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EFFICACY OF 25-HYDROXYCHOLECALCIFEROL
ON THE PREVENTION OF
TIBIAL DYSCHONDROPLASIA IN ROSS BROILER CHICKS

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EFFICACY OF 25-HYDROXYCHOLECALCIFEROL ON THE PREVENTION OF TIBIAL DYCHONDROPLASIA IN ROSS BROILER CHICKS

By

Martin F. Ledwaba

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ABSTRACT

EFFICACY OF 25-HYDROXYCHOLECALCIFEROL ON THE PREVENTION OF TIBIAL DYCHONDROPLASIA IN ROSS BROILER CHICKS

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Six experiments were conducted to study the effects of 25-hydroxycholecalciferol (25-(OH) D_3) on growth performance, incidence and severity of tibial dyschondroplasia (TD) and phytate phosphorus retention in Male Ross x Ross broilers grown in battery brooders. Experiment 1 was a 2 x 3 factorial design with 2 levels of ultraviolet (UV) light (no UV-light and UV-light) and 3 concentrations of 25-(OH)D_3. In Experiment 2 chicks were fed a TD-inducing diet and in Experiment 3-6 a normal broiler starter diet. In Experiments 2-6 all chicks received no UV-light and their diets were supplemented with various levels of 25-(OH)D_3 ranging from 0, 10, 18, 36, 40, 54, 70, 72 or 90μg/kg depending on the experiment. Experiment 6 included a grower phase, that consisted of birds transferred from the starter phase, in which a grower diet was fed supplemented with 25-(OH)D_3. The UV-light, 25-(OH)D_3 or the combination of both improved growth performance, phosphorus utilization and reduced rickets, severity and incidence of TD. Supplementation with 25-(OH)D_3 does not seem to improve performance in normal starter or grower broiler diets. Severity and incidence of TD decreased linearly only in Experiments 2 and 3 with 25-(OH)D_3 supplementation. From the data we conclude that the low TD incidence observed with 25-(OH)D_3 supplementation is partly due to Ross x Ross strain. Lower levels of 25-(OH)D_3 can increase phytate phosphorus in broiler starter diets and 40 μg/kg can improve phytate phosphorus retention in grower diets.

I dedicate this thesis to my grandfather France Ledwaba who I never got to meet before he passed and who never had the opportunity to learn how to read and write while he was alive.

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LIST OF ABBREVIATIONS

ATP- Adenosine triphosphate

BMP- Bone morphogenetic protein

CaBP- Calcium binding protein

DBP- vitamin D binding protein

ECF- Extracellular fluid

HTD- High TD incidence

LTD- Low TD incidence

NpP- Non-phytate phosphorus

P*i*- Inorganic phosphorus

PTH- Parathyroid hormone

TD- Tibial dyschondroplasia

TGFβ- Transforming growth factor-beta

VBP- Vitamin D binding protein

VDR- Vitamin D receptor

INTRODUCTION

Improved growth rates and feed efficiency have been the primary concerns for broiler breeders over the past years. Geneticists have selected for these economic traits and in turn nutritionists have made it possible to provide the necessary combination of nutrients to optimize those traits. As the meat-type commercial poultry industry grew, so have the birds used for production. As a result, metabolic parameters of the young growing birds have come under heavy pressure causing muscle growth and skeletal development to be out of synchrony. Skeletal deformities and leg weakness have become one of the major problems to the poultry industry from the standpoint of economics and welfare. Skeletal problems likely cause the broiler industry close to 120 million and the turkey industry 40 million dollars in losses every year (Sullivan, 1994).

Tibial dyschondroplasia (TD) is a common skeletal abnormality found in young rapidly growing meat-type poultry (ducks, broiler chickens and turkeys) and is influenced by nutrition as well as genetics (Riddel, 1981; Edwards, 1984). Tibial Dyschondroplasia is a bone abnormality arising from a growth disorder in endochondral long bone and lesions associated with TD were first observed by Leach and Nesheim (1965). In TD, ossification of the hypertrophic region of the growth plate does not occur. The cartilage which is responsible for long bone growth in young birds is not replaced by bone, but is instead retained, leaving an island of degenerative cartilage cells that are arrested in early hypertrophy (Hargest et al., 1985). The soft cartilage that remains in a TD growth plate may lead to distress to the bird and as a result they are reluctant to walk. Reluctance in walking often results in a change in feeding behavior and body weight gain is negatively

affected. As a result, the number of culls increases when birds become weak and are not reaching target weights (Morris, 1993). Squatting or sitting can increase breast blisters, the part of the bird that holds the most value in the poultry meat market. In addition, the bone is more prone to deformities and breakage especially during processing which can lead to downgrading of the carcass or condemnation (Burton et al., 1981).

Dietary supplementation with various Vitamin D_3 metabolites to low calcium diets as well as to diets adequate in calcium can reduce the incidence and severity of TD (Edwards, 1989, 1990; Rennie et al., 1993, 1995). The vitamin D_3 metabolite that is most commonly used as a feed additive and is available commercially today is 25-hydroxycholecalciferol (25-(OH)D_3). Feeding 25-(OH)D_3 provides growth stimulation in addition to TD prevention and the metabolite can improve phosphorus utilization in broiler chicks. Phosphorus pollution in the soil and water has been blamed partially on the poultry industry due to run-off from fields in which manure/litter has been used as fertilizer. Few studies have been published on the effectiveness of 25-(OH)D_3 on the digestibility of phosphorus (Applegate et al., 2000; Angel et al., 2001).

Previous studies on requirements for 25-(OH)D_3 in the diet of broiler chicks are mainly based on weight gain and feed efficiency (McNutt and Haussler, 1973; Cantor and Bacon, 1978; Yarger et al 1996). However, the inconsistent results in recent studies with 25-(OH)D_3 on TD incidence and severity suggest that the requirement for 25-(OH)D_3 might be substantially lower than recommended. The objective of this study is to provide more information on the effectiveness of 25-(OH)D_3 in reducing TD incidence and severity and improving phosphorus utilization without compromising performance specifically when a marginal calcium diet is fed (85%).

CHAPTER 1

LITERATURE REVIEW

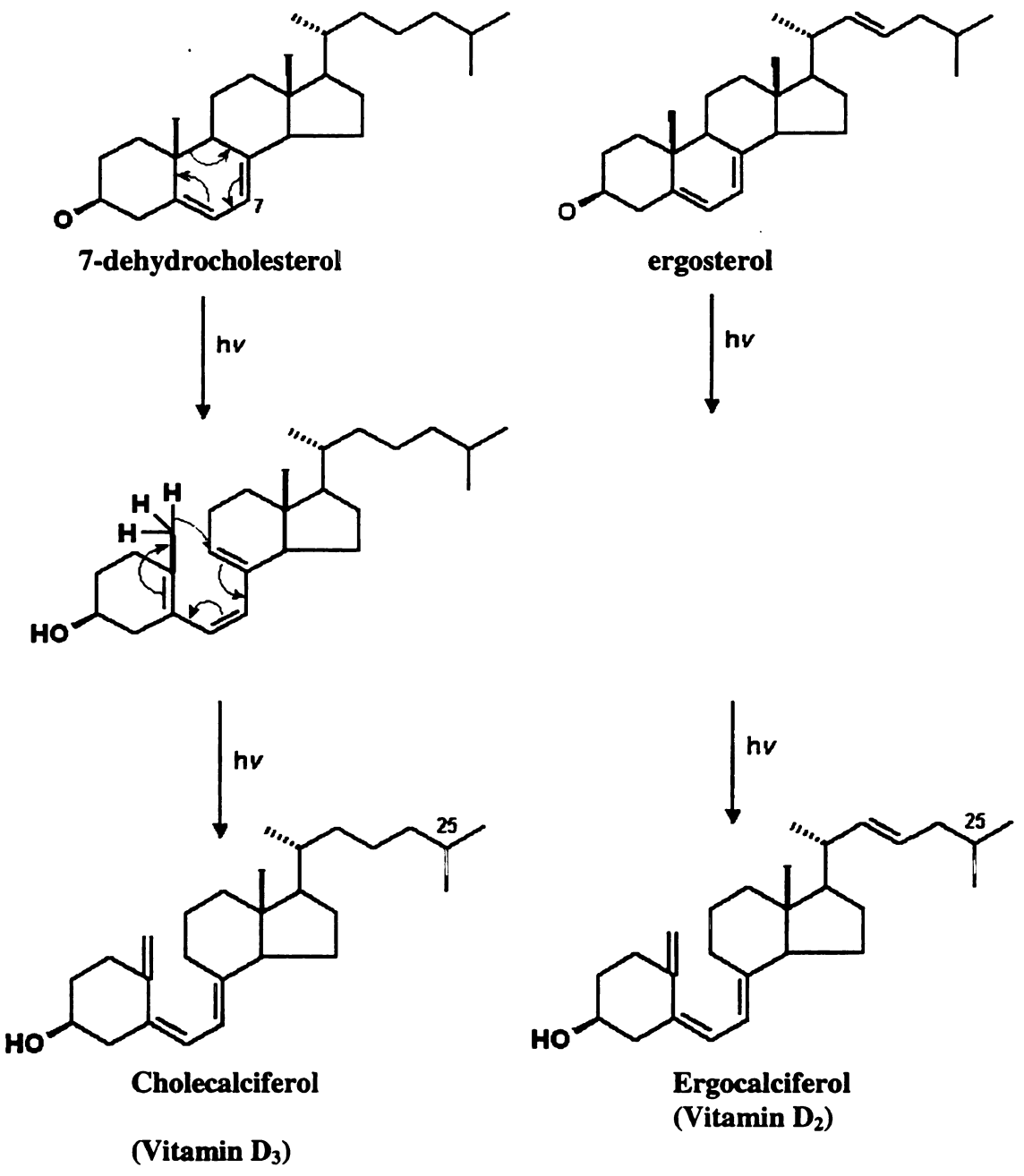
The Vitamin D System and 25-(OH)D_3

Vitamin D is the collective name given to a family of compounds or related sterols that exhibit anti-rachitic activity (curing or preventing rickets). The metabolite, 25-hydroxycholecalciferol, is one of the compounds that belongs to the vitamin D family. Vitamin D is characterized as a fat-soluble vitamin and was first named and discovered by McCullom in 1922 (McDowell, 1989). Before its discovery, vitamin D activity was linked to vitamin A. This was true until the findings that cod-liver oil, which was known to exhibit strong vitamin A and anti-rachitic activity, still preserved its anti-rachitic activity after all vitamin A had been destroyed in the oil.

