are seen.\(^ {113,119}\) Proliferating-prehypertrophied zones are normal lengths. Vitamin D-deficiency also results in defective mineralization of matrix so consequently there can also be metaphyseal changes similar to phosphorus deficiency.

Field rickets is a unique spontaneous form of rickets described only for turkey poult's.\(^ {103,150,205}\) The cause is usually not determined and this term has been used to imply that the ricketic lesions are due to causes other than nutritional deficiency of calcium, phosphorus or vitamin D.\(^ {205}\) Yet, there are no published reports proving that factors
other than these nutritional deficiencies are involved. In one report, adequate levels of biologically active vitamin D may not have been provided. In this latter report, a deficiency of intestinal calcium binding protein was suspected to be the cause. The clinical parameters and bone histological changes described for field rickets are similar to those caused by vitamin D-deficiency.

The pathogenesis of vitamin D-deficient bone lesions has not been established. Vitamin D and/or its metabolites may be directly required for normal bone and cartilage matrix formation and mineral deposition. Ricketic bone changes may be indirectly produced by hypocalcemia and hypophosphatemia due to reduced vitamin D stimulated intestinal transport of these minerals. In addition, ricketic bone lesions may be due to decreased bone matrix degradation (i.e., defective osteoclasts).

The importance of vitamin D for collagen and ground substance synthesis is unknown. In vitamin D deficiency, collagen chain composition is similar to that in vitamin D treated chicks but the rate of maturation of bone collagen appears to decrease. This is evidenced by increased lysine oxidase activity and hence increased potential lysine crosslinking and increased crosslinking ratios of dihydroxylysinonorleucine (DHLNL) to hydroxylysinonorleucine (HLNL). In vitamin D treated chicks during maturation and growth, lysine oxidase levels and DHLNL/HLNL ratios normally decrease. Collagen fibers of vitamin D-deficient growing chicks are less susceptible to proteolytic enzymes (pepsin and papain) than found in vitamin D treated chicks, suggesting that collagen fibers from deficient chicks are more
Furthermore, collagen synthesis can be inhibited by 1,25(OH)$_2$D$_3$, suggesting that this metabolite can directly control bone growth and development. The metabolism or synthesis of bone or cartilage ground substance, phospholipids, glycoproteins and proteoglycans are altered in vitamin D-deficiency. Proteoglycans from ricketic chick cartilage are smaller than proteoglycans found in vitamin D treated chicks, but this difference was thought to be due to differences in plasma calcium concentration and not due to vitamin D status. On the contrary, proteoglycans found in ricketic rat cartilage are increased in size. In addition, 1,25(OH)$_2$D$_3$ stimulates osteocalcin synthesis, suggesting that this metabolite is directly involved with synthesis of a compound that can regulate mineralization. Another direct role for vitamin D metabolites in cartilage and bone metabolism might be that chondrocytes and bone cells hydroxylate 25OHD$_3$ to 1,25(OH)$_2$D$_3$ or 24,25(OH)$_2$D$_3$. Autoradiographically, 1,24(OH)$_2$D$_3$ can be found in osteocytes and chondrocytes. In addition, cholecalciferol affects the metabolism of the zone of proliferation more than other growth plate regions. Vitamin D-deficient rat bones have reduced lysophosphatide and free fatty acid content but elevated cholesterol levels; correcting the rachitic status restores bone lipid composition. Hence, vitamin D may play a direct role in bone lipid metabolism independent of vitamin D effects on calcium homeostasis and mineral deposition.
The pathologic bone changes may indirectly be associated with the availability of calcium and phosphorus rather than a deficiency of vitamin D and/or its metabolites which then might secondarily affect the normal maturation and ossification of collagen, proteoglycans and cartilage. The decreased intestinal absorption of calcium in vitamin D-deficiency is probably not a structural or chemical difference in the intestinal brush border cytoskeleton. Recently, ricketic bone lesions were proposed to be caused by a deficiency of phosphorus which prevents growth and interlocking of hydroxyapatite crystals into ricketic bone. In vitamin D-deficiency bone matrix synthesis is reduced. When the hypocalcemia of vitamin D-deficiency is corrected bone matrix synthesis is restored, which suggests that serum calcium plays a regulatory role in determining rates of bone matrix formation and maturation. In addition, diets low in selected minerals can lead to decreased mineralization of bone, and rachitic bone placed in solution containing normal calcium and phosphorus calcifies.

In vitamin D-deficiency, osteoclastic bone resorption may be defective and in the rat there are decreased numbers of osteoclasts both of which may account for the buildup of osteoid and development of rachitic bone lesions. Yet, the buildup of unmineralized osteoid may simply inhibit osteoclastic removal. Besides effecting bones, vitamin D-deficiency has been shown to reduce hematopoietic bone marrow stem cells which could potentially lead to a reduction in number and/or function of bone resorbing monocytes and osteoclasts.
In vitamin D-deficient chicks, metabolic acidosis is a consistent finding which might have a direct or indirect effect on the metabolism of calcium, phosphorus or parathyroid hormone. Since the metabolism of vitamin D involves multiple systems, the pathogenesis is most likely complex and involves all or a portion of each of the above proposed mechanisms.
PATHOLOGY OF VITAMIN D DEFICIENCY IN GROWING TURKEYS

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ABSTRACT

Turkey poults were fed a vitamin D-deficient diet and examined for clinical signs and structural changes of bone and parathyroid glands. Deficient poults (group I) developed ricketic changes during days 10-14. Control (group II) poults received a weekly progressive increased oral treatment with vitamin D$_3$ and did not develop rickets. In group I, the proliferating-prehypertrophied zone of the growth plate increased significantly in the proximal tibiotarsus, but was only slightly elongated in the distal tibiotarsus. The unmineralized hypertrophic chondrocyte zone increased rapidly in conjunction with a reciprocal decrease in the length of the mineralized hypertrophic degenerative zone. This reciprocal exchange occurred faster in the proximal tibiotarsus than the distal tibiotarsus. Changes in relative lengths of these 3 zones were possibly due to faster growth rate in the growth plates of the proximal tibiotarsus. Based on observed morphologic changes, the metaphyseal blood vessels and associated marrow cells may induce differentiation of the proliferating-prehypertrophied zone into hypertrophic chondrocytes. Calcium/phosphorus ratios in femoral bone ash were increased in vitamin D-deficient poults; this suggested that phosphorus may be selectively released from ricketic bone. Other significant findings included: decreased bone ash, decreased total amount of femoral bone ash (calcium, phosphorus, magnesium), decreased bone length, decreased body weight, increased plasma alkaline phosphatase, normocalcemia, hyper-normophosphatemia, parathyroid
hyperplasia and degeneration and rapid depletion of vitamin D₃ metabolites—25(OH)D₃, 1,25(OH)₂D₃ and 24, 25(OH)₂D₃. Low 25OHD₃ and 1,25(OH)₂D₃ in group II poults in the early part of this experiment, suggests that 1400 IU of vitamin D₃/Kg of feed, which exceeds the NRC requirement, may not be an adequate level of vitamin D₃ for growing turkey poults.
INTRODUCTION

Leg weakness in turkeys results in significant economic losses. In addition to the 4-6% of male turkeys that are culled or die from skeletal deformities, depressed weight gains and removal of deformed limbs at processing are important. Although part of the losses are due to infectious disease, noninfectious causes such as vitamin D deficiency are major contributors to skeletal disease. In conventional, light-restricted confinement housing, turkeys receive insufficient quantities of vitamin D$_3$ via photolysis and must rely upon dietary supplement. Under these conditions, turkeys appear to be more vulnerable to low biopotent vitamin D$_3$ in their ration or interference with absorption of vitamin D$_3$ from their diet than chickens or other food-producing animals.

Pathological changes of experimental and natural vitamin D-deficient rickets are well documented in chickens, but there are few reports in turkeys. Avian rickets primarily occurs in the first month of life during the most rapid skeletal growth. Experimental avian rickets is characterized by lameness, retarded body growth, hypocalcemia, hypophosphatemia, elevated serum alkaline phosphatase, parathyroid hyperplasia, and severe bone distortion. Bone changes include elongation of epiphyseal growth plates, failure of mineralization of cartilage and osteoid, decreased bone ash, fibrous osteodystrophy, and increased numbers of osteoclasts. The term "field rickets" in poults implies that some unidentified factor or causes other
than nutritional deficiencies of calcium, phosphorus or vitamin D₃ are responsible for reduced bone calcification. In one report, field rickets was thought to be due to intestinal damage with secondary malabsorption and reduced calcium binding protein, but no direct evidence was provided that bioavailable vitamin D₃ was present in the ration.

The seco-steroid hormone vitamin D₃ and its metabolites promote normal skeletal development through their actions on bone, intestine and kidney in concert with parathyroid and calcitonin hormones which maintain calcium and phosphorus homeostasis. Vitamin D₃ is hydroxylated by hepatocyte endoplasmic reticulum to 25-hydroxyvitamin D₃ (25OH₃) and hydroxylated further to the biologically active form of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and the less active 24,25-dihydroxyvitamin D₃ (24,25-(OH)₂D₃) by renal mitochondria 25(OH)D-1-hydroxylase or 25(OH)D-24-hydroxylase enzyme systems. 1,25-dihydroxyvitamin D₃, like other steroid hormones, imparts its bioactivity by interacting with receptors in intestine, bone, and kidney. This activity can be expressed in increased blood calcium and phosphorus levels, and is the result of enhanced calcium and phosphorus absorption from the intestine, calcium and phosphorus resorption from bone and calcium reabsorption from glomerular filtrate. 13,16

This study was undertaken to characterize the temporal morphologic and clinical changes in experimental vitamin D deficient growing turkey poults. We compared the qualitative differences among bones with
different growth rates and characterized the effect of vitamin D deficiency on these differences.

**MATERIALS AND METHODS**

One-day-old male broad-breasted white turkey poults, obtained from a closed disease-free breeding flock, were divided into two equal groups and placed in battery brooders housed in separate windowless rooms lighted with soft incandescent lighting. Both groups were immediately fed a synthetic vitamin D deficient diet—Tekland, (Madison, Wisconsin) diet—TD 82391, which contained 1.4% total calcium, 1.1% total phosphorus and metabolizable energy of 3,260 K Cal/kg. Group I poults received 0.1 ml corn oil, orally, in daily doses beginning at day 1. Group II (control) poults received vitamin D₃ delivered in 0.1 ml corn oil, orally, in daily doses. Daily doses of vitamin D₃ were increased weekly in group II poults so that each poult received 1400 IU (35 ug) of vitamin D₃ per kilogram of predicted feed consumed. The predicted amount of feed consumed was based upon the predicted weekly feed consumption of commercial male turkeys.²⁹ The daily poult dose of vitamin D₃ for days 1-7 was 0.5 ug, days 8-14 was 1.0 ug, and days 15-21 was 2.0 ug. Three poults at day 1 and 3 poults from each group were necropsied at each sampling time (days 3, 7, 10, 14, 17, 21) for a total of 39 poults.
The poult were weighed and exsanguinated by cardiac puncture. Hematocrit and plasma calcium, phosphorus, alkaline phosphatase, 25OHD₃, 1,25(OH)₂D₃, and 24,25(OH)₂D₃ were determined as previously described. Femur, tibiotarsus and tarsometatarsus bone length were measured with precision calipers. Thyroid-parathyroid-ultimobranchial-thymus complex (removed en bloc) and frontal plane sections of proximal and distal tibiotarsus, and proximal tarsometatarsus were placed in 10% neutral buffered formalin. Fixed tissues were processed by routine paraffin techniques and 6 µm sections were cut. Undecalcified bone tissues were stained with hematoxylin and eosin (HE), von Kossa HE, periodic acid-Schiff alcian blue (PAS-AB) and Masson Goldner trichrome. The parathyroid glands were stained with HE, PAS-AB and Wilder reticulum stain. Femoral bone ash percent was determined from dried fat-free bone obtained by washing for 24 hours in a 3:1 diethyl ether to ethanol solution in a soxlet extractor. The ash was expressed as a percentage of the dried, fat-free bone weight. Total bone calcium, phosphorus and magnesium levels were determined after dissolving the femoral bone ash with 10 ml of 20% hydrochloric acid. Levels were determined from the diluent as previously reported and recorded as total bone calcium, phosphorus and magnesium (mg).

Computerized histomorphometric measurements were made using an Orthoplan microscope fitted with a drawing tube (Leitz, Wetzlar, Germany) in conjunction with a semi-automatic digitizing tablet (Bio Quant II from R & M Biometric, Knoxville, Tennessee). Length and perimeter measurements were made directly onto the tablet. The magnification at
the tablet surface was recalibrated daily with a 1 mm stage micrometer, and a 1 cm x 1 cm eyepiece reticle was used for proper alignment and orientation of all histologic measurements. The articular surface width of the proximal and majority of the distal tibiotarsus von Kossa--HE stained histological bone sections were divided into 3 equidistant measurement sites. Because central fibrocartilagenous regions made sampling difficult, the proximal tarsometatarsus and appropriate distal tibiotarsus bones were divided into 2 equidistant measurement sites at the medial and lateral articular condylar regions, leaving 4 measurement sites. The proliferating-prehypertrophied zone, unmineralized hypertrophied zone, mineralized hypertrophied degenerative zone, and metaphyseal marrow blood vessel lengths were sequentially determined along the long axis of each bone beginning at the previously described measurement points. A 2.5x objective lens and 10x ocular lenses were used for these measurements. The unmineralized hypertrophied zone began at the end of the proliferating-prehypertrophied zone when the chondrocytic lacunae first became rounded and lost their orderly column arrangement because of increased amounts of light basophilic extracellular ground substance. The mineralized hypertrophied degenerative zone began at the end of the unmineralized hypertrophied zone when von Kossa stained mineral deposits first became evident. This zone included metaphyseal primary spongiosa and abruptly ended when the degenerate chondrocyte lacunae outline were lost, usually where bony trabeculae formed secondary spongiosa. The metaphyseal vessel lengths originated at the distal end of the mineralized hypertrophied
degenerative zone and ended at the point of maximum penetration of the unmineralized hypertrophied zone. The total length represented the sum of the sequential length measurement of the proliferating-prehypertrophied zone, unmineralized hypertrophied zone, and mineralized hypertrophied degenerative zone. The mean for each individual bone and mean of means for each appropriate bone group and time period were recorded. The chondroclast index was also measured at the above measurement points and represented the total number of chondroclasts lining the perimeter of the hypertrophic cartilage tunneled out by the most proximal portion of the metaphyseal capillary loops divided by this same perimeter length (mm). Chondroclasts were multinucleated cells with abundant foamy vacuolated eosinophilic cytoplasm. Sixteen x objective lens and 10x occular lenses were used for this measurement and the most proximal edge of the cartilage tunnel was always aligned adjacent to the central edge of the microscopic field.