A more traditional cure for rickets, which was known long before the discovery of the nutritional cure from vitamin D, is the healing property of UV-light. However, the mutual relationship between vitamin D and UV-light in healing rickets was recognized after experiments showed that UV-light exposure increased production of vitamin D in livers of animals (Goldblatt and Soames, 1923) and stimulated vitamin D activity in certain foods (Steenbock and Black, 1924). Identification of the vitamin D form, vitamin D_2, occurred in 1931 and the precursor to vitamin D_3 (cholecalciferol), 7-dehydrocholesterol, was first isolated from the skin in pigs in 1937 (Deluca, 1982). It was not until the 1960's that studies showed Vitamin D_3 (the parent metabolite) could be metabolized to different metabolites and that at least one of these metabolites functioned as an active hormone (Deluca, 1979). The first metabolite of cholecalciferol to be identified and isolated was 25-hydroxycholecalciferol (Blunt et al., 1968).

The vitamin D molecule including all its metabolites, is a secosteroid and its structure consist of 4 rings of a cyclopentanoperhydrophenanthrene system in which one ring, the B-ring, has undergone fission and is opened, distinguishing it from classical steroids. The structure also consists of differing side chains from one secosteroid to another. (See figure 1-diagram of structure.) The most prominent secosteroids or forms of vitamin D are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). These two different forms of vitamin D are derived from pre-cursors known as ergosterol and 7-dehydrocholesterol, respectively. Only after irradiation by UV-light, are these pre-cursors characterized as a form of vitamin D (Norman, 1990). Ergocalciferol can be manufactured synthetically from plant sterols. Vitamin D_2 cannot be efficiently metabolized by birds and is therefore not considered a functional metabolite in this species (Valinetse and Bauman, 1981). In contrast, 7-dehydrocholesterol that is derived from cholesterol occurs naturally and is found in animal tissue. It can be synthesized in the liver during normal steroid production then transported to the skin by a transporter protein (Soares, 1984). Moreover, 7-dehydrocholesterol can be produced directly from cholesterol in the epithelial cells of the skin, as well as in the intestinal wall (Klasing, 1998). The vitamin D precursor, 7-dehydrocholesterol, is more common in the skin of the legs and feet of chickens than in the body (Koch and Koch, 1941). The oil of the preen-gland (uropygial gland) also contains some amounts of this pro-vitamin. During preening it may be spread onto the feathers, and is subsequently converted into vitamin D after which ingestion may occur, but in a very inefficient way (Taylor and Dacke, 1984). The conjugated double bond system (double bonds five to seven) in the B-ring of the

Figure 1. Diagram of Vitamin D_3 production from 7-dehydroxycholecalciferol by ultraviolet light

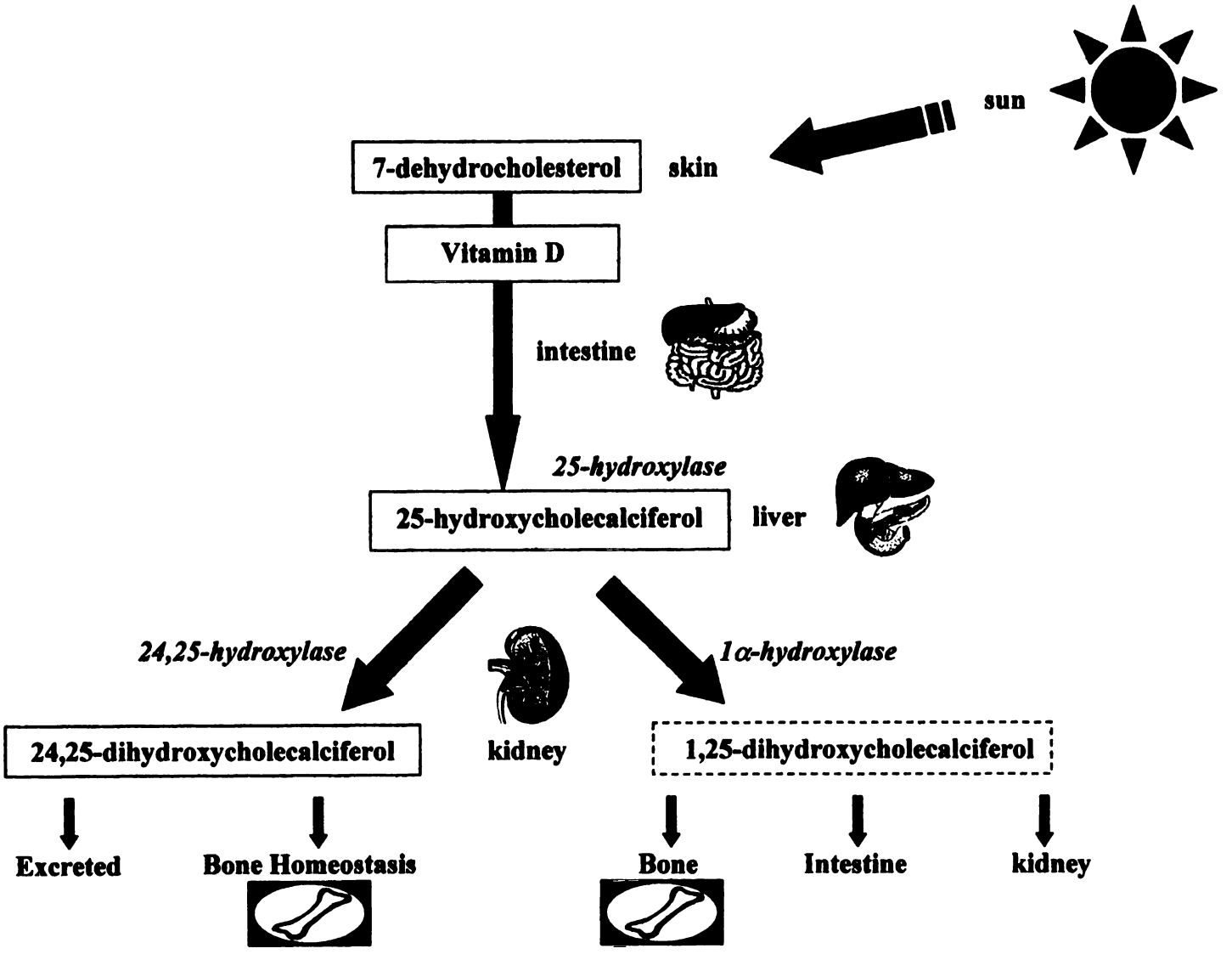


Adapted from Syed Nasrat Imam (2001).

provitamin structure allows absorption of UV-photons, preferably of wavelength 285-315 nm, transforming 7-dehydrocholesterol to the pre-vitamin D_3 (Norman, 1990). The final and complete cholecalciferol is only formed after 2-3 days of thermal isomerization which involves a series of transformations of its structure with rotations of the A-ring (McDowell, 1989).

The cholecalciferol that is formed in the skin is absorbed into the blood and transported by a binding protein (Gc globulin; group–specific component), which is mostly a gamma globulin in birds, but may be an alpha and beta globulin as well in some avian species (Dacke, 2000). The binding protein protects the vitamin from being inactivated by oxidation. Cholecalciferol has little metabolic activity and is highly hydrophobic. It can be stored in only limited amounts, especially in birds, mostly in adipose tissue from where it can be released slowly during vitamin D deficiency (Hurwitz, 1989). Cholecalciferol in circulation is rapidly carried to the liver and metabolized into 25-(OH)D_3. Although, the liver is the major site of the conversion, it may also take place in small amounts in the kidney and intestine (Tucker et al., 1973). During the transformation of cholecalciferol into 25-(OH)D_3, a hepatic enzyme, 25-hydroxylase, hydroxylates the 25th carbon of the side-chain (see figure 2.). The hydroxylation primarily takes place in the microsome, apparently because the microsomal enzyme has a low Km or a high affinity for its substrate, but it can also occur in the mitochondria especially during excessive levels of cholecalciferol. The 25-hydroxylase requires NADPH, molecular oxygen, cytochrome P450, and flavoprotein (Deluca, 1979). The enzyme is one of the P450 hydroxylases and its activity does not seem to be regulated by 25-(OH)D_3 or 1,25-dihydroxycholecalciferol (1,25$(OH)_2D_3$). The

Figure 2. Diagram of Vitamin D metabolism.



25-hydroxylase may be under limited control and an increase or decrease in dietary cholecalciferol or UV-light exposure results in a linear increase/decrease in plasma concentration levels of 25-(OH)D_3, respectively (Soares et al., 1995). This correlation has made circulating 25-(OH)D_3 an appropriate index for assessing vitamin D status in birds. The 25-(OH)D_3 metabolite is the major circulating metabolite, serving as the major storage form of all the vitamin D metabolites. In comparison to cholecalciferol, the 25-(OH)D_3 metabolite is absorbed more efficiently when these metabolites are given orally, 66.5% vs. 83.6%, respectively (Bar et al., 1980). The hydroxylation to 25-(OH)D_3 improves water solubility (Blunt et al., 1968) and the storage site where majority of 25-(OH)D_3 can be found, is in the blood. The half-life of 25-(OH)D_3 in blood is about 3 weeks (Holick, 1990).

In circulation, 25-(OH)D_3 is mostly bound to the vitamin D binding protein(VBP). The binding affinity of 25-(OH)D_3 towards the VBP is higher than most metabolites, especially 1,25$(OH)_2D_3$ and cholecalciferol (Soares et al., 1995). Vitamin D binding protein transports 25-(OH)D_3 to the kidney where it is further metabolized. In the kidney a second hydroxylation occurs at the 1 carbon position, and the enzyme responsible for converting 25-(OH)D_3 into 1,25$(OH)_2D_3$ is called 25-(OH)D_3-1-α-hydroxylase. This particular enzyme is found in the mitochondria of the proximal tubules of the kidney. This enzyme is a mixed function oxidase and requires 3 proteins; P450-cytochrome, iron-sulfur protein (renal ferrodoxin) and flavoprotein (Deluca, 1979). Similarly to 25-hydroxylase, NADPH fuels this particular system. The 1-α-hydroxylase is under very strict control and is found in very low levels under normal conditions. However, during vitamin D deficiency when hypocalcemia sets in, the parathyroid

glandsecretes parathyroid hormone (PTH), which in turn stimulates the activity of 1-α-hydroxylase. Hypophosphotemia also regulates this enzyme, but not through PTH. In addition, the enzyme reaction is product stimulated, its activity being modulated by $1,25(OH)_2D_3$ itself via a powerful feedback mechanism. The metabolite $1,25(OH)_2D_3$ is the metabolically active form of vitamin D and acts as a hormone in various tissues (Soares, 1984). Moreover, $1,25(OH)_2D_3$ is more rapid than $25\text{-}(OH)D_3$ in inducing the classical effects of vitamin D (DeLuca, 1973) and this might explain why $1,25(OH)_2D_3$ has a relatively short half-life of 4 to 6 hours in the blood (Groff and Gropper, 2000a). During periods of low circulating levels of $1,25(OH)_2D_3$, the activity is increased. Whereas, when circulating levels of $1,25(OH)_2D_3$ are in excess, activity is suppressed (Henry and Norman, 1984). During the latter circumstances, another hydroxylation is taking place, primarily in the kidney. This is also a mitochondrial enzyme, but this step involves 24-hydroxylation. The $1,25(OH)_2D_3$-24-hydroxylase has the ability to convert $25\text{-}(OH)D_3$ to $24,25(OH)_2D_3$ thereby preventing the formation of $1,25(OH)_2D_3$. The 24-hydroxylase enzyme can also be expressed at target tissue sites of $1,25(OH)_2D_3$. The enzyme is very important in reducing the life span of $1,25(OH)_2D_3$ initiating catabolism of the metabolite. The $24,25(OH)_2D_3$ metabolite is rapidly excreted in chickens and therefore believed to serve as an excretory route for excess $25\text{-}(OH)D_3$ (Holick et al., 1976). The metabolite, $24,25(OH)_2D_3$, also appears to play an important role in normal bone formation (Ornoy et al., 1978). It has been demonstrated that $24,25(OH)_2D_3$ has biological activity in the chondrocytes of the growth plate (Corvol et al., 1978; Suda et al., 1985). Atkin et al. (1985) suggested that $24,25(OH)_2D_3$ promotes cartilage maturation, differentiation and bone mineralization.

Many other hydroxylations can take place resulting in many different vitamin D metabolites, of which 35 or more have been isolated and chemically identified. However, the primary metabolites that support normal mineralization in bone and/or calcium and phosphorus homeostasis are $1,25(OH)_2D_3$, $24,25(OH)_2D_3$ and $25\text{-}(OH)D_3$.

The Effect of Vitamin D on the Intestine

Calcium Absorption

In order for bone to function properly the bone matrix must mineralize. Several minerals, but primarily calcium and phosphorus need to be in the blood to enter the bone fluid. Calcium (Ca) is in the form of Ca^{2+} or amorphous calcium phosphate $[Ca_3(PO_4)_2]$ in solution. This non-crystalline Ca will precipitate out as salts on the surface of the bone and eventually be converted into hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_6]$ (crystalline calcium) to complete the ossification process. Digestion and absorption of dietary calcium accounts for a large part of the calcium influx into the blood. This is especially true in young birds, which are still growing and have limited calcium stores in the skeleton. Calcium absorption takes place primarily in the small intestine particularly from the duodenum and jejenum. (Larbier and Leclercq, 1992). Calcium is absorbed in the intestine by two main mechanisms. One occurs by a passive, unregulated, also referred to as non-saturable mechanism, that takes place in between the cells or enterocytes by diffusion. This pathway is often used when calcium intake is elevated (Bronner, 1990). The other component involves the active energy dependent or saturable mechanism and is active when calcium levels are low in the blood. In this case the transport of Ca is across an enterocyte and is a protein-facilitated process, which is mediated by calcium binding

protein (CaBP or calbindin). Calcium binding protein serves as a protein carrier across the basolateral membrane and is regulated by the vitamin D metabolite 1,25-OH_2D_3 (Groff and Gropper, 2000b). When vitamin D deficient chicks were administered a dose of 1,25-OH_2D_3, the nuclear receptors of the vitamin D dependent CaBP (calbindin-$D_{28}K$) in the chick intestine were saturated with the hormone (Theofan et al., 1986). As a result, calbindin-$D_{28}K$-mRNA as well as CaBP levels were significantly increased.

When interacting with an intracellular nuclear vitamin D receptor protein (VDR) of its target tissue, in this case the intestine, 1,25$(OH)_2D_3$ can form a VDR-1,25$(OH)_2D_3$ complex. This complex can influence gene transcription by induction or suppression of promoter regions containing a VDR response element. Hence, 1,25$(OH)_2D_3$ can improve calcium absorption by interacting with the DNA of the enterocyte and promoting the synthesis of CaBP as mentioned (Groff and Gropper, 2000b). It is also believed that 1,25$(OH)_2D_3$ can stimulate Ca absorption directly by inducing changes in the basolateral membrane, a process known as transcaltachia (Nemere and Anthony, 1990). Although, fractional calcium absorption by the gut is fairly poor (about 30-50% of ingested calcium) 1,25$(OH)_2D_3$ has the potential to increase calcium absorption up to 75% (Aurnaud, 1990).

Phosphorus absorption

The active metabolite, 1,25$(OH)_2D_3$, not only plays a significant role in the calcium absorption process, but also in phosphorus absorption. Phosphorus can be absorbed from the jejenum in the small intestine. As with calcium, absorption of phosphorus can occur via two mechanisms; the passive unregulated mechanism which is

a diffusion process and/or the ATP-regulated process which is coupled to a transport system involving sodium (Larbier and Leclercq, 1992). The hormone, $1,25(OH)_2D_3$, is believed to accelerate this sodium dependent transport system (Klasing, 1998). The metabolite, $1,25(OH)_2D_3$, interacts with a receptor in the basolateral membrane of the duodenal loop which in turn triggers lysosomes present there. These lysosomal vesicles are believed to transport phosphate in a similar fashion in which calcium is transported during transcaltachia (Nemere, 1996). Also, other vitamin D metabolites such as $24,25(OH)_2D_3$ as well as $25\text{-}(OH)D_3$ may have specific receptors (Nemere, 1994). Vitamin D, via its active metabolite, appears to also improve phosphorus absorption by increasing the activity of alkaline phosphatase in the brushborder membrane (Groff and Gropper, 2000a). Alkaline phosphatase (zinc-dependent enzyme) helps in the hydrolysis of organic phosphates (Bikle et al., 1979). However, its role in phosphate transport across the membrane is unclear (Bikle et al., 1979). Davies (1970) reported a small, but gradual increase in the activity of alkaline phosphatase when phosphorus deficient diets (0.16% P, 1.0% Ca) were supplemented with 750 ICU (18.7 μg/kg) or 187.5 μg/kg of cholecalciferol (Davies et al., 1970). However, when phosphorus was adequate (0.48% available P) in the basal diet, vitamin D_3 supplementation did not increase the activity of alkaline phosphatase. The stimulation of alkaline phosphatase by vitamin D is minimal and it is difficult to explain the fact that alkaline phosphatase is mostly found in the duodenum where the pH is low (acidic), but phosphate absorption is highest in jejenum where the pH is high (alkaline) (Harrison and Harrison, 1961).

The Effect of Vitamin D on the Kidney

Understanding the ability of vitamin D to act in the kidney, as this tissue serves a major role in maintaining the phosphorus and calcium balance in the body is crucial. The kidney will excrete more than 80% of the phosphorus that is absorbed by the body. In the event of a phosphorus deficiency, reabsorption of phosphorus can help the body maintain a positive balance, especially since reducing renal excretion by as little as 50% is equivalent to increasing dietary intake of phosphorus by three times (Aurnaud, 1990). Calcitrol ($1,25(OH)_2D_3$) may stimulate reabsorption of phosphorus in the distal renal tubule. Showing that $1,25(OH)_2D_3$ directly stimulates renal reabsorption of phosphate has been difficult, because of the secondary effect caused by vitamin D *in vivo* i.e. increased serum calcium and serum phosphate as well as low levels of PTH (Maxwell and Kleeman, 1994b). In other words, when the body experiences low levels of phosphorus, there will be increased levels of ionized calcium causing PTH levels to decrease. This low level of PTH signals the removal of the PTH-block on renal phosphate reabsorption, leading to increased phosphate retention (Deluca, 1979). At the same time $1,25(OH)_2D_3$ increases due to the low phosphorus levels. There is confusion as to whether $1,25(OH)_2D_3$ stimulates the kidney directly or indirectly by lowering PTH and hence the removal of the PTH-block on kidney reabsorption of phosphate. Liang et al. (1982) has shown that isolated renal chick cells have increased phosphate uptake during administration of $1,25(OH)_2D_3$ *in vitro*. Also, in humans during failure of renal tubular reabsorption of phosphate due to disease there are corresponding low circulating levels of $1,25(OH)_2D_3$ (Bell, 1985). Furthermore, during prolonged absence of vitamin D, the hypophosphotemia (low plasma phosphorus) that often follows is partly due to the

increased clearance of phosphate as a result of reduced renal reabsorption of organic phosphate (McDowell, 1989). Nevertheless, the reduction in P excretion when vitamin D is administered could also be attributed to increased P deposition into the skeleton, an event that is also stimulated by vitamin D (Maxwell and Kleeman, 1994b).

Sutton & Dirks (1978) showed that $1,25(OH)_2D_3$ improves calcium reabsorption by the kidney and vitamin D dependent CaBP (calbindin-D28K) is also present in this tissue (Christakos et al., 1979; Roth et al., 1982). However, the majority of calcium (98%) that filters through the kidney is reabsorbed without the influence of $1,25(OH)_2D_3$ or PTH. The remaining 2% of the filtered load that is reabsorbed is believed to actually be stimulated by PTH and $1,25(OH)_2D_3$, both acting in concert, and not solely by $1,25(OH)_2D_3$ (Deluca, 1982). However, mineral reabsorption by the kidney can regulate plasma concentrations of calcium in the event of short term rises or falls in intestinal absorption and bone losses of calcium. When there is an increase in calcium absorption there must be a corresponding change in calcium excretion to maintain a steady state, this is often accomplished by a decrease in reabsorption by the kidney (Maxwell and Kleeman, 1994a). In order for bone mineralization to take place, one of the challenges is to maintain the mineral constituents of the extracellular fluid. This task is partly controlled by vitamin D stimulated tubular reabsorption of the kidney (Deluca, 1982).

The Effect of Vitamin D in Bone

In bone $1,25(OH)_2D_3$ may have various actions that are indirectly involved in mineralization, new bone formation, as well as bone resorption. Bone, which is comprised primarily of osteoblasts, osteoclasts and chondrocytes, actively responds to

endocrine hormones such as $1,25(OH)_2D_3$. Osteoblasts (bone cells) serve several functions, one of major importance is that of bone matrix synthesis. The bone matrix is made up largely of collagen I and many various bone proteins that are involved in the mineralization process. The capability of $1,25(OH)_2D_3$ to stimulate osteoblasts to synthesize osteocalcin, a localized vitamin K-dependent protein that acts as a chemo-attractant and recruits more osteoblasts into the bone matrix during matrix breakdown, enhances new bone formation. Osteocalcin is a calcium binding protein that can bind to hydroxyapatite and become part of the bone matrix prior to mineralization. Some of the osteocalcin is also released into the blood, and can serve as an indicator of osteoblast metabolism (Maxwell and Kleeman, 1994a).