Epiphyseal blood vessels which penetrated the proliferating-prehypertrophied zone were counted. The metaphyseal blood vessel which penetrated the deepest into the unmineralized hypertrophied zone was used as a reference point; those blood vessels which penetrated to within one square of an eyepiece reticle (.38 mm), set at the reference point, were counted and those branching within this range were counted as one. Blood vessel numbers were counted using 2.5x objective lens and 10x occular objective lenses.

Statistical analysis comprised means, standard deviation, standard error, two tailed student t-test and analysis of variance.
RESULTS

Between days 3-7, vitamin D-deficient pouls (group I) were depleted of detectable levels of endogenous 25OHD$_3$, 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ and from day 7 on significantly (P<.05) differed from vitamin D$_3$-treated pouls (group II) with elevated levels of alkaline phosphatase activity and decreased percentage bone ash (Figs. 1, 2; Table I). The 25OHD$_3$ levels also decreased to a significant low level and the 1,25(OH)$_2$D$_3$ levels remained depressed during the first 7 days in group II pouls; 24,25(OH)$_2$D$_3$ was undetectable during days 10-14. Clinical evidence of rickets developed rapidly during days 9-10; the pouls' hocks were adducted so that pouls walked with a waddle or hopping motion and required their wings when turning. Feed consumption markedly decreased after 10 days. Further, at day 10, significant differences (P<.05) appeared between groups in body weight, bone length of the femur, tibiotarsus and tarsometatarsus and amount of bone ash calcium, phosphorus, magnesium and calcium/phosphorus ratio. Hyperphosphatemia was marked and the calcium levels were slightly decreased, but normocalcemia prevailed throughout this experiment (Figs. 3, 4, Tables I, II). At day 14, vitamin D-deficient pouls were severely lame, had ruffled feathers, tended to sit on their haunches huddled under the heaters--suggestive of inability to control body heat, and made little effort to allude capture. By day 21, group I pouls were very lethargic and weakened, the death rate reached 2-3 poultis/day. Pouls did not have terminal hypocalcemic seizures. Their mean PCV was 36.7 ±
Fig. 1: Plasma levels of vitamin D₃ metabolites in vitamin D₃ deficient (●--●) and treated poult (□--□). Figure is mean ± SD of 3 poult per day except where asterisk, N = 2. Hash marks represents undetectable levels.
VITAMIN D DEFICIENT (VDD)

VDD + D₃

25(OH)D₃ ng/ml

1,25(OH)₂ pg/ml

24,25(OH)₂ ng/ml

DAYS

1 3 7 10 14 17 21
Fig. 2: Plasma levels of alkaline phosphatase activity in vitamin D-deficient (solid bar) and vitamin D$_3$ treated poult (open bar). Figure is mean ± SD of 3 poult per day. Asterisk at day 7 represents significant difference between groups (P < .05) with remaining days also significantly different.
Table I - Femoral bone ash (%) and bone ash calcium, phosphorus, calcium/phosphorus ratio and magnesium (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Bone Ash (%)</th>
<th>Calcium a</th>
<th>Phosphorus a</th>
<th>Ca:P</th>
<th>Magnesium a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>35.1 ± 1.4</td>
<td>7.4 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>2.2 ± 0.04</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Day 3b</td>
<td>34.8 ± 1.3</td>
<td>10.7 ± 1.4</td>
<td>5.0 ± 0.8</td>
<td>2.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>36.8 ± 2.6</td>
<td>8.8 ± 0.8</td>
<td>4.5 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Day 7</td>
<td>28.5 ± 0.7</td>
<td>14.0 ± 0.6</td>
<td>6.7 ± 0.3</td>
<td>2.1 ± 0.1</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>I</td>
<td>34.7 ± 2.9</td>
<td>18.1 ± 3.3</td>
<td>8.0 ± 1.0</td>
<td>2.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>26.4 ± 1.7</td>
<td>16.6 ± 4.9</td>
<td>7.1 ± 0.8</td>
<td>2.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>II</td>
<td>40.6 ± 1.9</td>
<td>34.0 ± 6.9</td>
<td>20.4 ± 2.8</td>
<td>1.7 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>23.9 ± 2.2</td>
<td>21.9 ± 5.7</td>
<td>9.0 ± 1.5</td>
<td>2.4 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>II</td>
<td>38.9 ± 1.3</td>
<td>50.6 ± 8.3</td>
<td>33.0 ± 3.2</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Day 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>21.3 ± 2.0</td>
<td>23.9 ± 4.1</td>
<td>9.6 ± 0.9</td>
<td>2.5 ± 0.2</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>II</td>
<td>44.5 ± 1.2</td>
<td>79.3 ± 3.1</td>
<td>50.3 ± 3.3</td>
<td>1.6 ± 0.1</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>21.1 ± 3.4</td>
<td>16.8 ± 0.8</td>
<td>7.1 ± 0.3</td>
<td>2.4 ± 0.1</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>II</td>
<td>46.4 ± 0.8</td>
<td>155.3 ± 16.0</td>
<td>90.0 ± 7.4</td>
<td>1.7 ± 0.04</td>
<td>3.8 ± 0.3</td>
</tr>
</tbody>
</table>

Figure is total amount (mg) derived from bone ash - 3 poult's/treatment/day.

I = Vitamin D deficient diet.

II = Vitamin D deficient diet + Vitamin D3 (control).

P < .05 between group I and group II.

P < .01 between group I and group II.

P < .001 between group I and group II.
4.7 versus group II PCV of 33 ± 2.7. All the remaining vitamin D-deficient poults died by day 25.

Long bone, beak, rib and vertebrae osteopathy, found at necropsy of group I poults, developed rapidly and progressively increased in severity. At day 10, bone and beak softness, pliability and ease of cutting was striking; also, focal petechial hemorrhages of proximal tibiotarsus growth plate were found in a background of slightly elongated metaphyseal white cartilagenous streaking. With time, cartilagenous elongation was the most prominent long bone change but it did not result in a significant increase in metaphyseal and epiphyseal diameter or flaring (Fig. 5). Costovertebral and costochondral junctions, first found at day 10, became nodular and the ribs were thickened, twisted and distorted. A lack of adipose tissue, cardiac serous atrophy, and pale fatty livers were present by day 21. The white nodular parathyroid glands of group I poults were more distinct but of similar size when directly compared to those of group II. Parathyroid glands from the vitamin D-deficient poults were considered enlarged when the marked body size difference between the two groups were considered.

In group II, the histological changes and morphometric measurements were similar between the proximal tibiotarsus and proximal tarso-metatarsus but corresponding distal tibiotarsus lengths were much less than the other two bones. The proliferating-prehypertrophied, mineralized hypertrophied degenerative zone, metaphyseal marrow blood vessel length, total growth plate length and osteoclast index, for group II, increased over time, peaked at day 14 and then usually stabilized
Fig. 3: Body weight of group I poults (solid bar), group II poults (open bar) and predicted male poult weight on commercial feed (grey bar). Figure is mean ± SD of 3 poults per day.
VITAMIN D DEFICIENT (VDD)

PREDICTED MALE POULT WEIGHT ON COMMERCIAL FEED

BODY WEIGHT (g)

VDD + D₃
Fig. 4: Calcium and phosphorus plasma levels, of vitamin D-
deficient (●--●) and vitamin D₃ treated poults (□--□).

Figure is mean ± SD from 3 poults per day except where asterisks, N = 2.
VITAMIN D DEFICIENT (VDD)
VDD + D₃
Fig. 5: Frontal sections of tibiotarsus at day 21. Vitamin D-deficient top, vitamin D₃ replete bottom. Marked cartilagenous elongation of metaphysis in deficient tibiotarsus (top).
(Tables II, III). The distal tibiotarsus occasionally showed a slightly different morphologic pattern from the other two bones (Tables II, III). The distal tibiotarsus proliferating-prehypertrophied zone was the one exception because it increased slightly to day 10 and then stabilized. Group II plasma alkaline phosphatase activity increased until day 14 and then decreased (Fig. 2). In all 3 bones, the unmineralized hypertrophied zone length increased only slightly during days 10-17. The metaphyseal blood vascular marrow space was similar in length as the mineralized hypertrophied degenerative zone or slightly longer, i.e. 0-.2 mm. The metaphyseal marrow blood vessel never penetrated the proliferating-prehypertrophied zone; yet, in both groups eosinophilic streaks of flattened cells occasionally connected and directly aligned metaphyseal and epiphyseal capillaries (Fig. 6).

In the vitamin D-deficient poult (group I), subtle histological evidence of rickets was first found at day 7; ricketetic changes were severe by days 10 and 14, corresponding with the clinical signs of lameness. These changes varied markedly among age matched poult, especially during days 10-14 (Tables II, III). The observed variation was not related to differences in total bone length or body weight. Moreover, the proximal tibiotarsus and proximal tarsometatarsus histological changes and patterns from the same poult varied little between themselves; however, the distal tibiotarsus had important differences from these two bones. Initially, the vitamin D-deficient poult's proximal tibiotarsus and proximal tarsometatarsus proliferating-prehypertrophied zone lengths became elongated; the proliferating-
prehypertrophied zone length continued to increase over time, but peaked at day 17 compared to day 14 for group II. Group I proximal tibiotarsus lengths were significantly longer (P<0.05) than group II proliferating-prehypertrophied lengths when averaging days 3-21 and using ANOVA (Table II). Surprisingly, group I distal tibiotarsus proliferating-prehypertrophied zone length initially elongated, but this difference was not statistically significant (P>.05) when compared to appropriate distal tibiotarsus control lengths (Table III). The distal proliferating-prehypertrophied margin of the vitamin D₃ treated poults (group II) was regular and straight versus the irregular margin which began at day 10 in all 3 group I bones because of the combination of a downward distal growth of the proliferating-prehypertrophied zone chondrocytes interdigitating between the proximal growth of marrow blood vessel spaces. By day 21, these changes appeared to be due entirely to the proximal growth of the metaphyseal marrow blood vessel spaces. The proximal growth of the metaphyseal marrow blood vessel spaces were always surrounded by a cap of hypertrophic chondrocytes and were usually a uniform distance from the proliferating-prehypertrophied zone (Figs. 6, 7). Another major ricketic change, which began at day 7, was the increase of the unmineralized hypertrophied zone length followed with a reciprocal decrease in length of the mineralized hypertrophic chondrocytes zone so that by day 14, only patchy areas of mineralized hypertrophic chondrocytes remained in the primary spongiosa region (Fig. 6, Tables II, III). Sequentially, the unmineralized hypertropic cartilage columns became twisted, thickened, and lost their orderly
Table II. Proximal tibiotarsus, epiphyseal and metaphyseal morphometric measurements (mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proliferating Zone Length</td>
<td>Unmineralized Zone Length</td>
<td>Mineralized Zone Length</td>
<td>Total Length</td>
<td>Metaphyseal Marrow Vessel Length</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.7 ± 0.03</td>
<td>0.2 ± 0.04</td>
<td>1.9 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>1.6 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>2.3 ± 0.6</td>
<td>2.0 ± 0.5</td>
<td>1.1 ± 0.7</td>
<td>5.3 ± 0.5</td>
<td>2.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.9 ± 0.1</td>
<td>0.5 ± 0.6</td>
<td>3.2 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>3.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>2.5 ± 0.2</td>
<td>2.9 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>6.2 ± 0.3</td>
<td>3.4 ± 0.5</td>
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<tr>
<td></td>
<td>1.9 ± 0.1</td>
<td>0.4 ± 0.02</td>
<td>4.6 ± 0.2</td>
<td>6.9 ± 0.1</td>
<td>4.8 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- a Figure is in mm from 3 pouls/treatment/day.
- b I = Vitamin D deficient diet.
- c II = Vitamin D deficient diet + oral vitamin D₃.
- d P < .05 between group I and group II.
- e P < .01 between group I and group II.
- f P < .001 between group I and group II.
<table>
<thead>
<tr>
<th>Group</th>
<th>Chondroclast*&lt;sup&gt;g&lt;/sup&gt; index</th>
<th>Total Number&lt;sup&gt;h&lt;/sup&gt; epiphyseal blood vessels</th>
<th>Total Number&lt;sup&gt;h&lt;/sup&gt; metaphyseal marrow spaces</th>
<th>Tibiotarsus&lt;sup&gt;h&lt;/sup&gt; bone length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 ± 1.8</td>
<td>5.0 ± 1.5</td>
<td>8.7 ± 1.9</td>
<td>39.0 ± 1.1</td>
</tr>
<tr>
<td>Day 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3.0 ± 0.7</td>
<td>6.3 ± 0.7</td>
<td>11.7 ± 0.9</td>
<td>43.3 ± 0.6</td>
</tr>
<tr>
<td>II</td>
<td>2.4 ± 0.6</td>
<td>5.0 ± 0.0</td>
<td>8.7 ± 0.3</td>
<td>42.3 ± 1.2</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.6 ± 0.2</td>
<td>6.3 ± 1.8</td>
<td>16.2 ± 0.3</td>
<td>47.3 ± 2.5</td>
</tr>
<tr>
<td>II</td>
<td>3.1 ± 0.9</td>
<td>8.3 ± 0.7</td>
<td>15.3 ± 0.3</td>
<td>47.7 ± 1.5</td>
</tr>
<tr>
<td>Day 10</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>5.2 ± 0.7</td>
<td>9.7 ± 1.5</td>
<td>14.0 ± 1.2</td>
<td>50.7 ± 2.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>3.0 ± 1.0</td>
<td>10.7 ± 1.5</td>
<td>16.0 ± 1.7</td>
<td>55.3 ± 1.5</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.9 ± 0.6</td>
<td>11.0 ± 3.4</td>
<td>15.0 ± 1.2</td>
<td>53.0 ± 1.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>3.4 ± 0.9</td>
<td>10.0 ± 3.1</td>
<td>17.3 ± 2.0</td>
<td>63.7 ± 1.8</td>
</tr>
<tr>
<td>Day 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.5 ± 0.7</td>
<td>11.0 ± 1.2</td>
<td>14.0 ± 1.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>55.7 ± 0.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>2.1 ± 0.9</td>
<td>13.3 ± 2.9</td>
<td>20.3 ± 1.6</td>
<td>65.3 ± 2.5</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.9 ± 0.8</td>
<td>10.7 ± 0.3</td>
<td>16.7 ± 2.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54.3 ± 3.1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>2.1 ± 0.8</td>
<td>13.0 ± 0.9</td>
<td>26.0 ± 2.3</td>
<td>76.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Figure is number of chondroclasts/mm hypertrophied cartilage; represents chondroclasts lining hypertrophied cartilage perimeter of the most proximal portion of the metaphyseal marrow vascular space from 3 poultstreatment/day.