In addition, alkaline phosphatase hydrolyses organic phosphate esters and pyrophosphate, which are known to inhibit the mineralization process. Osteoblasts are believed to express alkaline phosphatase and are in fact very rich in this enzyme. Osteoblasts can also produce growth factors such as transforming growth factor-β (TGF-β), a potent enhancer of bone formation and inhibitor of osteoclasts. Thus, $1,25(OH)_2D_3$ serves an important role in regulating the expression of genes that produce other localized transcription factors or bone morphogenetic proteins (BMPs), such as TGF-β, which induce differentiation of chondrocytes and osteoblasts (Maxwell and Kleeman, 1994a).

During long bone growth, as the shape of the bone changes or is altered, a process known as modeling is actively occurring. Modeling occurs through resorption and formation of bone at different sites. Resorption does not necessarily follow formation so the two processes are not coupled. If bone modeling does not take place in a normal fashion it can result in different skeletal abnormalities in young growing poultry

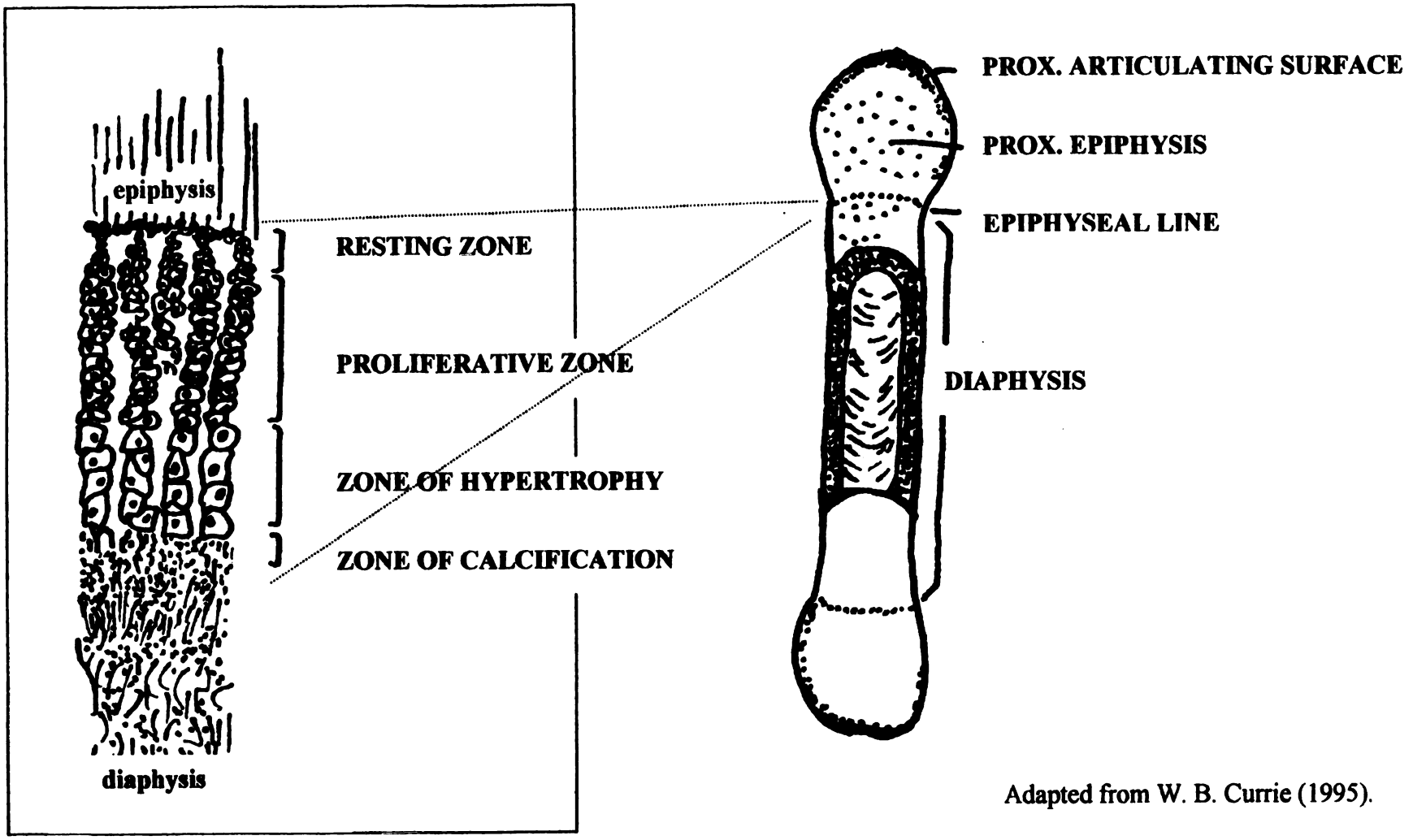
(Watkins, 1999). Bone resorption during modeling is carried out by osteoclasts. These are multinucleated cells derived from hemapoetic stem cells. The differentiation from stem cell to osteoclast is induced by $1,25(OH)_2D_3$ (Maxwell and Kleeman, 1994a). The principal effect of $1,25(OH)_2D_3$ on osteoclasts in this particular case is to increase the number of mature osteoclasts. Once the mature osteoclasts are formed, vitamin D receptors (VDRs) in these cells become absent and no further receptor binding of $1,25(OH)_2D_3$ occurs (Merke et al., 1986). Suda et al. (1992) found receptors for $1,25(OH)_2D_3$ in osteoblasts, but not in osteoclasts. The active metabolite, $1,25(OH)_2D_3$, can increase the activity of mature osteoclasts, but this is an indirect effect. This indirect effect on mature osteoclasts is mediated by $1,25(OH)_2D_3$ via its action on osteoblasts. In other words, $1,25(OH)_2D_3$ stimulates the osteoblasts to produce various molecules that recruit osteoclasts to activate bone resorption.

Endochondral ossification involves the conversion of cartilage into bone. The initial formation of cartilage occurs in three steps. First, mesenchymal cells proliferate. Second, once these cells begin to accumulate they produce extracellular matrix proteins, which induce condensation of these dividing cells. In the third step there is differentiation of the mesenchymal cells into chondrocytes. The chondrocytes secrete the cartilage extracellular matrix. When a long bone in the young chick grows, the center of the shaft turns into bone first. Cartilage is replaced by bone progressively outward towards the end of the shaft. At the distal end of the shaft there is a region which is always producing new cartilage as long as the bone grows. This front or region is referred to as the epiphyseal growth plate (see figure 3.). During the mineralization process chondrocytes are replaced by osteoblasts. Before this can take place, the new cartilage being formed in

the epiphyseal growth plate must undergo three phases. In a normal growth plate, chondrocytes also pass through three layers: 1) In the zone of resting cells, the chondrocytes have not yet reached full maturity. However, these are committed cells that are actively proliferating and can be characterized by their spherical shape; 2) In the zone of proliferating cells, the cartilage cells have differentiated into mature chondrocytes which are still actively proliferating, but are now depositing cartilage and therefore increasing the length of the bone. These cells have a flattened appearance and are arranged in a columnar fashion; 3) In this zone of hypertrophic cells, chondrocytes have once more differentiated into spherical cells, increasing in size mainly by fluid entering the cytoplasm. These cells have become large (interstitial growth) and are actively depositing calcium in the extracellular matrix. Chondrocyte proliferation in this region would normally be limited by apoptosis or programmed cell death. The hypertrophic chondrocytes are more vulnerable to the invasion of capillaries from the periosteum. This vascularization of the hypertrophic region allows blood to bring in more calcium and osteoblasts, which secrete bone matrix onto which the calcium can precipitate for calcification. This process is crucial for bone mineralization to take place, hence replacement of cartilage by bone (Hargest et al., 1985).

Certain vitamin D metabolites, especially $1,25(OH)_2D_3$ have a direct effect on these chondrocytes. The hormone, $1,25(OH)_2D_3$, stimulates differentiation of chondrocytes, promoting the normal development and maturation of cartilage. Farquharson et al. (1993) showed that $1,25(OH)_2D_3$ promotes proliferation as well as differentiation of chondrocytes *in vivo* and *in vitro*. Studies have shown that $1,25(OH)_2D_3$ and 24, $25(OH)_2D_3$ can be produced locally by chondrocytes in the growth plate (Suda et

Figure 3. Diagram of epiphyseal growth plate



Adapted from W. B. Currie (1995).

al., 1985). In a different study, Farquharson et al. (1995) isolated chondrocytes, which were cultured and then examined to see if chondrocytes had the ability to metabolize exogenous 25-(OH)D_3 The chondrocytes converted 25-(OH)D_3 to 24,25-$(OH)_2D_3$ but not to 1,25$(OH)_2D_3$. However, there is controversy over which is the main metabolite participating in chondrocyte differentiation and whether the metabolites produced endogenously (locally) or by renal synthesis are the one's that influence chondrocyte differentiation (Farquharson et al., 1995). Chondrocyte differentiation is also known to be stimulated by TGF-β, which is believed to be regulated by 1,25$(OH)_2D_3$.

Schwartz et al. (1988) showed that 1,25$(OH)_2D_3$ as well as 24,25-$(OH)_2D_3$ could directly and non-genomically (no protein synthesis involved) stimulate the activity of alkaline phosphatase of isolated rat chondrocyte membranes (Schwartz et al., 1988). Alkaline phosphatase is believed to be produced by matrix vesicles that are present in the cartilage matrix. Matrix vesicles are described by Wuthier (1988) as a lipid enclosed microenvironment produced by chondrocytes, that contain enzymes and ion-transport proteins for phosphorus and calcium (Ca^{2+}), important in making apatite. Also, 1,25$(OH)_2D_3$ stimulates calbindin synthesis by chondrocytes which leads to calcium accumulation and cartilage calcification. The vitamin D dependent CaBP has also been detected in bone tissue of chicks (Christakos and Norman, 1978) and in growth plate cartilage of chick tibias (Zhou et al., 1986).

Tibial Dyschondroplasia

TD is a non-infectious metabolic bone disease that is especially prominent in fast growing strains of meat –type poultry. Leach and Nesheim (1965) first described the

characteristics of the abnormality in 1965. The authors observed an abnormal cartilage formation in chicks that had been fed a purified basal diet (98 ICU/kg cholecalciferol). They described it as a mass of opaque cartilage irregular in size situated below the epiphyseal growth plate in the proximal end of the tibiotarsus and tarsometatarsus. Siller (1970) noticed a similar or identical cartilage abnormality in chicks of 5 weeks of age. After histological examination of the growth cartilage in the proximal region of the tibia of broilers, the investigator observed that there was a thickening of the hypertrophic cartilage layer and that this zone extended distally into the zone where normal ossification would take place. The chondrocytes of the mass of the abnormal cartilage appeared shrunken and degenerative which extended into the region of diaphysis replacing the trabecular bone that would normally form there. The investigator added that the metaphyseal areas were less vascularized by blood vessels. He named this bone disease Tibial Dyschondroplasia. Similar results were observed by Itakura and Goto (1973) in heavy strain broiler chicks and by Wise and Nott (1975) in meat type ducks. Several studies on commercial broiler chicks confirmed that the characteristics of TD, especially the failure of vascularization of the excessive mature cartilage (pre-hypertrophic chondrocytes) (Riddell, 1975b; Riddell et al., 1971). Poulos et al. (1978) found that pre-hypertrophic chondrocytes in TD lesions did not reach full maturity or hypertrophy. Hargest et al. (1985) actually saw a 40% reduction in cell size of chondrocytes in the hypertrophic region of TD lesions. Furthermore, this group detected necrotic cells adjacent to the early hypertrophic cells of the growth plate. Some apoptotic cells were found occasionally in less severe lesions, but were totally absent in severe lesions indicating that chondrocytes have the capacity to undergo normal physiological

death if conditions are favorable. Leach and Lilburn (1980) observed that chondrocytes of TD lesions had reduced oxidative capacity compared to normal tissue. Similar results were obtained by Hargest et al. (1985), who saw signs of energy depletion and low calcium and phosphorus in chondrocytes of the hypertrophic region. Hargest et al. (1985) further demonstrated that growth plate cartilage in TD lesions have very low calbindin and alkaline phosphatase concentrations when compared to normal growth plates. The low or absent levels of calbindin in TD lesions may explain the lack of intracellular calcium deposits in the bones affected by TD (Xu et al., 1991). Many biochemical studies of the tibial growth plate were conducted in the 1990's in an attempt to understand the mechanism of TD. However, the exact mechanism and primary factor involved in the development of TD remains unclear. Orth et al. (1991) hypothesized that the increased collagen crosslinks observed in the TD lesion inhibited enzymes such as collagenases which are important in the degradation of matrix, allowing the cells to expand (undergo hypertrophy), and aid in vascularization for metaphyseal blood vessels to invade.

Ohyama et al. (1997) suggested that TD development also involved a defect in apoptosis (programmed cell death). The signals that trigger the transition from hypertrophy to apoptosis are still under investigation. Nevertheless, there is overwhelming evidence and a general agreement amongst most investigators that the cartilage plug that accumulates in the TD lesion is a consequence of an interruption in the timing and sequence of the episodes that takes place during long bone growth. The inability of chondrocytes to undergo hypertrophy only permits them to reach a transitional state with no apoptosis, leaving a poorly mineralized and inadequately

vascularized cartilage in the metaphysis. The cartilage accumulation is due to a lack of degradation rather than an increase in the rate of production of chondrocytes.

After Leach and Neisheim's discovery in 1965, more reports of cartilage abnormality were reported in the 1970's from England to Australia, Canada, Japan, South Africa and the USA. Although Leach and Neisheim (1965) did not notice any signs of leg weakness or lameness in their chicks with TD lesions at 2-4 weeks of age, the majority of these reports associated this defect with lameness and leg deformities of meat-type poultry causing a decline in commercial value especially after processing. Siller (1970) might not have identified the cartilage abnormality had he not observed signs of unusual posture, squatting, lameness and reluctance to walk in broilers at 5 weeks. Riddell (1971) observed the lesion in broilers of commercial flocks only after having performed radiographic studies at 9 weeks. They had initially seen clinical signs such as reluctance to move, stilted gate, marked bowing of the tibia and even birds in a crippled state. Radiography that was repeated on these chickens at 17 weeks of age showed partial resorption of the abnormal cartilage and by 21 weeks it was completely gone. In 1972 at a Japanese poultry farm, inside barns that had no windows, rapidly growing heavy breeds showed clinical signs of TD at 35 days of age. Signs included leg deformity and unusual gait (Itakura and Goto, 1973). Since the 1970's, the growth rate of birds has increased almost two-fold and the clinical signs of TD may not always be detected before processing. However, clinical signs such as lameness, severe leg weakness, squatting (sitting on hocks), abnormal posture and reluctance to move are often associated with TD and are still occasionally seen in commercial flocks. Bowing of the tibial head has also been attributed to TD (Riddell, 1975b), but this may also occur due to excessive weight.

If lesions persist this may lead to fractures and weight loss (Poulos et al., 1978). Prasad (1972) observed a 25% weight loss in afflicted birds compared to normal birds. However, birds that are afflicted with a cartilage abnormality usually have similar body weights compared to birds without the cartilage abnormality. Under commercial conditions, birds that have a severe cartilage abnormality and as a result have difficulty in moving, often do not reach the feeders. Hence, the result is reduced feed efficiency and body weight.

More often TD lesions are subclinical and such lesions may only be seen after closer examination of the growth plate either by the naked eye, histopathology, or by using radiography or a lixiscope. Thorp et al. (1991) argued that naked eye assessment is an inadequate means of identifying TD. The authors noticed that the lesions that demonstrated the classical characteristics of TD, as examined by the naked eye, actually did not exhibit the proper cellular morphology native to TD after histopathologic examination. Nevertheless, many of the early studies have been assessed by the naked eye and are often still performed this way. Tibial Dyschondroplasia lesions may first appear at 14 days of age (Leach and Nesheim, 1965; Riddell, 1975a; Poulos *et al.*, 1978; Kling, 1985). In more recent studies, (Edwards, 1985) detected TD lesions in birds as early as 7 days of age while Elliot et al. (1992) observed TD lesions in birds at 6 days of age. The TD condition is reversible and spontaneous resorption of the TD lesion can occur after 6 weeks of age (Poulos et al., 1978).

The incidence of TD in broiler chicks varies tremendously from flock to flock. Different frequencies have been reported since the 70's. Siller (1970) stated that the incidence of clinical characteristics of TD in the fowl were in the range of 1-2 % as suggested by breeders in England. After having visited several commercial poultry farms

in the USA in 1971, Prasad et al. (1972) reported about 30-40% incidence of clinical signs of TD. Itakura and Goto (1973) reported approximately 1-5% incidence at a Japanese poultry farm of about 28,000 broiler chickens reared in windowless houses. However, subclinical cases of TD might have existed in these flocks, but were not included in those numbers. Hemsley (1970) described the incidence of subclinical TD to be greater than 7 % in broiler chickens in Australia. Similar results were observed in Western Canada (Riddell et al., 1971). When 200 birds in Australia were randomly selected from each of 3 different processing plants and examined for TD, incidences of 35%, 27% and 14% were reported (Burton et al., 1981). Consideration to the difference was given due to the different weight and age of birds from one plant to another. The weight and age at which the birds are being examined will inevitably influence the incidence of TD, but genetic factors also play a major role. Leach and Neisheim (1965) actually increased the incidence of TD through genetic selection. The authors developed a high incidence strain of chickens with 41 % TD incidence and a low incidence strain with 16% TD. Since then other investigators have verified this and genetically manipulated chicken's predisposition to the development of TD (Riddell, 1976; Sheridan et al., 1978).

The initial observation by Leach and Neishem (1965), that males are more susceptible to TD than females has been observed by most studies involving tibial dyschondroplasia. Friedman (1977) stated that the abnormality occurred exclusively in males. Wise and Jennings (1972) observed the same thing in ducks as well as chickens. However, Riddell et al. (1971) did not see any marked differences between females and males. The reason for the high incidence of TD in males of meat-type poultry may be the

faster growth rate that exists in males compared to females. Overall, the issue of growth rate and body weight gain was initially thought to be one of the primary factors in the development of TD. Riddell (1971) noticed that endochondral bone growth took place the fastest in the proximal tibiotarsus and proximal tarsometatarsus indicating that the fast growth in meat type chickens could be a cause of TD (Riddell et al., 1971). Wise and Jennings (1972) also discussed the possibility that TD was caused by a rapid early growth rate by chicks and that this weight exerts excessive pressures on the growth plate cartilage resulting in a failure of metaphyseal blood vessel penetration (Wise and Jennings, 1972). Riddell (1975b) tested the hypothesis that TD might be caused by the excessive weight by inducing unilateral lameness in broiler chicks. Tendons of the tibiotarsal joint were severed on one leg whereby more weight was placed on the other leg due to the resulting limp. Results showed that weight was not the primary factor in the development in TD. A weight -bearing study conducted by Cook et al. (1980) confirmed that weight had no direct effect on TD incidence. In another experiment, oat hulls were added to a commercial broiler starter diet to slow growth of chicks and in which the growth plates were measured, then compared to chicks fed only the starter diet (Riddell, 1975a). There was only a small correlation between growth rate of the growth plate and the development of TD. Riddell (1975a) suggested that growth rate is only a contributing factor rather than a primary factor. However, Poulos et al. (1978) noticed that when broilers were fed a high-energy diet, the birds had a higher growth rate and a higher incidence of TD than birds fed a low energy diet which had lower growth rate and a low incidence of TD. The author was not clear on whether the high incidence of TD was due to higher growth rates or due to the calcium levels which appeared to be higher

in the high-energy diet after analysis, 1.63% Ca vs 1.43% Ca, respectively (Poulos et al., 1978). The author believed that excessively high calcium levels might increase TD incidence.

Nevertheless, studies where feed restriction has been implemented on commercial broiler chicks (Huff, 1980; Edwards and Sorensen, 1987; (Roberson et al., 1993) the TD incidence was significantly reduced. However, in the study by Lilburn et al. (1989) the body weights of these fasted birds at 28 days of age were not less than the birds that were fed ad libitum (control). This is in contrast with Edwards (1987) who saw a 15% reduction in BW gain at 20 days of age and Roberson et al. (1991) who saw a 6% reduction, both correlated with reduced TD incidence. Roberson et al. (1991) also noticed a greater accumulation of Ca in the growth plates of feed deprived birds. The timing of the deprivation seems to be important in reducing the incidence of TD, suggesting that feed deprivation is most effective during the starter period between ages 4 to 20 days (Edwards and Sorensen, 1987) or 14 through 21 (Roberson et al., 1991). Elliot et al. (1994) reported that feed withdrawal improved bone mineralization and decreased TD incidence as well as severity. The author also reported seeing significantly increased plasma $25(OH)D_3$, which is positively correlated with dietary calcium and dietary vitamin D unlike $1,25(OH)_2D_3$, which is negatively correlated. Sufficient supply of calcium in the diet will decrease plasma $1,25(OH)_2D_3$. Feed withdrawal will do the same, indicating that calcium utilization is enhanced under these conditions. This would explain the improved bone mineralization and possibly Roberson's (1991) observation of Ca accumulation in growth plates of feed deprived chicks. Slowing the growth rate may result in a very low incidence of TD and may even prevent it from occurring. However,

in the meat-type industry, this is undesirable as higher body weights give higher dressing percentage and convert into more profit.