<sup>h</sup>Figure is number of chondroclasts/mm hypertrophied cartilage.

Figure is from 3 poultstreatment/day.
<table>
<thead>
<tr>
<th>Group</th>
<th>Proliferating-(^a) prehypertrophied zone length</th>
<th>Unmineralized(^a) hypertrophied zone length</th>
<th>Mineralized(^a) hypertrophied zone length</th>
<th>Total(^a) length</th>
<th>Metaphyseal marrow vessel length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.5 ± .04</td>
<td>0.3 ± .02</td>
<td>0.7 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Day 3(^b)</td>
<td>0.7 ± .07</td>
<td>0.3 ± .03</td>
<td>1.4 ± 0.2</td>
<td>2.4 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Day 7(^c)</td>
<td>0.4 ± .02</td>
<td>0.1 ± .01</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Day 10</td>
<td></td>
<td></td>
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<td>Day 14</td>
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<tr>
<td>Day 17</td>
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<td></td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Figure is in mm from 3 poult\(s\)/treatment/day.

\(^b\) I = Vitamin D-deficient diet.

\(^c\) II = Vitamin D-deficient diet + oral Vitamin D<sub>3</sub>.

\(^d\) P < .05 between group I and group II.

\(^e\) P < .01 between group I and group II.
<table>
<thead>
<tr>
<th>Group</th>
<th>Chondroclast Index</th>
<th>Number of Epiphyseal Blood Vessels</th>
<th>Number of Metaphyseal Marrow Spaces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.2 ± 0.7</td>
<td>5.7 ± 1.3</td>
<td>11.7 ± 2.3</td>
</tr>
<tr>
<td>Day 3</td>
<td>1.9 ± 0.4</td>
<td>7.7 ± 1.7</td>
<td>11.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>2.3 ± 0.9</td>
<td>5.7 ± 0.3</td>
<td>12.3 ± 1.7</td>
</tr>
<tr>
<td>Day 7</td>
<td>2.3 ± 0.1</td>
<td>9.3 ± 2.4</td>
<td>16.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>2.3 ± 0.3</td>
<td>5.7 ± 1.2</td>
<td>18.3 ± 2.7</td>
</tr>
<tr>
<td>Day 10</td>
<td>4.0 ± 0.5</td>
<td>9.0 ± 0.0</td>
<td>16.3 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>3.5 ± 0.2</td>
<td>10.3 ± 1.9</td>
<td>23.0 ± 2.9</td>
</tr>
<tr>
<td>Day 14</td>
<td>3.0 ± 0.4</td>
<td>10.7 ± 3.2</td>
<td>16.7 ± 2.2d</td>
</tr>
<tr>
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<td>3.4 ± 0.4</td>
<td>7.0 ± 1.2</td>
<td>24.0 ± 0.0</td>
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<tr>
<td>Day 17</td>
<td>2.8 ± 0.8</td>
<td>11.3 ± 2.2</td>
<td>22.3 ± 1.5d</td>
</tr>
<tr>
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<td>2.4 ± 0.2</td>
<td>7.7 ± 0.7</td>
<td>29.7 ± 1.8</td>
</tr>
<tr>
<td>Day 21</td>
<td>2.7 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>24.3 ± 1.3e</td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.2</td>
<td>11.7 ± 2.2</td>
<td>37.7 ± 1.2</td>
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*Figure is number of chondroclasts/mm hypertrophied cartilage; represents chondroclasts lining hypertrophied cartilage perimeter of the most proximal portion of the metaphyseal marrow vascular space from 3 poult's/treatment/day.*

*Figure is from 3 poult's/treatment/day.*
Fig. 6: Proximal tibiotarsal, undecalcified bones.

Histological changes present in central region of each bone, von Kossa-HE. Top row, vitamin D$_3$ repleted poults, necropsy day in right hand corner. Bottom row, vitamin D-deficient poults, equivalent necropsy day as section above. E, epiphysis; P, proliferating-prehypertrophied zone; B, epiphyseal capillary; U, unmineralized hypertrophied zone; M, mineralized degenerating hypertrophied zone; V, metaphyseal marrow blood vessel length; epiphyseal - metaphyseal vascular streaking (arrowhead, day 14). In deficient poults, note central metaphyseal cartilage remnants (X, days 7 - 10); receding loss of mineral with reciprocal increase in amounts of unmineralized hypertrophic cartilage; scalloped distal margin of proliferating-prehypertrophied zone (arrow, day 10); metaphyseal blood vasculature branching; mineralization surrounding proximal metaphyseal blood vessels (open arrow, day 21).
Fig. 7: Enlargement of Fig. 6, vitamin D-deficient bottom row, day 10, near region of arrow. Von Kossa-HE.

Fig. 7a. Prominent scalloped proliferating-prehypertrophied distal margin.

Fig. 7b. Necrosis and hemorrhage of twisted cartilage column.

Fig. 8: Enlargement of Fig. 6, vitamin D-deficient and vitamin D₃ repleted poult's, day 21. a) b) Secondary metaphyseal trabeculae. Differences in osteoid seam thickness (arrow) lining well-mineralized trabeculae, solid osteoid trabeculae (arrowhead), marrow space cellularity and stromal components between vitamin D-deficient (a) and vitamin D₃ repleted poult's (b).
progression to primary and secondary spongiosa. Proximal tibiotarsus focal necrosis and hemorrhage occurred in areas of most severely twisted cartilage columns and this change was found only during day 10 (Fig. 7).

Additional histologic ricketic changes included progressively thickened osteoid seams which lined the hypertrophied cartilage columns and primary and secondary spongiosa. This change was present by day 10. By day 21, a band of newly formed secondary trabeculae, subadjacent to the cartilage columns, was composed of solid sheets of osteoid and trapped osteocytes; yet, the remaining secondary trabeculae still retained a major portion of their mineral (Fig. 8). During days 10–14, an apparent increased number of osteoclasts or chondroclasts were noted within metaphyseal marrow spaces of the cartilage columns; the chondroclast index measuring the number of chondroclasts actually lining the proximal portion of the cartilage columns was increased but did not differ significantly (P<.05) between groups I and II at day 10. Also, beginning at day 14, but especially by day 21, many of the osteoclasts and chondroclasts appeared inactive because of their rounded shape, distinct cellular borders, nonvacuolated bright eosinophilic cytoplasm and condensed hyperchromatic nuclei in comparison to group II osteoclasts whose corresponding morphology was just the opposite. At days 10–17, sheets of increased numbers of plump spindle-shaped osteoblasts were found within the metaphyseal marrow space, lining the periosteum and surrounding central fibrocartilagenous tendon attachment sites of the proximal tarsometatarsus and distal tibiotarsus. Compared to controls, group I metaphyseal marrow blood vessel space became more branched,
increased in size, was of similar length except for a lack of a peak at day 14, and decreased in total numbers (Fig. 6, Tables II, III). The total numbers of metaphyseal marrow blood vessel spaces in both group I and group II were quite variable, both between similar bones and different bones, with the distal tibiotarsus containing the greatest number followed with the proximal tarsometatarsus then the proximal tibiotarsus (Tables II, III). Furthermore, by day 21, the metaphyseal marrow spaces and diaphysis regions were markedly hypocellular and consisted primarily of loose spindle-shaped mesenchymal cells, vascular sinuses, a nonacid mucopolysaccharide ground substance, and sparse populations of erythroid and myeloid cells (Fig. 8). In all 3 bones, a solid sheet of metaphyseal and diaphyseal unmineralized cartilage cone which was removed by day 14 in group II, persisted as a small remnant in group I until day 21 (Fig. 6). Within this sheet of cartilage, central mineralization occurred only where blood vessels had invaded and this form of hypertrophic cartilage mineralization and removal was much less prevalent in group I than in group II. In group I, distal tibiotarsus condylar and proximal tarsometatarsus central secondary ossification centers had ricketic changes similar to the growth plates. Metaphyseal and diaphyseal cortical endosteal spaces progressively increased in size from day 14. In the deficient poult's, patchy areas of mineralized hypertrophic chondrocytes surrounded the most proximal metaphyseal blood vessels at day 21 (Fig. 6) in group I poult's. Parathyroids were single or double round lobes which were caudal to the thymus and thyroid glands. Delicately encapsulated, parathyroid
Fig. 9. Vitamin D-deficient poult, day 21, HE.

a) parathyroid lobes (P) containing ultimobranchial body (U) in a notch adjacent to the carotid artery and as a cap. Atrophied thymus (arrow), duct space (arrowhead). b) enlargement of a. Glandular ultimobranchial body acini, ciliated duct (D), parathyroid lobule (P). c) enlargement of b. Numerous vacuolated degenerate chief cells and indistinct lobulation.

Fig. 10. Vitamin D₃ treated poult day 21 HE. a) large cap of ultimobranchial body (U) and smaller lobe of parathyroid (P) adjacent to carotid artery (A) and cranial lobe of parathyroid (P). Note, numerous duct spaces (arrowheads). b) enlargement of a. glandular ultimobranchial bodies acini (U), duct spaces (arrowhead) and parathyroid lobules (P). c) enlargement of b. Lack of chief cell cytoplasmic vacuolation and distinct lobulation.
glands usually incorporated an ultimobranchial body (delicate acini structures) within a medial notch or as a peripheral cap (Figs. 9, 10). Rarely, ultimobranchial bodies were found free. Ducts, whose lining epithelium varied between pseudostratified columnar to simple flat epithelium were found free in the serosa surrounding parathyroid and ultimobranchial bodies and located within the parenchyma of these organs.

Parathyroid chief cells of deficient poult's were hyperplastic and, especially at days 10-14, tended to be organized and had palisades perpendicular to the delicate fibrovascular stroma as thin elongate spindle shaped cells. At the end of the experiment (days 17-21), vacuolar degeneration of hyperplastic chief cells was widespread (Fig. 9). Lobular detail was slightly less distinct in the vitamin D-deficient group. No obvious differences in numbers of mitotic figures or differences between ultimobranchial acini were detected between groups. Chief cells in group II were plump and disorganized. Marked thymic atrophy was an ancillary finding at day 21 in the vitamin D-deficient poult's.
DISCUSSION

Young growing poults appear to have a greater sensitivity to mineral deficiency than young growing chicks. In this experiment, rapid depletion of endogenous vitamin D₃ metabolites was quickly followed by severe rickets. The surprisingly low levels of plasma vitamin D₃ metabolites in the repleted poults during the first week might be due to partial failure of lipid absorption in young poults. This could affect vitamin D₃ absorption. Furthermore, the retrograde flow of digesta, which is important for turkeys total absorption of nutrients, might not be fully developed in young poults. If adequate vitamin D₃ is absorbed, the liver vitamin D₃ 25-hydroxylase system may not be fully developed or adequate during this period. During the latter periods of this experiment, the plasma levels of 25OH₃D₃, and 1,25(0H)₂D₃ in the vitamin D₃ treated poults were reduced compared to the plasma levels of corresponding metabolites in older turkeys.

The absence of 24,25(OH)₂D₃ in plasma during days 10-14 in control poults and elevated 1,25(OH)₂D₃ suggests that renal 24 hydroxylase was inhibited with subsequent maximum production of 1,25(OH)₂D₃. In this experiment, the plasma levels of vitamin D₃ metabolites obtained with individual oral dosing with 1400 IU of vitamin D₃/kg of feed, suggested that the NRC requirement of 900 IU of vitamin D₃/kg of feed may not be adequate for young poults on pre-starter diets. Other authors agree with this hypothesis. This early predisposition to low plasma levels of vitamin D₃ metabolites if combined with
gastrointestinal diseases, inadequate mixing of feeds, vitamin D₃ of low biological potential or availability, may be responsible for a larger portion of the noninfectious causes of turkey leg weakness than previously thought. The differences noted between the so-called field rickets and vitamin D₃-deficient rickets might also be explained on this basis.²¹,³⁵,³⁷,⁴⁴

The marked hyperphosphatemia noted during days 10-14 (Fig. 4) in the group I poults might have been selective bone loss of phosphorus. This is supported by the finding of a significantly increased bone ash calcium phosphorus ratio (Table I). The ossification defect in avian rickets has recently been proposed to be a phosphorus-related calcification defect which further prevents growth and interlocking of the apatite crystals.⁴⁰ Alternatively, the marked hyperphosphatemia at day 10 may be due to proximal tibiotarsus bone necrosis, cardiac muscle damage during cardiac puncture or erythrocyte hemolysis during collection and handling. The normocalcemia to slightly reduced levels of calcium and elevated levels of phosphorus in our experiment, contrasts with other authors findings of hypocalcemia and hypophosphatemia in avian rickets.⁴,¹¹,⁴⁶ Yet, the clinical findings, gross changes and light microscopic morphologic changes we described are similar to other authors descriptions of vitamin D₃-deficient avian rickets.¹¹,²⁶,³⁷ If the poult's would have lived longer than 25 days, they most likely would have developed hypocalcemia and hypophosphatemia.