In addition to the amount of feed given, the composition of a diet fed to broilers may contribute significantly to the development of TD. Several nutrients when deficient or in excess have been shown to induce TD: an excess of methionine metabolites such as homocysteine, cysteine and cystine, but not methionine by itself (Orth et al., 1992); an excess of histidine (Andrews et al., 1989); an excess of chloride (Leach and Neisheim, 1965); an excess or deficiency of vitamin D including some of its metabolites (Edwards, 1984); an excess or deficiency of calcium and phosphorus (Edwards and Veltmann, 1983). The nutrients that make the most impact on TD development, when their levels are increased or decreased individually or at the same time in the diet, are vitamin D, calcium and phosphorus. When Leach and Neisheim first observed TD, the diet that was fed to these chicks appeared to be adequate for all nutrients. However, when a diet composed of corn and soybean meal was supplemented with minerals and vitamins and fed to broilers, it prevented the occurrence of the cartilage abnormality. Despite the fact that they tested the levels of individual vitamins and minerals in the diet, they were unsuccessful in identifying a single nutritional factor affecting the occurrence of the cartilage abnormality. Groth and Frey (1966) observed that the calcium and phosphorus in the bone ash of chick tibias showed a relationship with the mineral content of the diet.

Leach and Neisheim (1965) realized that changing the mineral mixture of the diet had an effect on the acid-base and/or anion/cation balance, which affected the TD incidence. A high chloride (anion) diet increased TD incidence and sodium bicarbonate (cation) decreased TD incidence. The possibility that an imbalance in calcium to

phosphorus ratio was the cause of TD or at least the cause of leg weakness was considered (Poulos et al., 1978). Hedhammer (1974) had reported that high intakes of calcium had resulted in osteochondrosis in dogs. The lack of studies on calcium and phosphorus role in the incidence of TD prompted Edwards and Veltmann (1983) to conduct studies in this area. These authors observed that when a basal diet (1100 ICU/kg cholecalciferol) with high calcium levels was fed to broiler chicks, TD incidence decreased. Whereas when phosphorus was increased, TD incidence increased. Low levels of calcium and high levels of phosphorus increased TD incidence, showing 39% TD incidence with 0.8% calcium and 0.75% phosphorus present in the basal diet. Phosphorus seems to upset the acid balance in the similar way as high chloride diets. Lilburn et al. (1989) also noticed a strong positive relationship between levels of available phosphorus and TD and expanded on the acid balance theory. In another study (Edwards, 1984) confirmed that an increased calcium: phosphorus ratio was related to a decrease in TD incidence and that if calcium was increased in combination with a phosphorus increase, thus maintaining a 2: 1 ratio, it would result in a decrease in TD incidence (13% TD incidence with 1.1 calcium (Ca) and 0.55% available phosphorus (aP)). In other words, it is the ratio of Ca: aP, rather than total calcium plus phosphorus in the diet that is of prime importance in the expression of the TD lesion. Riddell (1987) also confirmed the observation that an increased calcium: phosphorus ratio in the diet reduced TD incidence and severity and that an increasing phosphorus level relative to calcium is the cause of greater incidence of TD rather than low calcium levels (Riddell and Pass, 1987). The author reported 27% TD incidence in 4 week old chicks fed a starter diet containing 1.4 % Ca and 0.75% aP compared to 70% TD incidence in chicks fed a diet containing 0.8%

Ca and 0.75% aP. Roberson et al. (1993) showed a significant decrease in TD incidence and severity when the Ca : P ratio was kept at about 2:1. Although, this study exhibited TD incidences higher than normal the authors attributed this to the fact that the battery brooder had no fluorescent lights and likely the amount of cholecalciferol (400 ICU/kg) was inadequate under such conditions.

The effects of cholecalciferol in the diet, presence of ultraviolet light or the combination of the two and its relationship with calcium and phosphorus levels on the development of TD are nutritional and environmental factors that have been studied extensively. The relationships of these factors have been long known to be highly important in growth performance and bone development of chicks. Most of the early studies attempting to establish vitamin D requirements were focusing on its effect on phosphorus utilization as well as its antirachitic effects since vitamin D deficiency was linked to rickets and therefore the parameters measured were often weight gain, feed efficiency and bone ash. Waldroup et al. (1963) conducted a series of experiments with broiler chickens in battery cages without any UV-light and fed basal diets with various Ca : P ratios and levels of cholecalciferol. This group noticed that when phosphorus was low (0.48 %) and Ca : P ratio was 1.4 : 1 or 1.8 :1 the body weight and bone ash was depressed compared to a 1 : 1 ratio. However, if phosphorus was increased, there was a need for increased ratios in order to see an increase in bone ash and body weight. As mentioned earlier, this finding was later found to pertain to TD incidence as well. Waldroup et al. (1964) also noticed that increasing levels of vitamin D were beneficial in significantly increasing both body weight and bone ash, suggesting that the optimum level of vitamin D_3 to achieve this improvement for broiler chicks was 826 ICU/kg

compared to NRC (1960) recommended levels of 200 ICU/kg. Waldroup (1965) concluded that if calcium levels were below 1.0%, cholecalciferol levels needed to be higher than 200 ICU/kg.

With the discovery and isolation of other vitamin D metabolites in the late 1960's, several studies were conducted on chicks using some of these various metabolites. McNutt & Haussler (1973) fed leghorn cockerelles (male chicks) a vitamin D deficient diet and supplemented the birds with cholecalciferol, 25-(OH)D_3 or 1,25$(OH)_2D_3$. Results showed that 25-(OH)D_3 gave a better growth response than either cholecalciferol or 1,25$(OH)_2D_3$. The 25-(OH)D_3 metabolite was also more effective at increasing percent bone ash over the control diet than the other two metabolites, at least at lower levels of supplementation. The results suggested that overall, 25-OHD_3 seemed to be the most nutritionally effective metabolite. McNaughton et al. (1976) attempted to establish the 25-(OH)D_3 requirement in broiler chicks and reported a significant increase in tibia ash when 25-(OH)D_3 was added to a diet low in phosphorus (0.45%), compared to when cholecalciferol was added in similar amounts (198 ICU or 300 ICU/kg). However, no differences in bodyweights were observed. Cantor and Bacon (1978) saw a significant improvement in weight gain and feed:gain when feeding broilers chicks 25-(OH)D_3 compared to similar levels of cholecalciferol (1.25, 2.50 or 5.0 μg/kg).

The prevention of TD in poultry had not been extensively studied in the 1970's although there were speculations on the involvement of vitamin D metabolism in the development of TD. Prasad (1971) attempted to eliminate the abnormal cartilage formation by adding dicalcium phosphate and/ or adding cholecalciferol to the drinking water, but without success. Sunde (1975) conducted several experiments on commercial

male poults and looked at the effects of cholecalciferol and 25-$(OH)D_3$ on leg disorders. The author did not clearly indicate that the leg disorder studied was TD. However, he referred to similar clinical signs often associated with the leg abnormality in broilers, assuming it to be the same condition observed by Hemsley (1970). Differences in incidence of the leg disorders in 3-4 week old poults supplemented with 10 µg/kg of 25-OHD_3 or 25 µg/kg of cholecalciferol were not great. However, Sunde (1975) stated that the diet containing 25-OHD_3 appeared to give lower leg disorder incidence, and increased bone ash and body weights. The author speculated that genetically there was an impaired ability to convert cholecaliferol to 25-$(OH)D_3$ or 1,25$(OH)_2D_3$ by young birds. Saveur & Mongin (1978) also proposed that vitamin D metabolism played a part in the development of TD. These authors had noticed that the metabolic acidosis reduced the kidney's ability to convert 25-$(OH)D_3$ to 1,25$(OH)_2D_3$ by 40% *in vitro* and *in vivo*. Metabolic acidosis impairs normal cartilage maturation. In addition, vitamin D deficiency was clearly linked to rickets, and the similarity between TD and rickets seemed great at that time. Edwards began to test the effects of vitamin D metabolites on the incidence and severity of TD in the early 1980's. Broiler chicks that had received 0.2 ml of either 24, 25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ each day showed no response (Edwards, 1984). The initial conclusion that there was an impaired conversion of 25-$(OH)D_3$ to 1,25$(OH)_2D_3$, leading to a deficiency of this active metabolite and leads to a higher TD incidence was disputed at that time by this author.

Edwards (1989) conducted a study in which broiler chicks were fed a TD inducing diet (0.75% Ca and 0.76% P) supplemented with cholecalciferol and/or 25-$(OH)D_3$ or 1,25$(OH)_2D_3$. The effects on bone ash and the development of TD on birds

that had received no vitamin D by 16 days of age were described as very dramatic despite the fact that they had received UV-light. When a vitamin D deficient basal diet was supplemented with either 1100 ICU/kg cholecalciferol, 10 μg/kg $25(OH)D_3$ or 10 μg/kg $1,25(OH)_2D_3$, all significantly reduced the TD incidence from 69% to 42, 28 or 3%, respectively in one experiment. Supplementation of $25(OH)D_3$ did not show any evidence of being more effective in reducing incidence or severity of TD, increasing bone ash, increasing body weight gain or improving feed efficiency than supplementing cholecalciferol at these levels. The metabolite $1,25(OH)_2D_3$ significantly reduced TD incidence over the other two metabolites. However, in another experiment, supplementation of either vitamin D metabolite did not significantly improve any of the measured parameters.

25-Hydroxycholecalciferol Supplementation

The availability and the cost of producing vitamin D metabolites in the 1970's and early 1980's had hindered investigators from conducting more studies on the potential of usage of $1,25(OH)_2D_3$ or $25\text{-}(OH)D_3$ as a supplement in commercial poultry diets. However, by the 1990's it was clear that overall, $25\text{-}(OH)D_3$ and $1,25(OH)_2D_3$ had many potential benefits including reduction in TD incidence and severity. Along with the rapid growth of the poultry industry, problems such as environmental contamination due to excess phosphorus in the manure has increased tremendously. This has become a large issue especially in agricultural states practicing intensive livestock production. Much of this can be blamed on the organically bound phosphorus called phytate or phytic acid. Monogastric animals, such as chicks, cannot efficiently utilize the phytate and as a result,

phosphorus excretion can be very high in the feces. Poultry diets are mainly composed of cereal grains that often have high phytate content ranging from 60 to 80% of the total phosphorus found in the plant (Simon et al., 1990). In addition, because of the importance of P in energy metabolism and bone development it must be present in feed in sufficient quantities to meet the bird's requirement. This has traditionally been achieved by supplementing inorganic phosphorus, which results in increased P excretion (Sebastian et al., 1998). Simply reducing the levels of phosphorus in the diet will compromise the requirements to achieve optimum performance and bone development, possibly leading to bone abnormalities such as tibial dyschondroplasia (van der Klis and Versteegh, 1999).