Similar ossification of hypertrophic chondrocytes adjacent to the distal border of the proliferating-prehypertrophied zone (Fig. 6, day 21)
has also been reported in a case report of vitamin D-deficient rickets. This finding suggests, in light of parathyroid exhaustion by day 21, that ossification of cartilage matrix in vitamin D-deficient growing poults may be possible if adequate phosphorus and calcium are supplied to bone.

In this study, the major bony changes of vitamin D-deficiency occurred in the growth plate and metaphyseal regions, as reported by others. The bone lesions developed in a temporal sequence (which varied according to growth rate differences of individual bone), e.g., the greater elongation of the proximal end of the tibiotarsus compared to the distal end is partly due to its faster growth rate. This faster rate probably reflects the larger size of the normal proximal tibiotarsus proliferating-prehypertrophied zone. The growth rate of any particular bone has been reported to be determined by the size of its proliferative zone because the cellular proliferative rate, at least in mammals, is similar in different growth plates. The early elongation of the proliferating-prehypertrophied zone in all 3 bones coincided with significant depletion of endogenous vitamin D metabolites. These associated events, led us to speculate that in addition to the growth rate effect, the deficiency of vitamin D or one of its metabolites may also play a role in the elongation of the proliferating-prehypertrophied zone length. The metabolism of the zone of proliferation has been reported to be more affected by cholecalciferol than other growth plate regions and chondrocytes have been reported to hydroxylate 25OHD₃ to 24,25(OH)₂D₃.
This study led us to postulate that the metaphyseal capillaries or associated marrow space cells possibly can induce differentiation of the proliferating-prehypertrophied zone chondrocytes into hypertrophic chondrocytes. This assumption is based on the morphologic appearance in the vitamin D-deficient pouls of hypertrophic chondrocytes always surrounding metaphyseal capillaries in the irregular scalloped distal border of the proliferating-prehypertrophied zone and at day 21 the decreasing length of the proliferating-prehypertrophied zone is possibly associated with increased branching of the metaphyseal capillaries. This hypothesis would also support the theory that the increased length of the proliferating-prehypertrophied zone in avian rickets might be because of a delay in its maturation and hypertrophy. The delayed maturation could have also partially contributed to the elongation of the proliferating-prehypertrophied zone in our study, especially if the cellular effects, which are discussed below, are considered. Furthermore, in this study, the statistically significant (P<.05) greater number of metaphyseal blood vessel spaces found in the vitamin D$_3$ repleted poult bones was attributed to the larger size of the control poult bones and not necessarily a biological phenomenon of vitamin D-deficiency.

The continuous growth of group I proximal tibiotarsus proliferating-prehypertrophied zone until day 17 and then the rapid decrease in length compared to the peak at day 14 in the group II pouls emphasizes that multiple time periods need to be morphologically evaluated or invalid conclusions concerning the osseous changes associated with vitamin D-
deficient rickets might be made from single examinations. The cause of
group II proximal tibiotarsus and proximal tarsometatarsus steady
increase in growth plate length measurements until day 14 is not known;
however, these changes may be environmentally induced because the pouls
were housed in the brooder until day 14 and then released into a room
with concrete floors.

Rapid accumulation of metaphyseal unmineralized hypertrophic
chondrocytes was one morphologic hallmark of rickets in our study. Under
our experimental conditions, it was impossible to determine if this
change was because of maturation of proliferating chondrocytes or the
loss of mineral. Because this change occurred at different rates between
the proximal tibiotarsus and distal tibiotarsus, and with parathyroid
hyperplasia and normocalcemia, we assumed that both processes are
operative. It was not possible to determine from this experiment the
cause of the disproportionate loss of mineral from the growth plate, but
apparent minimal loss from the metaphyseal secondary trabeculae. There
may be differences in types of mineral deposited between these two areas
such as bushite, amorphous CaP\textsubscript{0.4} or octacalcium-phosphate\textsuperscript{2,40} versus
the more resistant hydroxyapatite in the secondary trabeculae. The layer
of osteoid lining the secondary trabeculae may also prevent osteoclast
resorption.\textsuperscript{32}

In the vitamin D-deficient pouls, the persistence of the meta-
physeal cone of hypertrophic chondrocytes and abundant accumulation of
unmineralized hypertrophic chondrocytes in large trabeculae indicated
that this form of chondrocytes and matrix may be resistant to
chondroclast resorption. This resistance may be caused by the masking of chemotactic matrix material\(^2\) with a layer of osteoid which might decrease the number of regional monocytes or effect their further differentiation into osteoclasts;\(^14,30\) however, the chondroclastic index in our experiment did not demonstrate a decrease in number of chondroclasts. The unmineralized osteoid layer could have reduced osteoclast removal\(^3\) or as noted toward the end of this experiment the osteoclasts could have been inactivated, possibly because of parathyroid exhaustion. This defect may be caused by a direct deficiency of vitamin D\(_3\) or one of its metabolites in its role of stimulating monocyte differentiation,\(^1,6\) or as a metabolite for osteoblasts.\(^2\) The increased numbers of undifferentiated spindle-shaped cells noted in the deficient poult's marrow spaces may be caused by vitamin D\(_3\) deficiency which then might secondarily affect the differentiation of the proliferative-prehypertrophied chondrocytes.
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ULTRASTRUCTURAL PATHOLOGY OF VITAMIN D DEFICIENCY IN GROWING TURKEYS

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Bone (proximal tibiotarsus) and parathyroid chief cells were examined from growing turkey poults fed a vitamin D-deficient diet and from control poults which were given the same diet and a progressively increased oral dose of vitamin D₃. Parathyroid chief cells were metabolically inactive at Day 1. Throughout the remainder of the experiment, control poults had mostly metabolically active chief cells containing abundant rough endoplasmic reticulum, Golgi apparatus, and secretory granules. Vitamin D₃-deficient poults had hyperplastic parathyroid chief cells from day 10, characterized by progressively increasing electron lucency in cytoplasm. Cytoplasmic volume was expanded, Golgi complexes were enlarged and dilated, and were associated with numerous vesicles, vacuolar spaces, and prosecretory granules. Rough endoplasmic reticulum cisternae were abundant and irregularly dilated at one end. The number of mature secretory granules were reduced. Contiguous chief cell plasma membranes were tortuous and interdigitated. By day 21, clear widened intercellular spaces were found and chief cells contained numerous cytoplasmic vacuoles and vesicles and individual transitional clear chief cells were found. Osseous changes in vitamin D-deficient poults consisted of a lack of provisional mineralization from day 10. Cartilage matrix vesicles (80-113 nm in diameter) and bone matrix-like vesicles (53-133 nm in diameter) were present in both groups and mineralization was associated with these structures. Other minor osseous
differences were interpreted to be nonspecific for vitamin D₃-deficient rickets.

...
INTRODUCTION

In vitamin D deficiency, parathyroid hyperplasia and abnormal bone growth have been documented in avian species. Ultrastructural changes of secondary hyperparathyroidism have been reported in avian vitamin D$_3$ deficiency, or dietary hypocalcemia. In vitamin D deficiency, hyperplastic chief cells usually have irregular plasma membranes with complex interdigitation of contiguous cells, expanded cytoplasmic volume, decreased numbers of mature secretory granules and expanded or increased numbers of cytoplasmic organelles associated with protein synthesis and packaging of secretory products. The ultrastructural features of normal avian parathyroid glands appears to be morphologically similar to the parathyroid glands of many other species, except that the number of mature secretory granules are claimed to be fewer in number than in most mammals. Chief cells from parathyroids of normal growing chickens and normal laying hens have characteristics of active secretion, probably because of the high rate of calcium metabolism during their rapid bone growth phase or egg laying cycle.

In vitamin D deficiency, osteoclast, osteoblast, osteocyte and chondrocyte changes are thought to be minimal when compared to controls treated with vitamin D$_3$. A sequential ultrastructural study of ricketic bone combined with parathyroid ultrastructural changes is needed to characterize events underlying the changes seen at the light microscopic level. It may help to define the pathogenesis of the osseous
changes found in avian vitamin D₃-deficient rickets. This mechanism remains elusive even though the biochemical metabolism of vitamin D is well understood.¹⁴,³⁷ These sequential morphologic changes may also provide additional input into the mechanisms of endochondral ossification.

The purpose of this study was to record the parathyroid gland and osseous changes found during the sequential development of vitamin D₃ deficient rickets in growing turkeys. Light microscopic findings are described in our paper on vitamin D₃-deficient rickets in growing turkeys.²¹
MATERIALS AND METHODS

The vitamin D₃-deficient diet and experimental design used to produce the vitamin D₃-deficient ricketic poults and vitamin D₃ treatment protocol for control poults were recently reported; the parathyroid glands and proximal tibiotarsus used in this experiment were obtained from these same turkey poults. Poults were necropsied and examined at days 1, 3, 7, 10, 14, 17 and 21.

At necropsy, the left proximal tibiotarsus from the first randomly selected poult of each treatment group during each time period was immediately cut using a razor blade into thin midfrontal slabs, approximately 1-2 mm thick. From this cut, a second 2-3 mm wide section was cut from the central portion and then further cut into a minimum of 4 blocks from the region primarily subadjacent to but partially including the growth plate band. All cuts were trimmed under fixative. The right parathyroid-ultimobranchial body-thymus complex was removed en bloc from the second poult of each treatment group and time period. Because the parathyroid gland was not easy to identify grossly, a minimum of three 1 mm³ blocks were taken from the most appropriate regions. The parathyroid and undecalcified proximal tibiotarsal growth plate were fixed in 2.5% glutaraldehyde, washed overnight, stained in osmium tetroxide, rinsed in cacodylate buffer for one hour, processed through graded ethanols and propylene oxide and embedded in epoxy resins (Epon).

A minimum of two blocks from the proximal tibiotarsus were cut in a plane parallel to the proximal-distal axis of the growth plate using a
diamond knife. Usually, the most proximal portion of capillary invasion into hypertrophic cartilage was found by light microscopic examination of 1 μm toluidine blue stained sections which were then further processed for transmission electron microscopic examination. Likewise, 1 μm sections of the parathyroid glands were cut. Occasionally, it was necessary to return to the parathyroid complex and resample because the parathyroid gland was not found on initial sectioning. Thin sections stained with uranyl acetate and lead citrate were examined with a Philips 410 electron microscope (Philips Electronics, Eindhoven, The Netherlands) at 60 kV.
RESULTS

At day one, parathyroid chief cells were in closely packed solid nests which were surrounded by a continuous basement membrane, capillaries and spindle shaped mesenchymal cells. Most chief cells had evidence of metabolic inactivity (Fig. 1). The predominant chief cell was small and cuboidal, with an electron lucent cytoplasm and had a high nuclear to cytoplasm ratio. Its cytoplasm contained abundant lipid vacuoles but few organelles. There were few Golgi complexes and those present had tightly apposed saccules with few vesicles or prosecretory granules associated with the maturing face. Most of these chief cells had small rough endoplasmic reticulum cisternae and scattered ribosomes. Low numbers of chief cells contained a few large (.3-.4 um in greatest diameter) membrane bounded round, electron-opaque, secretory granules. Plasma membranes were tightly apposed and straight.

During days 7-21, chief cells from the vitamin D₃ replete poult were composed of nests of tightly packed elongate to polygonal cells. The majority of chief cells appeared metabolically active, e.g., they contained abundant and well-developed cytoplasmic organelles concerned with protein synthesis and secretion. Chief cell cytoplasmic volume was expanded and its nuclear to cytoplasmic ratio was estimated to be reduced when compared to day 1. Cytoplasmic organelle proportion and morphology varied considerably among chief cells. The cytoplasmic matrix of some chief cells had a slightly increased electron density. These cells contained a greater amount of dispersed and aggregated profiles of rough
Fig. 1: Parathyroid chief cells day 1. Closely apposed cuboidal cells have small cytoplasmic volumes with high nuclear to cytoplasmic ratio, few cytoplasmic organelles, few mature secretory granules (arrow), lipid droplets (arrowhead) and unruffled plasma membranes. Bar = 1.0 um
endoplasmic reticulum and increased amounts of aggregated ribosomes. The rough endoplasmic reticulum profiles were usually short, irregularly dilated, and contained granular grey precipitate. In fewer cells, the rough endoplasmic reticulum cisternae were parallel, dilated, and organized into lamellar arrays. More electron lucent chief cells contained prominent expanded Golgi complexes with greater numbers of associated and nonassociated vesicles and prosecretory granules, and lesser amounts of rough endoplasmic reticulum cisternae and ribosomes.

The presence and number of mature secretory granules varied between these cells during days 10-21. Usually, the more electron lucent cells contained high numbers of small (0.13-0.26 μm) pleomorphic (dumbbell and oblong) and elongated secretory granules (Fig. 2). Whereas, other cells contained lesser numbers of round, larger (.3-.4 μm) secretory granules; however, secretory granules could usually be found in all chief cells examined. At day 7, secretory granules were judged to occur infrequently and were of similar size when compared to day one. Coated vesicles associated with Golgi complexes were scattered throughout the cytoplasm. Cytoplasmic lipid vacuoles were commonly seen and were numerous in some cells. Chief cell plasma membranes for the most part were relatively straight without interdigitation, but chief cells could be found with tortuous interdigitated plasma membranes.