For many years vitamin D has been known to have the property of improving phytate phosphorus digestibility (Mellanby, 1950). When cholecalciferol was added to a diet low in P and Ca and fed to broiler chicks, the hydrolysis of P-phytate was enhanced and phosphorus utilization was improved up to 77% (Mohammed et al., 1991). Studies on phytate retention with other metabolites have showed positive results on this criterium. McNaughton and Murray (1990) indicated that when 25-(OH)D_3 was added to the feed it was more effective in utilizing P than when cholecalciferol was added in similar amounts. Supplementation with 1,25$(OH)_2D_3$ has also proved to be effective in increasing phosphorus retention significantly from 31 to 68% (Edwards, 1993).

Supplemention of high levels of cholecalciferol to poultry feeds has not been able to yield the same positive results as lower levels of 25-(OH)D_3 or 1,25$(OH)_2D_3$. The relative activities amongst cholecalciferol, 25-(OH)D_3 and 1,25$(OH)_2D_3$ have been documented. Their potency can be placed in the order of 1,25$(OH)_2D_3$ as being the most potent, and then 25-(OH)D_3 and cholecalciferol as the least potent. These studies show

that when compared to cholecalciferol, the 25-$(OH)D_3$ metabolite tend to be in the range of about 1 to 4 times as active (McNaughton et al., 1976; Sunde, 1975) while 1,25-$(OH)_2D_3$ range from 2 to 13 times as active depending on the criteria measured (Boris, 1977; Edwards, 1989; Haussler and Rasmussen, 1972; Myrtle and Norman, 1971). As far as reducing TD incidence, 1,25-$(OH)_2D_3$ has been the most successful metabolite, and the most efficient dosage level in TD prevention from a safety standpoint appears to be as low as 5 μg/kg (Rennie et al., 1993). Although, 10 μg/kg of 1,25-$(OH)_2D_3$ has been shown to be more consistent in reducing both genetically induced TD (Thorp et al., 1993) and nutritionally induced TD (Edwards, 1989, 1990; Roberson and Edwards, 1994), this particular level is regarded as slightly toxic. Rennie (1993) saw tendencies of hypercalcemia and some growth depression with higher levels (10 μg/kg) of 1,25-$(OH)_2D_3$. In combination with high calcium diets broilers are especially sensitive to 1,25-$(OH)_2D_3$ (Edwards et al., 1992; Rennie et al., 1995). Due to the potency of 1,25-$(OH)_2D_3$ there are major safety concerns for using this metabolite as a dietary supplement in poultry feeds. In addition 1,25-$(OH)_2D_3$ is quite expensive to produce, a tightly regulated hormone, and currently is not approved for use in commercial poultry feeds. In contrast, 25-$(OH)D_3$ is not considered a hormone and can be produced fairly cheap today and is available commercially. Therefore, 25-$(OH)D_3$ has been considered a good alternative to 1,25-$(OH)_2D_3$ as a vitamin D supplement in feed. Comparative studies between 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$ have demonstrated that 25-$(OH)D_3$ can be fed up to 690 μg/kg for long periods of time without adverse effects (Yarger et al., 1995). Rennie and Whitehead (1996) fed up to 250 μg/kg of 25-$(OH)D_3$ and observed a reduction in TD incidence from 21% to 5% with no negative side effects. Full renal

tubular calcification was observed in chicks, but only after levels as high as 1000 μg/kg 25-(OH)D_3 had been fed for 14 days. However, minor signs of renal tubular calcification had been observed with as little as 100 μg/kg 25-(OH)D_3. Supplementation of 25-(OH)D_3 to poultry diets to reduce TD has often given inconsistent and inconclusive results.

The development in technology allowed for the synthesis of 25-(OH)D_3 in kilogram quantities in the 1990's. Amoco Bioproducts Corporation began formulating 25-(OH)D_3, into hydrogenated vegetable oil-based beadlets and mixing it into a premix using ricehulls as a carrier (Yarger et al., 1995). The basal level recommended for 25-(OH)D_3 was in the range of 50 to 70 μg/kg. However, 69 μg/kg was ultimately chosen as the appropriate level based on studies conducted by Yarger et al. (1995) who showed that supplementing feed with 25-(OH)D_3 at 69 μg/kg is adequate for maximal body weight gain and feed efficiency when the basal diet contains no cholecalciferol. However, the only study based on TD was conducted by Edwards (1989) who had observed that 10 μg/kg 25-(OH)D_3 reduced TD incidence and severity in low calcium diets, but results were inconsistent. Rennie and Whitehead (1996) later concluded that higher concentrations than Edwards (1989) were needed to effectively reduce TD. The authors noticed that 75 μg/kg significantly reduced TD incidence to 10% with diets lacking in cholecalciferol, but adequate in calcium. In 1996, Mireles et al. (1996) fed broilers 68.9 μg/kg from day 0 for 21 days and reported a significant reduction in TD incidence. Furthermore, TD severity progressively decreased when fed over a period of 49 days. When Mitchell et al. (1997) fed broilers selected for low incidence of TD (LTD) several different levels of 25-(OH)D_3, the group demonstrated that supplementing with as low as 5 μg/kg 25-(OH)D_3 to high calcium (1.0 % Ca) diets significantly reduced TD incidence

and severity. The highest supplementation level of 40 μg/kg 25-(OH)D_3 in the study showed that TD incidence was not significantly different from supplementing 5 μg/kg. The author claimed that 10 μg/kg 25-(OH)D_3 was adequate to obtain maximal bone ash and decreased TD incidence in 16 day LTD line chickens, provided that the diet contained a basal level of 1100 ICU/kg cholecalciferol (Mitchell et al., 1997).

At Auburn University, a study was conducted in the attempt to examine the effectiveness of 25-(OH)D_3 in reducing the incidence of TD in LTD line chicks selected aggressively against TD for nine generations. This group of scientists reported lower TD incidence and severity when the diet was supplemented at 68.9 μg/kg (Zhang et al., 1997). Four week old male broilers exhibited about 6.5 % TD when fed a diet supplemented with 68.9 μg/kg compared to 14.6 % TD incidence for birds fed only the basal diet which contained sufficient cholecalciferol (2200 ICU/kg). This is in contrast with Roberson (1999) who saw no reduction in TD severity or incidence in 17 day broiler chicks, fed a diet sufficient in calcium (0.95%) supplemented with low to high levels of 25-(OH)D_3 (23,46, 69, 92 or 250 μg/kg). There are arguments on whether the level of 68.9 μg/kg 25-(OH)D_3 is the correct level to satisfy performance and TD incidence as pre-existing patents have recommended levels below 68.9 μg/kg (Dudley- Cash, 1996).

The objective of this study was to study the effects of 25-hydroxycholcalciferol on the incidence and severity of TD and phosphorus utilization in broiler chicks and to estimate the level needed to reduce TD and improve phosphorus utilization when chicks are fed a normal broiler diet. Based on the previous studies we hypothesized that dietary supplementation of 25-(OH)D_3 will reduce the incidence of TD in broilers at a lower

level than the manufacturer's recommendations of 69 µg/kg regardless of whether the birds are fed a normal broiler diet or TD inducing diet.

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CHAPTER 2

Materials and Methods

General Procedure

A total of 240 one-day-old male broiler chicks were used for each experiment. Chicks were procured from a commercial hatchery and were of Ross X Ross strain except for Experiment 2 in which Ross X Arbor Acres chicks were obtained. All research was approved by the All-University Committee on Animal Use and Care of Michigan State University in accordance with the Guide for the Care and Use of Agricultural Research and Teaching Animal Care. Ten chicks per pen were assigned randomly to each of 24 pens in an electrically heated battery brooder[1] housed in a room without windows. Room temperature was maintained at approximately at 23°C. Incandescent lighting was continuous in the room and fluorescent light[2] (15 watt cool white bulb) was continuous in the battery pens. Feed and water were provided ad libitum in all experiments. The compositions of the corn-soybean meal based diets are listed in Table 1. The 25-hydroxycholecalciferol premix was donated by Monsanto[3]. Chromic oxide was used as an external indicator at 0.10 % of the basal diet to determine phytate phosphorus retention.

In Experiment 1, 12 pens randomly selected out of a total of 24 pens had plastic filters[4] designed to block ultraviolet radiation from fluorescent light to establish whether the filters work under conditions of the experiment. Chicks were fed a basal diet that

[1]Petersime Incubator Co., Gettysburg, OH 54328.
[2]OSRAM SYLVANIA, LTD., Danvers, MA 01923
[3]Monsanto Animal Nutrition, Naperville, IL 60563
[4]University Products, Inc., Holyoke, MA 01041

Table 1. Composition of the experimental diets

Ingredients and analysis	Starter diet		Grower diet
	TD-inducing	Normal	Normal
	%		
Ground yellow corn	56.61	51.00	61.13
Soybean meal (dehulled)	35.00	39.00	31.12
Soybean oil	5.00	6.07	4.32
Dicalcium phosphate	1.86	1.70	1.21
Limestone	0.28	0.98	1.06
Salt	0.45	0.45	0.40
DL-methionine	0.20	0.20	0.16
Trace mineral mix[2]	0.25	0.25	0.25
Vitamin pre-mix[1]	0.25	0.25	0.25
Chromic oxide	0.10	0.10	0.10
Calculated Composition			
Protein	21.6	23.0	20.0
ME, kcal/kg	3200	3200	3200
Calcium	0.65	0.85	0.76
Total Phosphorus	0.72	0.69	0.58
Nonphytate Phosphorus	0.48	0.45	0.35

	Experiment						
	1	2	3	4	5	6	6
Analyzed Composition						Starter	Grower
Calcium	0.75	0.67	0.85	0.88	0.86	0.94	0.80
Total Phosphorus	0.78	0.73	0.70	0.71	0.67	0.70	0.62
Phytate Phosphorus	0.22	0.25	0.28	0.27	0.23	0.27	0.25

[1]Vitamin premix provided per kilogram of diet: vitamin A (all-trans-retinyl acetate), 5500 IU; vitamin E (all-rac-α-tocopheryl acetate), 11 IU; menadione (as menadione sodium bisulfite), 1.1 mg; riboflavin, 4.4 mg; Ca pantothenate, 10 mg; nicotinic acid, 44 mg; choline chloride, 600 mg; vitamin B12, 0.1 mg; vitamin B_6, 3 mg; thiamin (as thiamin mononitrate), 2.2 mg; folic acid, 3 mg; biotin, 0.3 mg; and *cholecalciferol 1100 ICU (TD inducing diet) and 2200 ICU (normal diet).