In vitamin D₃-deficient poult's, from day 10 on, chief cell hyperplasia and hypertrophy were evident. Chief cells were closely packed, elongated or irregularly polyhedral, and the majority were markedly enlarged. Contiguous cell plasma membranes became tortuous and
Fig. 2: Parathyroid chief cells, vitamin D$_3$ replete poult, day 21. Note relative straight contiguous plasma membranes with uncomplicated interdigitations, numerous pleomorphic secretory granules (SG), prosecretory granules (P), lipid vacuoles (L), Golgi complex (G), and rough endoplasmic reticulum (arrows). Bar = 1 um.
intricately interdigitated with occasional cells having complicated infoldings. Adjacent to the perivascular spaces and in scattered areas (especially by day 21) the intercellular spaces were widened and contained grey granular material or were eccentrically dilated with clear spaces. The cytoplasmic volume of most cells was a greater proportion of the total cell volume than occurred in control poult. Chief cell cytoplasmic organelle proportion and morphology did not vary as extensively between cells (as was noted in the control poult) because their cytoplasm appeared progressively more electron lucent with increasing time when compared to control poult. By day 21, a predominant change was the high number of smooth membrane vesicles and variable sized vacuolar spaces primarily associated with enlarged and dilated complex Golgi apparatus but were also randomly scattered throughout the cytoplasm (Fig. 3). Other causes of increased cytoplasmic electron transparency included: short eccentrically dilated or vacuolated rough endoplasmic reticulum cisternae, decreased numbers of ribosomes, and decreased numbers of secretory granules. At day 21, chief cells were nearly degranulated, but a few large (.3-.4 um or rarely .5-.6 um) randomly located secretory granules were found in most chief cells. Prosecretory granules and some coated vesicles could usually be found associated with expanded Golgi complexes and also scattered throughout the cytoplasm. Cytoplasmic lipid vacuoles were fewer than found in control poult. In group I, at day 21, occasional cells interspersed among chief cells had a prominent electron lucent cytoplasm and rare cytoplasmic organelles. These cells were interpreted to represent tran-
Fig. 3: Parathyroid chief cells, vitamin D₃ deficient poult, day 21. Large area of cytoplasm contains dilated Golgi complex (G), smooth membrane-lined vacuolar spaces (V), vesicles (arrow), expanded dilated rough endoplasmic reticulum cisternae (arrowhead), large mature secretory granules (SG), prosecretory granules (P), adjacent stromal capillary (C) and apposing contiguous widened basal laminae (L). Bar = 1 um
Fig. 4: Parathyroid chief cells, vitamin D$_3$-deficient poult, day 21. Transitional clear cell with distinct electron lucent cytoplasm and few cytoplasmic organelles. Mitochondria (M), mature secretory granules (G), plasma membrane with widened intercellular spaces (arrow), and background of ribosomes. Bar = 1.0 um
sitional clear cells (Fig. 4).

Minor ultrastructural morphologic differences were detected between the vitamin D₃ deficient poult's and the vitamin D₃ treated poult's when chondrocytes or their matrix, osteoblasts, osteoid, chondroclasts or osteoclasts were compared in the area of proximal metaphyseal capillary invasion of hypertrophic cartilage. A lack of provisional mineralization was obvious from day 10 on in the vitamin D₃ deficient poult's, and from day 14 on a slightly increased number of osteoblastic and chondrocytic intracytoplasmic lipid vacuoles were detected. A slightly greater population of undifferentiated plump spindle and round stem cells whose cytoplasm contained fewer organelles was found in vitamin D₃ deficient poult's marrow spaces especially during days 10-14 (Fig. 5). By day 21, similar cells were seen whose cytocavitary network was dilated and vacuolated. Osteoclasts or chondroclasts from the deficient poult's, located in marrow spaces from day 17 on, appeared inactive because of their condensed electron opaque cytoplasm, cortical distribution of small cytoplasmic vacuolar spaces, fewer cytoplasmic organelles and irregular short cytoplasmic projections (Fig. 15). Mature osteoblasts, 2-3 cells thick, could be found lining hypertrophic chondrocyte matrix indicating that hypertrophic chondrocytes could be found more distal from the proliferating-prehypertrophied zone than occurs in vitamin D₃ replete groups.

In both groups, hypertrophic chondrocyte's cytoplasm contained identifiable mitochondria, electron dense amorphous debris and ribbons of irregularly dilated endoplasmic reticulum cisternae filled with electron
Fig. 5: Tibiotarsus, marrow space, vitamin D-deficient poult, day 10. Numerous undifferentiated round stem cells with few cytoplasmic organelles. Mononuclear phagocytic cell (P), containing primary and secondary phagolysosome (arrow), adjacent hypertrophic, chondrocytic matrix (X), metaphyseal capillary (C) and presumed osteoblast (O). Bar = 1.0 um
Fig. 6: Enlargement of mononuclear phagocytic cell (P) and secondary phagolysosomes in Fig 5. Note, pseudopodia partially surrounding grey granular debris (arrow). N (nucleus). Bar = 0.5 um
Fig. 7: Metaphysis, capillary invasion of hypertrophic chondrocytes, matrix and matrix vesicles from the proximal tibiotarsus of vitamin D₃ replete poult, day 7. Matrix vesicles (arrow) are most prominent in longitudinal wall (LW). Hypertrophic chondrocyte: M, mitochondria; ribbons of RER arrowhead; electron dense cytoplasmic debris. Bar = 1.0 µm.

Insert. Enlargement of pleomorphic matrix vesicles. Bar = 0.5 µm.
dense granular material (Fig. 7). The nuclei of hypertrophic chondrocytes were irregular in shape and contained condensed granular chromatin. The plasmalemma was thin and irregular in outline and was often pulled away from the perilacunar matrix. The perilacunar matrix was most prevalent in the degenerating hypertrophic zone and was composed of evenly dispersed fine granular material lacking fibrils (Figs. 8, 9).

Degenerating hypertrophic chondrocytes contained fewer identifiable cytoplasmic organelles and large electron lucent spaces when compared to hypertrophic chondrocytes (Figs. 8, 9). In both groups, in the area of metaphyseal capillary invasion of hypertrophic chondrocyte associated matrix, lining capillary endothelial cells had short pseudopodia-like projections extended into the adjacent matrix and aggregated matrix material was noted within endothelial gap junctions. The hypertrophic and degenerating hypertrophic chondrocytes were surrounded by a distinct septal wall of interterritorial matrix (Figs. 7-9). The interterritorial matrix was composed of a background of electron dense granules which were both free and attached to thin interlacing randomly oriented aperiodic microfibrils; microfibrils were only found in the interterritorial matrix. This matrix also contained pleomorphic (round to oblong) membrane bounded structures suggestive of matrix vesicles. The matrix vesicles varied in size from 80-113 nm in diameter. They contained central electron dense cores and usually were surrounded with a thin electron lucent halo (Fig. 7). Matrix vesicles were most prevalent in central portions of longitudinal walls, which was the major region of
Fig. 8: Degenerative hypertrophic chondrocytes and provisional calcification of longitudinal wall. Vitamin D₃ replete poult, day 21. Longitudinal wall of provisional calcification (L) with no obvious matrix vesicles surrounding area of provisional calcification, loss of chondrocyte cytoplasmic organelles, with large electron lucent spaces, ribbons of RER (arrow) and perilacunar granular aggregates (P). Bar = 1.0 μm
Fig. 9: Enlargement of Fig. 8. Aperiodic microfibril (arrow). Bar = 0.5 um
provisional calcification and occasionally they were found to contain needle-like deposits of mineral. Adjacent to the areas of provisional calcification, few matrix vesicles were found in the vitamin D₃ replete poults (Figs. 8, 9).

In both groups, osteoblasts appeared to differentiate from a progression of metaphyseal marrow space spindle shaped cells containing few cytoplasmic organelles to well-differentiated, cuboidal, nonpolarized metabolic active osteoblasts containing abundant cytoplasmic organelles (Figs. 10-13). Mature osteoblasts, arranged one to two cells thick, contained large Golgi complexes and abundant expanded granular endoplasmic reticulum cisternae, which contained fine grey deposits and were partially wrapped around mitochondria. Numerous cytoplasmic projections, filled with microfilaments, penetrated deeply into the adjacent osteoid (Figs. 11, 13). Fine granular aggregates of electron dense material were found more often in the terminal osteoblastic cytoplasmic projections in vitamin D₃ replete poults than in deficient poults (Figs. 12, 14). The mature osteoblasts primarily lined a layer of osteoid but in regions of early osteoid formation they lined the noncalcified border of the cartilage matrix. In areas where nonmineralized osteoid lined the cartilage matrix, a definable border was present between them, indicating that osteoid minimally penetrated the cartilage matrix layer (Fig. 12).

Round vesicle-like structures (53-133 nm), which contained central electron dense cores and were occasionally surrounded by electron lucent halos, were found within the interfibrillar space of collagen osteoid
Fig. 10: Proximal tibiotarsus metaphyseal marrow space cells, vitamin D\textsubscript{3} replete poult, day 14. Spindle shaped cells, suggestive of osteoblastic precursors (P), contain few cytoplasmic organelles versus well-differentiated osteoblasts (O) with abundant RER (arrow) and mitochondria (M). Metaphyseal capillary (C), mineralized osteoid region (MO). Bar = 1.0 um
Fig. 11: Osteoblasts adjacent to degenerative hypertrophic chondrocytes in vitamin D-deficient poult, day 14. Osteoblasts (O) 1-2 cells deep, prominent osteoblastic cytoplasmic projection (P), thick osteoid layer (X), aggregates of chondrocyte matrix vesicles (arrow) in twisted cartilage longitudinal wall (L). Bar = 1.0 um
Fig. 12: Enlargement of Fig. 11, near osteoblast cytoplasmic projection (P). Round variable sized membrane bound electron dense vesicle-like structures (short arrow), abundant periodic collagen osteoid fibers (arrowhead), cartilage matrix and nonperiodic microfibrils (long arrows). Bar = 0.5 μm
Fig. 13: Mineralization of osteoid calcification front, in vitamin D₃ replete poult, day 10. Osteoblastic electron lucent cytoplasmic region (L) and multiple cytoplasmic projections (P). Electron dense amorphous mineral deposits in osteoid (arrows). Osteocyte (OC) surrounded by osteoid. Bar = 1.0 um
Fig. 14: Enlargement of calcification front, Fig. 13.

Early needle-like mineral deposits on periphery of electron dense vesicle-like structures (arrow) within collagen (osteoid) interfibrillar space. Osteoblast cytoplasmic projection (P) and terminal osteoblastic cytoplasmic projections contains aggregated fine electron dense granules (arrowhead). Bar = 0.5 um
fibers of the calcification fronts of the control poult's and in the osteoid layer in deficient poult's lacking mineral deposition (Figs. 12, 14). Similar structures were not found in osteoblastic cytoplasm. Needle-like mineral crystals were found on these vesicle-like structures (Fig. 14). They were fewer in number compared to the numbers of matrix vesicles in cartilage interterritorial matrix. In early calcified bone matrix, random nodular areas of increased electron density (associated with crystalline mineral growth), appeared to correspond with similar random areas of vesicle-like structures deposited in non-mineralized osteoid of deficient poult's. Mineral spicules associated with collagen fibers or interfibrillar spaces could be found without obvious vesicle-like structures.

Mononuclear phagocytic cells from deficient poult's contained electron dense degenerate chondrocytic remnants within prominent secondary lysosomes (Fig. 6). This also occurred in vitamin D₃ repleted poult's, but to a lesser extent. Chondroclasts from both groups (but especially in the deficient poult's) had pseudopodia-like extensions around similar electron dense chondrocyte remnants. These chondroclasts lacked obvious ruffled borders but instead had well-developed clear zones when compared to osteoclasts from repleted poult's undergoing osteolysis (Fig. 15).
Fig. 15: Proximal tibiotarsus marrow space osteoclasts, chondroclastic phagocytosis of chondrocyte electron dense debris and osteoclastic osteolysis from vitamin D₃ deficient and vitamin D₃ replete poults.

Fig. 15a: Vitamin D₃ deficient poult, day 21. Inactive osteoclast with condensed electron dense cytoplasm, cortical cytoplasmic small vacuolar spaces and few delicate cytoplasmic projections adjacent cartilage matrix. Bar = 1.0 um

Fig. 15b: Vitamin D₃ replete poults, day 14. Osteoclastic ruffled border (R) clear zone (CZ) and numerous large cytoplasmic vacuolar spaces. Bar = 1.0 um

Fig. 15c: Vitamin D₃ deficient poults, day 21. Compare chondrocytic electron dense debris within chondroclast pseudopodia versus osteoclast osteolysis in Fig. b. Note prominent clear zone (CZ) surrounding cytoplasmic membrane invagination and lack of obvious ruffled border, mitochondria (M). Bar = 1.0 um
In this study, chief cell hyperplasia and hypertrophy, first noted at day 10 in the vitamin D-deficient poults, corresponded with the prominent clinical, gross and light microscopic ricketic changes that we recently reported. Tortuous plasma membranes with complex interdigitations may be an indicator of chief cell secretory activity associated with effects of decreased ambient calcium concentration. Similar plasma membrane changes found early in this study and when correlated with the slightly depressed plasma levels of calcium, suggest that turkey chief cells are highly sensitive to slight changes in plasma calcium levels. Similar chief cell plasma membrane changes have been described in vitamin D deficient or dietary hypocalcemic hyperparathyroidism.

Prosecretory granules most likely contain the secretory product responsible for the normocalcemic to slightly depressed calcium levels that we found in the vitamin D-deficient poults because chief cells were nearly degranulated of mature secretory granules. In both of our groups, most secretory granules were similar to sizes (0.1-0.4 um) reported in chickens. The rare larger secretory granules (0.5-0.6 um) found in the deficient poults may represent forms found in degenerate or exhausted cells incapable of secreting this material. The number of mature secretory granules in chicks is reported to be low. Comparing the number of secretory granules that we found in the vitamin D repleted turkeys to these reports, turkey chief...
cells evidently contain higher numbers of secretory granules than those of the chicken. The numbers of secretory granules in chief cells can vary markedly between closely related species since they are reported to be numerous in mice but infrequent in rats. During embryonic development or in newborn animals, inactive chief cells predominate, which supports our finding in day 1 poult that inactive chief cells prevail.

Chief cell vacuolar degeneration during days 17-21 was a predominant light microscopic finding in vitamin D3 deficient poult. Based upon our ultrastructure findings, this change is probably due to the coalescence of cytoplasmic vacuoles, widened intercellular spaces, and presence of transitional clear cells. Chief cell cytoplasmic vacuolation and appearance of transitional clear cells also suggested that a final stage of parathyroid exhaustion had been reached, with an expected onset of hypocalcemia to quickly follow if these poults had lived longer. Pale chief cells, similar to our transitional clear chief cells, have been described in normal laying hens. Consequently, transitional clear chief cells may not represent an exhausted chief cell but may be a normal cell type occasionally found in avian parathyroid glands.

In this study, the majority of chief cells in the vitamin D3 treated poults were in the active stage of their secretory cycle. Chief cells from normal growing chickens, or laying hens are in an active stage of their secretory cycle, probably due to rapid bone growth or egg laying. In growing chickens, cytoplasmic organelle proportion
varies among chief cells which compares similarly to this study. We did not see the four distinct chief cell types recently reported in vitamin D$_3$ deficient chickens.\textsuperscript{38} Our results agree with other ultrastructural descriptions of bony changes in vitamin D deficiency of chickens\textsuperscript{10,11} or mammals,\textsuperscript{3,8,15} and normal avian growth plates.\textsuperscript{12,20,24} Large amounts of intracytoplasmic lipid were found in all zones of chicken ricketic chondrocytes;\textsuperscript{10} yet this contrasts with our finding of minimal intracytoplasmic lipid and with reports in ricketic rats whose cartilage lipids are depleted.\textsuperscript{23} Other changes in ricketic chondrocytes were chains of intracytoplasmic vesicles,\textsuperscript{10} mitochondria loaded with calcium and phosphate,\textsuperscript{6} increased amounts of rough endoplasmic reticulum and increased numbers of mitochondrial profiles.\textsuperscript{15} Some of these changes were thought to be due to interference with normal cartilage lipid metabolism or secondarily to inefficient oxidative energy production.\textsuperscript{10,15} In swine osteochondrosis, similar ultrastructural changes are present and are interpreted as variable and nonspecific for osteochondrosis.\textsuperscript{17} Osmium ferrocyanide used as a secondary fixative to improve matrix components of hypertrophic zone chondrocytes\textsuperscript{16,17} may have shown additional differences between groups than with the glutaraldehyde fixative used in this experiment.

The extracellular chondrocyte matrix vesicles and matrix-like vesicles in bone calcification fronts described in this study are the first report in growing turkeys. Besides being slightly smaller, these structures are similar to structures described by other
To verify both of these structures as matrix vesicles, ultrastructural histochemistry and biochemical analysis will be needed, especially when there is controversy regarding matrix vesicles involvement with mineral deposits in bone. Matrix vesicles exist in bone where large amounts of osteoid exist, as in the area that we examined, but not where the ratio of mineralized to unmineralized osteoid is high, such as in lamellar bone. In some types of early bone formation, matrix vesicle direct involvement beyond the initiating phase of mineralization appears to be minimal. In our study, osteoid that was mineralized in the calcification fronts without the presence of obvious matrix vesicles, might be due to sectioning artifact and serial sections might reveal matrix-like vesicles. Needle-like mineral crystals associated with matrix-like vesicles in interfibrillary spaces in our study agrees with other studies in rapidly growing bone. In a review, Wuthier suggested that secondary nucleation of collagen follows independent of matrix vesicle mineralization so that calcifiable collagen does not primarily serve as initiator but rather as receptor and propagator of mineralization. However, others argue that calcifiable forms of collagen induce calcification of bone and that mineral deposits are associated with the periodicity of collagen and are independent of matrix vesicles.

Moreover, the osteoid matrix-like vesicles in our study may represent a portion of osteoblastic cytoplasmic projections that were often found penetrating deep into the osteoid layer. Especially, since cartilage matrix vesicles are thought to form from hypoxic chondrocytes by budding
from cell processes and are membrane bound extracellular structures containing membrane bound phosphotases (primarily alkaline phosphatase), and calcium, phosphorus, actin and lipids. Cartilage matrix vesicles from ricketic rats are morphologically similar and alkaline phosphatase activity is retained when compared to vitamin D₃ replete rats; furthermore, the initial site of mineralization occurs in matrix vesicles of healing ricketic cartilage. When rat cartilage and bone matrix vesicles are compared, cartilage has greater enzyme activity and protein content than bone probably because cartilage contains more matrix vesicles than bone per gram of tissue, similar to what we estimated. In addition, other enzyme parameters were similar, suggesting to these same authors and in a recent review that primary mineralization of bone and cartilage matrix vesicles may undergo similar mechanisms.

Mononuclear phagocytic cells play a role in bone remodeling. Monocyte chemotactic factors are released from resorbed or remodeling bone. In this study, chondrocyte electron opaque matrix material found in mononuclear phagosomes may also be chemotactic for monocytes. The irregular branching of metaphyseal capillaries found in vitamin D deficiency may be due to mononuclear phagocytic cells and chondroclasts removing chondrocyte and matrix debris that normally would be filled with mineralized osteoid but in vitamin D-deficiency, these areas are filled with branching capillaries and nonmineralized osteoid. In this study, chondroclasts also may have been induced by this same material to produce only clear zones with the
absence of easily recognizable ruffled borders; however, osteoclast clear zones can exist without ruffled borders, or the ruffled border may have been present in another plane of section. The inactive osteoclasts and degenerate marrow space cells seen near the end of this experiment in the vitamin D₃ deficient poults may be the metabolic consequences of starvation, dehydration and parathyroid exhaustion and not directly due to a lack of vitamin D. Osteoclasts had no significant ultrastructural changes in thyroparathyroidectomized rats repleted with high doses of parathyroid extract and fed a low calcium, vitamin D-deficient diet.
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PATHOLOGY OF VITAMIN D DEFICIENCY
IN VITAMIN D₃ METABOLITE REPLETED GROWING TURKES

O.R. HEDSTROM, R.L. HORST, AND N.F. CHEVILLE
Bone and parathyroid structural changes and clinical signs were examined in 3 groups of day-old turkey poult and clinical signs were fed a vitamin D-deficient diet for 5 weeks. Poult received progressively increased oral repletion: vitamin D₃, group I; 1,25(OH)₂D₃, group II; 1,25(OH)₂D₃ combined with 24,25(OH)₂D₃, group III. Group I total femoral calcium and phosphorus levels, standardized for body weight, were similar to group III but were greater (P<.05) than group II. Beginning at weeks 2-3, groups II and III had mild clinical and early histomorphometric rickett changes. Elongated proliferating-prehypertrophied zone with irregular distal border were found in bone growth plates and elevated alkaline phosphatase levels (P<.05 during weeks 4-5). Also, groups II and III histomorphometric growth plate lengths (unmineralized hypertrophied zone, mineralized hypertrophied zone, metaphyseal marrow vessel length and total growth plate length) were usually longer than comparable group I lengths. All 3 groups had normocalcemia, normophosphatemia, no evidence of endochondral ossification defects, no differences in parathyroid chief cells, no statistically significant body weight or bone ash differences, and no evidence of lameness. In groups II and III endogenous levels of 250HD₃ were depleted by week 1; likewise, in group II, 24,25(OH)₂D₃ levels were depleted by week 1. These studies suggest that 24,25(OH)₂D₃ has biologic significance as a vitamin D₃ hormone because it synergistically restores bone levels of calcium and phosphorus in growing
turkeys comparable to vitamin D₃. In addition, these studies show that vitamin D₃ is more effective than 1,25(OH)₂D₃ and that 24,25(OH)₂D₃ provides no synergism in preventing early bone and clinical ricketic changes.
INTRODUCTION

Vitamin D, a steroid hormone, requires hydroxylation before its activity can be manifested in the intestine, bone or kidney. Hepatocytes hydroxylate Vitamin D$_3$ to 25-hydroxyvitamin D$_3$ (25OHD$_3$) followed by renal hydroxylation of 25OHD$_3$ into 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) or 24,25-dihydroxyvitamin D$_3$ (24,25(OH)$_2$D$_3$). 13,38 1,25-dihydroxyvitamin D$_3$ is currently believed to be the major vitamin D$_3$ metabolite responsible for the physiologic actions of vitamin D: calcium and phosphorus absorption from the intestine, 13,19,49 and calcium and phosphorus reabsorption from bone. 18

Considerable controversy exists as to the role that these metabolites play in skeletal mineralization. Some authors report that ricketic rats$^{32}$ and chicks$^{9,46}$ can be cured by administration of 1,25(OH)$_2$D$_3$ alone. This metabolite increases tibial ash weight in chicks more than other vitamin D$_3$ metabolites. 4 However, some authors suggest that 1,25(OH)$_2$D$_3$ by itself cannot reproduce all of the beneficial osseous changes of vitamin D$_3$ in chicks$^{8}$ or rats$^{2,17,20}$ and it cannot support embryonic chick development.$^{23,45}$

Some authors claim 24,25(OH)$_2$D$_3$, usually in combination with 1,25(OH)$_2$D$_3$, is an essential vitamin D$_3$ metabolite for proper bone metabolism,$^{3,25,33}$ healing of rickets in chicks,$^{15,41}$ ricketic chick fracture repair,$^{14,39}$ healing of rickets in rats$^{2,20}$ embryonic chick development,$^{23}$ calcium homeostasis,$^{39}$ cartilage growth$^{11}$ and
differentiation, and maintenance of chick parathyroid gland size. Recently, a synthetic sterol (24,24-difluoro-25 hydroxyvitamin D₃) which cannot be hydroxylated at carbon 24 and hence converted to 24,25(OH)₂D₃ has been used to test the significance of 24 hydroxylation for its vitamin D physiological functions. The biologic activity of this synthetic sterol is similar to 25OHD₃ in terms of calcium and phosphorus homeostasis, promotion of osseous mineralization and treatment of rickets in growing rats or in offspring of rats maintained for 2 generations with this sterol. In addition, this sterol supports chick embryonic development. Based on these studies, 24,25(OH)₂D₃ appears not to have vitamin D₃ metabolic activity; in addition, some authors argue that 24-hydroxylation may represent the initial step in a degradative pathway.

This study compared vitamin D₃, 1,25(OH)₂D₃ and 1,25(OH)₂D₃ combined with 24,25(OH)₂D₃ for their capability to prevent qualitative changes of vitamin D deficiency in growing turkey poults. Furthermore, we wanted to compare structural and clinical variations in vitamin D-deficient poults repleted with vitamin D₃ or these same metabolites. This is important because the interaction of these two active vitamin D₃ metabolites needs further study to clarify their role in skeletal homeostasis.
MATERIALS AND METHODS

Experimental design and environmental conditions for this experiment were similar to our recent report;19 this experiment ran concurrently. Briefly, one-day-old male broad breasted white turkey poults were started on a synthetic vitamin D-deficient diet--Tekland, (Madison, Wisconsin) diet--TD82391; this diet was highly rachitogenic.21 The poults were divided into 3 groups of 20 poults per group and housed in separate rooms. Beginning at day one, oral treatment delivered in 0.1 ml of corn oil, was as follows: group I (control) received vitamin D-deficient diet plus progressively increased doses of vitamin D₃; group II received progressively increased doses of 1,25(OH)₂D₃; group III, received increased doses of 1,25(OH)₂D₃ combined with 24,25(OH)₂D₃. The daily dose of vitamin D₃, for group I poults was as follows: week 1 (0.5 ug), week 2 (1.0 ug), week 3 (2.0 ug), week 4 (3.0 ug) and week 5 (4.0 ug). Group I poults from weeks 1, 2, and 3 were the same poults used for controls in our recent report.23 Group II poults received 1,25(OH)₂D₃ twice daily for a total daily dosage as follows: week 1 (0.1 ug), week 2 (0.2 ug), week 3 (0.4 ug), week 4 (0.6 ug) and week 5 (0.8 ug). Group III poults received 1,25(OH)₂D₃ twice daily in the same dosage regimen as group II but also received 24,25(OH)₂D₃ for a total daily dosage as follows: week 1 (0.28 ug), week 2 (0.56 ug), week 3 (1.12 ug), week 4 (1.68 ug) and week 5 (2.24 ug).

Three poults per group per week were weighed and exsanguinated by cardiac puncture. Levels of plasma calcium, phosphorus, alkaline
phosphatase, 250HD$_3$, 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ were
determined as previously described.$^7,28,42$ Femur, tibiotarsus and
tarsometatarsus lengths were determined with precision calipers.
Thyroid-parathyroid-ultimobranchial thymus complex (removed en bloc) and
frontal plane sections of proximal and distal tibiotarsus and proximal
tarsometatarsus were placed in 10% neutral buffered formalin and further
processed as described.$^{21}$ Parathyroid and proximal tibiotarsus from
each group during weeks 1, 3 and 5 were processed for transmission
electron microscopic examination as previously described.$^{22}$

After necropsy, one femur was removed, stripped of tissue and stored
frozen (15°C) until analyzed. Bones were boiled in water for 1 hour,
dried for 24 hours at 75°C, defatted by washing in a soxhlet extractor
for 24 hours in a 3:1 diethyl ether to ethanol solution, dried for 24
hours at 100°C, weighed, ashed at 600°C for 48 hours and then
reweighed. Bone ash was dissolved in 10 ml of 20% hydrochloric acid and
then total bone calcium and phosphorus levels were determined from the
diluent as previously reported.$^7,42$ The data were standardized to 100
grams of the poult's body weight to facilitate comparison among treatment
groups.

Bone histomorphometric measurements were made using a Orthoplan
microscope fitted with a drawing tube (Leitz, Wetzlar, Germany) in
conjunction with a semiautomatic digitizing tablet (Bio Quant II from R
and M Biometric, Knoxville, Tennessee). Measurement of the
proliferating-prehypertrophied zone, unmineralized hypertrophied zone,
mineralized hypertrophied degenerative zone, total growth plate and
metaphyseal marrow blood vessel lengths, and chondroclast index have been described. Likewise, the method for counting total numbers of epiphyseal and metaphyseal blood vessels were described in this same report.

Statistical analysis comprised analysis of variance, means, standard deviation, and standard error. Groups II and III were each compared to the control (I).
RESULTS

A few important clinical and bone differences were found between groups. Group I (control) total femoral calcium and phosphorus levels (standardized for body weight) were significantly \((P<.05)\) greater than group II \((1,25(OH)_2D_3)\) but not for group III \((1,25(OH)_2D_3\) plus \(24,25(OH)_2D_3)\) (Table I). Group I total bone ash (standardized for body weight) did not differ significantly \((P<.05)\) from group II or group III but was greater than group II near the end of this experiment. These data were averaged over the five week period because there was little difference found from week to week. Plasma alkaline phosphatase levels were similar between poult's from each treatment group until week 3, after which groups II and III values were greater than group I (Fig. 1); this difference was statistically significant \((P<.05)\) for both groups II and III during weeks 4 and 5.

In the remaining clinical parameters, no significant differences were detected between groups. During this entire experiment, there was no evidence of lameness or leg disorders in any group. Significant body weight differences \((P<.05)\) were not detected between groups; yet, individual group II poult's beginning at week 3, had greater variation in size and weight (note S. D. Fig. 2) than did the more uniform poult's from the other 2 groups. As expected, group II and III poult's were depleted of detectable endogenous plasma levels of \(25OHD_3\) by week 1 and group II poult's were depleted of detectable endogenous plasma levels of \(24,25(OH)_2D_3\) by week 1 (Fig. 3). Except for some slight variation
<table>
<thead>
<tr>
<th>Group</th>
<th>Bone Ash (mg/100 g Body Wt)</th>
<th>Bone Ash Calcium (mg/100 g Body Wt)</th>
<th>Bone Ash Phosphorus (mg/100 g Body Wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>514.7</td>
<td>8.9 ± .22</td>
<td>1.7 ± .07</td>
</tr>
<tr>
<td>II</td>
<td>470.9</td>
<td>8.6 ± .22</td>
<td>1.5 ± .07</td>
</tr>
<tr>
<td>III</td>
<td>522.4</td>
<td>9.1 ± .22</td>
<td>1.7 ± .07</td>
</tr>
</tbody>
</table>

*Figure is total amount (mg) derived from bone ash and standardized per 100 grams of body weight, 3 poultstreatment/week averaged over 5 week period.

1 = Vitamin D deficient diet + vitamin n3 (control).
2 = Vitamin D deficient diet + 1,25(OH)2D3.
3 = Treatment groups with asterisk differ significantly (P<.05).

## Table I - Femoral bone ash and bone ash calcium and phosphorus (mean ± SEM)
Fig. 1: Alkaline phosphatase plasma levels in vitamin D$_3$ repleted (open), 1,25(OH)$_2$D$_3$ repleted (stippled) and 1,25(OH)$_2$D$_3$ combined with 24,25(OH)$_2$D$_3$ repleted poult (solid). Figure is mean ± S.D., 3 poult per week.
Fig. 2: Body weight of vitamin D$_3$ repleted (open), 1,25(OH)$_2$D$_3$ repleted (stippled), 1,25(OH)$_2$D$_3$ combined with 24,25(OH)$_2$D$_3$ repleted (solid) and predicted male poult weight$^{37}$ on commercial feed (lines). Figure is mean ± S.D., 3 poultts per week.
PREDICTED MALE POULT WEIGHT ON COMMERCIAL FEED

AGE IN WEEKS

BODY WEIGHT (grams)
during the first 2 weeks, the plasma levels of 1,25(OH)₂D₃ in groups II and III were similar to age-matched control levels and group III plasma levels of 24,25(OH)₂D₃ were also similar to age-matched control plasma levels. Control poult’s plasma calcium and phosphorus levels, usually remained slightly lower than levels found in groups II and III, which rebounded up and down (Fig. 4).

At necropsy, there were no differences found among age-matched poult’s from each treatment group. Long bones were straight and were judged to be of similar strength when cut or broke. Femoral, tibiotarsal, and tarsometatarsal bone length measurements from all 3 groups increased in length but no significant length differences were found among age-matched groups (Table II). Costochondral and costovertebral junctions were straight with narrow distinct boundaries. Parathyroid glands from all 3 groups became enlarged as the poult’s grew but no size differences or irregularities were detected among groups.

In both groups II and III, subtle histologic and morphometric evidence of rickets were found. Proliferating-prehypertrophied zone lengths of the proximal and distal tibiotarsus of groups II and III were significantly longer (P < .05) than similar control lengths when averaged over the 5 week period (Tables II-III, Fig. 5). The differences in length between the three groups were first found at week 3. In addition, the distal proliferating-prehypertrophied zone margin was irregular in both groups (Figs. 5, 6). This irregularity began at week 2, increased slightly in severity by week 5 but varied in degree between individual poult’s in either group. The distal margin of the proliferating-
1,25(OH)₂D₃

24,25(OH)₂D₃

25(OH)D₃ (ng/ml)

Age in weeks

161
Fig. 3: Plasma levels of vitamin D₃ metabolites in vitamin D₃ repleted (Δ—Δ), 1,25(OH)₂D₃ repleted (●—●) and 1,25(OH)₂D₃ combined with 24,25(OH)₂D₃ (○—○) repleted poult.s. Figure is mean ± S.D., 3 poult.s per week except where asterisks, N=2. Hash marks represents undetectable levels.
Fig. 4: Calcium and phosphorus plasma levels in vitamin D₃ repleted (△—△), 1,25(OH)₂D₃ repleted (●—○) and 1,25(OH)₂D₃ combined with 24,25(OH)₂D₃ (○—○) repleted poults. Figure is mean ± S.D. from 3 poults per week except where asterisk, N=2.
prehypertrophied zone for the control poult s always remained regular and straight. The proximal tibiotarsus unmineralized hypertrophied zone length, mineralized hypertrophied zone length, total growth plate length and metaphyseal marrow vessel length of group III were significantly longer (P < .05) than lengths from control poult s (Table II). Similar statistical significance (P < .05) was found for the proximal tibiotarsus of group II as in group III except that the mineralized hypertrophied and metaphyseal marrow vessel length differences were not statistically significant (P > .05) when compared to appropriate control lengths. Similar group II and III distal tibiotarsus lengths were longer than control lengths but statistical significance (P < .05) was found only for group III total length (Table III). The length differences (except the proliferating-prehypertrophied zone) for both bones when compared to appropriate control lengths may be associated with the elongated proliferating-prehypertrophied zone length.

Histologically, there were no detectable differences among all 3 groups in terms of bone development, growth or endochondral ossification (besides the changes noted above). In all 3 groups, at all time periods, provisional mineralization of cartilage trabeculae appeared similar and these trabeculae were straight and regular, metaphyseal blood vessels remained relatively straight, osteoid layers lining cartilage and osseous trabeculae were thin, metaphyseal and diaphyseal cartilage cones were removed by week 2 and secondary ossification centers were similar between groups. Metaphyseal blood vessel spaces were never seen penetrating the proliferating-prehypertrophied zone, eosinophilic streaks were
Fig. 5: Five-week-old poult's proximal tibiotarsus, undecalcified bones, von Kossa - HE, histological changes taken from central region of each bone.

Fig. 5A,B,C: Compare vitamin D3 repleted poult (A) proliferative-prehypertrophied zone length (P) and distal margin (arrows) with 1,25(OH)2D3 repleted (B) and 1,25(OH)2D3 combined with 24,25(OH)2D3 repleted poult's (C). Note adequate mineralization in mineralized degenerating hypertrophied zone (m) in all 3 groups. E, Epiphysis; u, unmineralized hypertrophied zone; v, metaphyseal marrow blood vessel length; b, epiphyseal capillary; epiphyseal-metaphyseal vascular streaking arrowhead.
Fig. 6: Enlargement of figure 5, distal margin of proliferative-prehypertrophied zone; A, vitamin D$_3$; B, 1,25(OH)$_2$D$_3$; C, 1,25(OH)$_2$D$_3$ combined with 24,25(OH)$_2$D$_3$. Note irregular distal margins in groups B and C compared to straight and regular distal margin in control (A).
Table II - Proximal tibiotarsus, epiphyseal and metaphyseal morphometric measurements (mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Proliferating-prehypertrophied zone length</th>
<th>Unmineralized hypotrophied zone length</th>
<th>Mineralized hypotrophied zone length</th>
<th>Total length</th>
<th>Metaphyseal marrow vessel length</th>
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<tbody>
<tr>
<td>I</td>
<td>1.44 ± .11</td>
<td>0.28 ± .03</td>
<td>3.65 ± .16</td>
<td>5.36 ± .22</td>
<td>3.75 ± .17</td>
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<tr>
<td>II</td>
<td>1.77 ± .11*</td>
<td>0.42 ± .03*</td>
<td>4.04 ± .16</td>
<td>6.23 ± .22*</td>
<td>4.11 ± .17</td>
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<tr>
<td>III</td>
<td>1.84 ± .11*</td>
<td>0.39 ± .03*</td>
<td>4.21 ± .16*</td>
<td>6.44 ± .22*</td>
<td>4.39 ± .17*</td>
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</table>

*Figure is in mm from 3 pouls/treatment/week, averaged over 5 week period.

I = Vitamin D deficient diet + vitamin D3 (control).

II = Vitamin D deficient diet + 1,25(OH)2D3.

III = Vitamin D deficient diet + 1,25(OH)2D3 and 24,25(OH)2D3.

* = Treatment groups with asterisk differ significantly (P < .05) from group I (control). Groups II and III are compared to group I.
Table II Continued and Tibiotarsus Bone Length (mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Chondroclast (^g) index</th>
<th>Total Number (^h) epiphyseal blood vessels</th>
<th>Total Number (^h) metaphyseal marrow spaces</th>
<th>Tibiotarsus (^h) bone length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (^b)</td>
<td>2.66 ± .26</td>
<td>12.6 ± .81</td>
<td>24.9 ± 1.1</td>
<td>75.0 ± 2.7</td>
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<td>II (^c)</td>
<td>2.50 ± .26</td>
<td>14.8 ± .81</td>
<td>26.5 ± 1.1</td>
<td>72.7 ± 2.7</td>
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<tr>
<td>III (^d)</td>
<td>2.36 ± .26</td>
<td>15.1 ± .81</td>
<td>26.3 ± 1.1</td>
<td>73.0 ± 2.7</td>
</tr>
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</table>

\(^g\)Figure is number of chondroclasts/mm hypertrophied cartilage; represents chondroclasts lining hypertrophied cartilage perimeter of the most proximal portion of the metaphyseal marrow vascular space from 3 poults/treatment/week, averaged over 5-week period.

\(^h\)Figure is from 3 poults/treatment/week, averaged over 5-week period.
Table III - Distal tibiotarsus epiphyseal and metaphyseal morphometric measurements (mean ± SEM)

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<thead>
<tr>
<th>Group</th>
<th>Proliferating-\textsuperscript{a} prehypertrophied zone length</th>
<th>Unmineralized\textsuperscript{a} hypertrophied zone length</th>
<th>Mineralized\textsuperscript{a} hypertrophied zone length</th>
<th>Total\textsuperscript{a} length</th>
<th>Metaphyseal\textsuperscript{a} marrow vessel length</th>
</tr>
</thead>
<tbody>
<tr>
<td>I\textsuperscript{b}</td>
<td>0.85 ± .05</td>
<td>0.17 ± .01</td>
<td>2.16 ± .11</td>
<td>3.18 ± .14</td>
<td>2.17 ± .13</td>
</tr>
<tr>
<td>II\textsuperscript{c}</td>
<td>1.04 ± .05\textsuperscript{*}</td>
<td>0.23 ± .01</td>
<td>2.22 ± .11</td>
<td>3.49 ± .14</td>
<td>2.33 ± .13</td>
</tr>
<tr>
<td>III\textsuperscript{d}</td>
<td>0.99 ± .05\textsuperscript{*}</td>
<td>0.19 ± .01</td>
<td>2.47 ± .11</td>
<td>3.64 ± .14\textsuperscript{*}</td>
<td>2.51 ± .13</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Figure is in mm from 3 poult\textbackslash t/treatment/week, averaged over 5-week period.

\textsuperscript{b} I = Vitamin D deficient diet + vitamin D\textsubscript{3} (control).

\textsuperscript{c} II = Vitamin D deficient diet + 1,25(OH)\textsubscript{2}D\textsubscript{3}.

\textsuperscript{d} III = Vitamin D deficient diet + 1,25(OH)\textsubscript{2}D\textsubscript{3} and 24,25(OH)\textsubscript{2}D\textsubscript{3}.

\textsuperscript{*} Treatment groups with asterisk differ significantly (P < .05) from group I (control). Groups II and III are compared to group I.
<table>
<thead>
<tr>
<th>Group</th>
<th>Chondroclast$^g$ index</th>
<th>Number$^h$ epiphyseal blood vessels</th>
<th>Number$^h$ metaphyseal marrow spaces</th>
</tr>
</thead>
<tbody>
<tr>
<td>I$^b$</td>
<td>2.45 ± .14</td>
<td>10.6 ± .72</td>
<td>36.7 ± 2.08</td>
</tr>
<tr>
<td>II$^c$</td>
<td>2.33 ± .14</td>
<td>11.2 ± .72</td>
<td>33.7 ± 2.08</td>
</tr>
<tr>
<td>III$^d$</td>
<td>2.33 ± .14</td>
<td>13.0 ± .72</td>
<td>37.1 ± 2.08</td>
</tr>
</tbody>
</table>

$^g$Figure is number of chondroclasts/mm hypertrophied cartilage; represents chondroclasts lining hypertrophied cartilage perimeter of the most proximal portion of the metaphyseal marrow vascular space from 3 poults/treatment/week, averaged over 5-week period.

$^h$Figure is from 3 poults/treatment/week, averaged over 5-week period.
occasionally found aligning epiphyseal and metaphyseal blood vessels and metaphyseal blood vessel spaces usually preceded the mineralized hypertrophic degenerative zone by 0-0.5 mm.

Ultrastructurally, no differences were observed among treatment group's chondrocytes or their matrix, matrix vesicles which we previously described, osteoblasts, osteoid, chondroclasts or osteoclasts when compared in the area of proximal metaphyseal capillary invasion of hypertrophic cartilage.

Histologically, parathyroid glands and ultimobranchial bodies appeared similar among all 3 groups during each time period. Chief cells were organized into distinct cords and lobules surrounded by delicate fibrovascular stroma. Chief cells were plump and piled up.

Ultrastructurally, chief cells appeared similar among each treatment group at each time period examined. Chief cells were polygonal to columnar and were tightly packed into lobules. The majority of chief cells usually contained an abundance of cytoplasmic organelles, which suggested that they were metabolically active. The amount and proportion of cytoplasmic organelles, secretory granules, and lipid vacuoles varied between individual chief cells.
DISCUSSION

Our finding that 24,25(OH)$_2$D$_3$ when combined with 1,25(OH)$_2$D$_3$ restores bone calcium and phosphorus levels similar to treatment with vitamin D$_3$ implies a significant biologic role for 24,25(OH)$_2$D$_3$. Bone mineralization was greater in human patients with nutritional osteomalacia or dialysis osteomalacia treated with a combination of 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ than 1,25(OH)$_2$D$_3$ alone. A combination of both metabolites appears to be essential in avian species for adequate bone metabolism, calcium and phosphorus homeostasis, egg hatchability or reduction in parathyroid gland size in ricketic chicks. Other authors disagree with the suggestion that 24,25(OH)$_2$D$_3$ is an important vitamin D$_3$ metabolite because they could not find significant differences between controls and rats treated with the synthetic sterol 24,24-difluro-25-hydroxyvitamin D$_3$ which cannot be converted to 24,25(OH)$_2$D$_3$. Yet, in only one of these papers was bone ash calcium levels quantified (no differences were found between 25OHD$_3$ and synthetic sterol treated rats) and if this analysis were completed in the remainder of these studies, results similar to ours may have been found. In future vitamin D metabolite studies, bone ash calcium and phosphorus levels should be quantified, because bone ash results frequently are equivocal and analysis for these micronutrients provides additional data concerning total mineralization. Furthermore, in some of these same rat studies, data were not compared to a vitamin D$_3$ treated
group as in our study. Such a comparison may have shown important
differences from the synthetic analogue treated rats. Also, direct
comparisons between turkeys and rats may not be appropriate because of
turkeys' faster growth rates and other species differences.

In this study, the restored bone levels of calcium and phosphorus
are most likely caused by the synergistic affect of 24,25(OH)$_2$D$_3$ to
1,25(OH)$_2$D$_3$ because detectable levels of 250HD$_3$ were gone by week
1. Furthermore, chicks given 24,25(OH)$_2$D$_3$ simultaneously with
1-alpha-hydroxyvitamin D$_3$ do not convert 24,25(OH)$_2$D$_3$ into 1,24,25
trihydroxycholecalciferal. Therefore, the additive effect of 24,25-
(OH)$_2$D$_3$ is probably not due to this conversion. Yet, rats appear to
require 1-hydroxylation of 24,25(OH)$_2$D$_3$ before intestinal calcium
absorption can occur.

The mechanism of action that 24,25(OH)$_2$D$_3$ has on bone is not yet
clearly defined. Only a few studies which support this metabolite as
being physiologically important, provide a proposed mechanism of action.
One mechanism might be that 24,25(OH)$_2$D$_3$, at least in man, stimulates
intestinal absorption of calcium which then could secondarily be
available for osseous mineralization, because increased plasma or urinary
levels of calcium are not found. In addition, 24,25(OH)$_2$D$_3$ combined
with 1,25(OH)$_2$D$_3$ can decrease avian parathyroid gland size and DNA
content, and others suggest that 24,25(OH)$_2$D$_3$ can decrease para-
thyroid hormone secretion, but point out that some of the results are
conflicting. Parathyroid hormone and 1,25(OH)$_2$D$_3$ act in concert to
cause osseous release of calcium and phosphorus from bone. If 24,25-
(OH)₂D₃ reduced parathyroid hormone secretion, then a build up of calcium and phosphorus levels in bone may occur. Unfortunately, parathyroid hormone levels were not measured in this experiment.

Furthermore, when both metabolites are administered together, they can restore bone resistance to the calcemic action of parathyroid hormone in uremia.¹⁴

This study demonstrates that the antiricketic effect of vitamin D₃ is more effective than 1,25(OH)₂D₃ and that 24,25(OH)₂D₃ has no synergism in preventing some of the clinical and bony rachitic lesions found in growing turkeys fed this same diet.²¹ These ricketic changes (elongated proliferating-prehypertrophied zone, irregular distal border of this zone, and elevated alkaline phosphatase levels) may be caused by a deficiency of other vitamin D metabolites because plasma calcium and phosphorus levels were normalized and no obvious endochondral ossification defects were present. In this experiment, plasma levels of 25OHD₃ were not detected in either metabolite repleted group; this deficiency (or possible deficiency of other vitamin D₃ metabolites) may be responsible for the retarded differentiation of the proliferating-prehypertrophied zone into hypertrophic chondrocytes. This implies a direct role for 25OHD₃ in bone development of turkeys and not necessarily a secondary role through hydroxylation into 1,25(OH)₂D₃ or 24,25(OH)₂D₃ followed with elevated calcium and phosphorus levels for potential ossification. Chondrocytes¹⁰ or bone cells,²⁹ in vitro, can hydroxylate 25OHD₃ to 1,25(OH)₂D₃ or 24,25(OH)₂D₃, and the lack of this metabolism in our study may affect differentiation
of the proliferative zone. Some authors\textsuperscript{43,45} suggest that 1,25(OH)\textsubscript{2}D\textsubscript{3} and 24,25(OH)\textsubscript{2}D\textsubscript{3} can affect chondrocyte differentiation, and rat proliferative chondrocytes were thought to be the target cells with specific receptors for 24,25(OH)\textsubscript{2}D\textsubscript{3}.\textsuperscript{16} These data do not support the role suggested for 25OHD\textsubscript{3} in our study. Furthermore, our study conflicts with Dickenson \textit{et al.},\textsuperscript{14} who found that 1,25(OH)\textsubscript{2}D\textsubscript{3} normalizes the proliferating-prehypertrophied zone length in ricketic-treated chicks.

It was our impression, that if this study was continued beyond 5 weeks, other statistically significant findings associated with rickets in our previous study\textsuperscript{21} (decreased body weight and bone ash) would have been found in the 1,25(OH)\textsubscript{2}D\textsubscript{3} repleted poult but not in the 24,25-(OH)\textsubscript{2}D\textsubscript{3} repleted poult. If this were true, then additional credibility would have been found for 24,25(OH)\textsubscript{2}D\textsubscript{3} combined with 1,25(OH)\textsubscript{2}D\textsubscript{3}, making 24,25(OH)\textsubscript{2}D\textsubscript{3} an essential vitamin D\textsubscript{3} metabolite. Ricketic chicks treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} restore plasma calcium and phosphorus and bone ash values, but fail to restore body weight.\textsuperscript{12} In this same experiment, chicks treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} combined with 24,25(OH)\textsubscript{2}D\textsubscript{3} restored all of these values and bone breaking strength was similar to chicks treated with vitamin D\textsubscript{3}.

Using our dosage regimen in both the vitamin D\textsubscript{3} metabolite repleted groups (especially weeks 3-5) we achieved physiologic plasma levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} and 24,25(OH)\textsubscript{2}D\textsubscript{3} similar to levels found in vitamin D\textsubscript{3} treated poult. This implies that the ricketic changes and lowered calcium and phosphorus levels in bone were not produced by an
inadequate or excessive dosage of $1,25(OH)_2D_3$. However, the levels of $25OHD_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ found in this study were considerably less than Horst et al. found in turkeys; poults in our study were younger than turkeys used in this report and with increasing age, the metabolite levels of our poults increased.
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This study partially confirms other authors' suggestion that growing poults have an acute sensitivity to mineral and vitamin D-deficiency. This sensitivity probably accounts for much of the leg weakness occurring in the field. Gastrointestinal diseases, inadequate mixing of feeds, vitamin D₃ of low biological potential or availability, or aflatoxins which can potentiate the effects of vitamin D-deficiency can potentially cause rickets. If these conditions are combined with the early predisposition to low plasma levels of vitamin D₃ metabolites found in this study, ricketic changes may not be florid but could be subclinical. Subclinical rickets in turkeys or broilers is probably more common than recognized. In healing rickets, remodeling may reduce the expression of gross deformities, but some bone lesions may persist and could cause complications such as an arthropathy or conformation problems that later could lead to leg weakness. Because of turkey poults' acute sensitivity to mineral or vitamin D-deficiency, poults may be better models to study endochondral ossification than chicks or rats which are primarily used.

In this study, quantification of bone ash micronutrients provided valuable data which helped to explain some of the findings or show important treatment differences. The bony skeleton contains approximately 99% of the calcium and phosphorus supplies of the body and for the most part is lined by a single layer of osteocytes. A minimal change in this vast surface area might escape morphologic detection but potentially
could have an important effect on calcium-phosphorus homeostasis. Therefore, investigators dealing with metabolic bone diseases should not analyze just for percent bone ash, but should analyze bone ash from appropriately selected sites for micronutrient levels of calcium and phosphorus. Furthermore, in metabolic bone disease studies involving growth plates, this region should be examined on a temporal basis because erroneous impressions and data may be collected from examining a few random time periods. This is further supported by the finding in this study that faster growth rates of different growth plates can affect histomorphometric interpretations.

Chondrocyte matrix vesicles and matrix-like vesicles in bone calcification fronts ultrastructurally described in this study provides further support for the importance of matrix vesicles in mineralization of cartilage and woven bone described for other species. However, to further document these structures as matrix vesicles, ultrastructural histochemistry and biochemical analysis will be needed.

The exact role that 24,25\((\text{OH})_2\)D\(_3\) plays in bone mineralization is not known. When 24,25\((\text{OH})_2\)D\(_3\) is combined with 1,25\((\text{OH})_2\)D\(_3\) bone mineralization is increased in humans\(^{10}\) similar to what was shown in this study. It was not possible to determine because of the experimental design of this study if 24,25\((\text{OH})_2\)D\(_3\) was directly involved with chondrocyte and/or osteoblast metabolism or indirectly involved by increasing intestinal absorption of minerals required for mineralization. Chondrocytes\(^{52}\) and osteoblasts\(^{99}\) can directly metabolize 25OHD\(_3\) to 24,25\((\text{OH})_2\)D\(_3\) or 1,25\((\text{OH})_2\)D\(_3\). Furthermore,
the ricketic changes found in groups III and IV in this study (elongation of the proliferating-prehypertrophied zone) may be caused by a lack of 25OHD$_3$ because chondrocytes may need this metabolite for their complete differentiation.

The studies in this dissertation have produced a better understanding of the interactions among vitamin D$_3$ deficiency, vitamin D$_3$, 1,25(OH)$_2$D$_3$, and 24,25(OH)$_2$D$_3$ metabolites in growing turkey poults. Important questions remain unanswered. Vitamin D$_3$ toxicity studies need to be conducted so that the optimum dosage of vitamin D$_3$ during the first weeks of life can be determined. Turkey poults may need to be started on a very high dosage of vitamin D$_3$ that tapers down with increasing age. Bone ash calcium and phosphorus standards need to be established in normal growing turkeys during weekly intervals of their production life span. If leg weakness problems develop, this standardized data might be useful for comparison in an attempt to determine the cause of the leg weakness condition. Vitamin D-deficient poults repleted with vitamin D$_3$ or minimal supplements during days 10-14 might provide a valuable model to study the mechanisms of endochondral ossification. If the metabolite repletion study was carried out longer than five weeks, would other ricketic changes have been found for group III poults (1,25(OH)$_2$D$_3$ repleted)?


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ACKNOWLEDGMENTS

Keldah, Anika and Ingrid provided joy and contentment during the struggle of this experience and they made it worthwhile. I acknowledge the sacrifice, companionship and support of Bette. My mother and father showed me how to persevere which gave me strength to see this work through.

I sincerely thank Dr. N. F. Cheville for helping me grow as a pathologist and as a person. I appreciate the opportunity, patience, guidance and most of all his friendship that he provided for this dissertation.

The effort and support that members of my committee, Drs. Ron Horst, John Kluge, Randall Cutlip and Jerry Sell, afforded me for this dissertation are appreciated.

I am grateful for the excellent training in veterinary pathology that I received at Iowa State University from the Department of Veterinary Pathology and Veterinary Diagnostic Laboratory.

Finally, the director and excellent personnel at the National Animal Disease Center are acknowledged for the support, resources, and facilities which made this dissertation possible.