[2]Mineral premix supplied per kilogram of diet: manganese, 120 mg; zinc, 100 mg; iron, 60 mg; copper, 10 mg; iodine, 2.1 mg; selenium, 0.1 mg.

contained no cholecalciferol and which was low in calcium and high in phosphorus [0.65% calcium and 0.48% non-phytate phosphorus (npP) by calculation], a diet known to induce TD in broiler chicks (Edwards and Veltmann, 1983). The vitamin D_3 metabolite, 25-hydroxycholecalciferol [25-(OH)D_3] was added to the basal diet at 0, 10 or 70 μg/kg. The experiment was a 2 x 3 factorial design with 2 levels of ultraviolet light (no light and light) and 3 concentrations of 25-(OH)D_3 (0, 10 and 70 μg/kg). Four pens were randomly assigned to each treatment group and the experiment lasted for 17 days.

Experiment 2 was designed to determine the effect of dietary supplementation of 25-(OH)D_3 on tibial dyschondroplasia when a TD-inducing diet is fed. Ross X Arbor Acres male chicks were used in this experiment. Fluorescent tubes in all of the 24 pens were covered with plastic filters providing no source of ultraviolet light for any of the chicks. The TD-inducing diet fed in this experiment contained 1100 ICU/kg cholecalciferol and 25-(OH)D_3 was added at 0, 10, 40 or 70 μg/kg for 17 days. Each treatment was fed to six pens of 10 chicks and was represented on each of the six decks of the battery-brooder.

Experiment 3 was conducted to determine the effects of supplementing 25-(OH)D_3 on the development of TD when a normal broiler diet was fed to broiler chicks. The experimental design was identical to Experiment 2 except the cockerels used in this experiment were Ross X Ross and a normal broiler starter diet [0.85% calcium and 0.45% non-phytate P (npP)] was fed containing 2200 ICU/kg cholecalciferol for 20 days.

Experiments 4 and 5 were conducted to further determine the effects of supplementing a wider range of 25-(OH)D_3 at even intervals on the development of TD when a normal starter broiler diet containing 2200 ICU/kg was fed to broiler chicks. In

both of these experiments, 4 pens of 10 chicks each were randomly assigned each treatment. Ross X Ross chicks were fed supplemental 25-(OH)D_3 at 0, 18, 36, 54, 72 or 90μg/kg for 20 days.

Experiment 6 was conducted to determine if 25-(OH)D_3 needed to be fed past the starter period to prevent TD. Ross X Ross chicks were fed the starter diet described in Experiments 3-5 for 17 days. Twelve pens/treatment were fed either 0 or 40 ug/kg 25-(OH)D_3. From 18 to 35 days, a grower diet formulated to contain 0.76% calcium, 0.35% non-phytate phosphorus and 2200 ICU/kg cholecalciferol was fed to birds selected from the starter period based upon proximity to the treatment body weight average. Pen-mates from two starter pens per treatment were mixed in a grower pen at 7 birds/pen. There were 4 pens/treatment for a total of 28 birds/treatment. Dietary treatments in Experiment 6 were: 1) 0 ug/kg 25-(OH)D_3 from 1 to 35 days of age, 2) 40 ug/kg 25-(OH)D_3 from 1 to 35 days of age, and 3) 40 ug/kg 25-(OH)D_3 from 1-17 days and 0 ug/kg from 18-35 days of age.

Bone

At the end of each experiment or phase, chicks were killed by cervical dislocation. The right tibia of each bird was cut longitudinally at the proximal head of the metaphysis to examine for the presence of TD. Tibias were scored for degree of severity on a scale from 0 to 3, with score 3 representing the most severe case of TD (Edwards and Veltmann, 1983). All the number 3 scores were calculated on a percent basis and a TD severity index was calculated using two different scoring systems. Scoring index number 1 defined the total TD score in each individual pen divided by the total number of

birds affected with TD per pen (Edwards, 1984). Scoring index number 2 defined the total TD score in each individual pen divided by the number of birds in each pen whether birds were affected with TD or not. The left tibia of each bird were collected and kept for bone ash analysis according to the AOAC (1984) method. The tibiae were fat extracted with ethanol and then ether before ashing at 600°C overnight.

In order to examine one of the problems associated with TD, such as leg weakness, all the birds were subjected to a water-bath leg weakness test at 34 days of age. Birds were placed standing in a water-bath filled with 3 cm of water kept at 28°C. Each bird was observed for 90 seconds and the time spent standing and sitting was recorded. A scoring system for the severity of lameness was developed on a scale from 0-3 (score 3 representing the most severe case of lameness). Score 0 was assigned to birds standing the entire 90 seconds; score 1 for birds making 1 attempt to sit, but standing for the remainder until 90 seconds; score 2 for birds making 2 attempts to sit and standing for the remainder until 90 seconds; score 3 for birds making 3 or more attempts to sit during 90 seconds or remain sitting for entire 90 seconds. The incidence of leg weakness and number 3 scores were calculated on a percent basis.

Feed

Feed samples were first ground by a Wiley Mill[5] using a 1mm screen then by a 1093 Cyclotech[6] using a 0.5 mm screen. Samples were digested in a MARS 5[7] microwave. The feed was analyzed for chromic oxide (Willams et al. 1962), calcium (Clinical Application of Atomic Absorption Emission Spectroscopy, 1972) and

[5]Arthur H. Thomas Co., Philadelphia, PA.
[6]FOSS TECATOR, AB Hoganas, Sweden, S-2632
[7]Microwave Accelerated Reaction System, CEM Corporation, Mathews, NC 28106

phosphorus (Gomori, 1942) content using a UNICAM 989 atomic absorption spectrophotometer[8]. Phytate phosphorus content of the feed was determined by a DU 7400 spectrophotometer[9] as described by Latta and Eskin (1980). Feed consumption was recorded throughout the trial and chicks were weighed once a week to calculate feed efficiency (gain: feed).

Excreta

Excreta samples were obtained by collecting fresh excreta from each pen over a 24-hour period at the end of each experiment or phase. The excreta was dried at 50° C and ground as previously cited for feed before digested in the microwave. Chromic oxide was measured in the manure according to the previously cited method by flame atomic absorption spectrophotometry[8]. Excreta were analyzed for phytate phosphorus content by the method described by Latta and Eskin (1980). Phytate phosphorus retention was calculated using the chromic oxide balance method as described by Edwards and Gillis (1959).

Blood

Blood samples were obtained at 13 days of age via cardiac puncture from one chick per pen. The blood was centrifuged for 15 minutes to extract serum and analyzed for serum phosphorus (Goldenberg and Fernandez, 1966) and serum calcium (Moorhead and Biggs, 1974) concentrations using a DU 7400 spectrophotometer[9].

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[8]Thermo Electron Corporation, Franklin, MA 02038
[9]BECKMAN, Fullerton, CA

Statistical Analysis

Data was analyzed by Analysis of variance (ANOVA) and the SAS General Linear Model Procedure. Experiment 1 was analyzed as a 2 x 3 factorial using 2-way ANOVA in which main effects of light and 25-OH D3, and interaction between light and 25-(OH)D_3 was incorporated into the model. Treatment means were separated by Bonferroni's multiple comparison test. To assess the fit of a regression model to the data, a lack-of-fit test was performed. Data were subject to nonlinear regression when appropriate. The breakpoint between the descending line and the plateau was determined using the DUD method of the NLIN procedure (Robbins et al.1979) to estimate the requirement for 25-(OH)D_3 to minimize TD incidence and severe lesions.

Results

Experiment 1

Overall, exposure of birds to ultraviolet light resulted in higher 17-day body weight ($p<0.001$), gain: feed ($p=0.027$), serum calcium ($p=0.016$), and bone ash ($p<0.001$), and lower rickets and TD incidence ($p<0.001$), severity score #2 ($p<0.001$) and severe lesions ($p=0.002$) than birds that were subjected to filtered fluorescent lights (Tables 5 and 6). Phytate phosphorus retention (PP) increased ($p=0.034$) by the presence of ultraviolet light. The effect was especially strong when the diet was deficient in vitamin D increasing the phytate phosphorus retention from 32.9 to 56.4%. Serum calcium concentration in birds fed the vitamin D deficient diet in pens with filtered lights was extremely low. This response was not observed in any of the other groups where serum calcium levels appeared normal and were influenced by other treatments in this experiment. Serum phosphorus levels were significantly higher in the birds subjected to filtered fluorescent lights with no cholecalciferol in the diet compared to any other treatment group except for birds consuming 70 μg/kg 25-(OH)D_3 and exposed to ultraviolet light in this experiment.

The overall effects of addition of 25-(OH)D_3 to the diet resulted in increased bone ash and gain: feed ($p<0.001$) and decreased rickets, TD incidence, severity score # 1 and 2 ($p<0.001$) and number 3 scores ($p=0.003$) (Tables 5 and 6). Serum calcium, bone ash and gain: feed only increased at 10μg/kg while body weight increased and TD incidence decreased ($p<0.001$) with each level of 25-(OH)D_3. However, TD severity (severity score # 1 and 2, and number 3 scores) decreased ($p<0.001$) only at 70 μg/kg 25-(OH)D_3. Rickets was eliminated in chicks with the addition 70μg/kg 25-(OH)D_3 to the

diet, but also at 10μg/kg provided that ultraviolet light was present. The interaction ($p<0.001$) is a significant decrease with ultraviolet light when no 25-(OH)D_3 was added, but no significant effects otherwise. Similarly, there were significant interaction effects between addition of 25-(OH)D_3 and ultraviolet light exposure for TD incidence, severity score #2, number 3 scores and bone ash ($p=0.031$, $p=0.028$, $p=0.02$ and $p<0.001$) (Table 6). The highest supplementation level was needed to significantly decrease the TD incidence and severity when chickens were deprived of ultraviolet light. However, when the ultraviolet light was present 10 μg/kg was sufficient in significantly reducing TD incidence and even eliminated severe lesions. Significant interactions were also observed for body weight and gain: feed ($p<0.001$). The addition of 10 μg/kg 25-(OH)D_3 to the diet increased ($p=0.013$) phytate phosphorus retention. Further increasing the level of 25-(OH)D_3 to 70 μg/kg resulted in phytate phosphorus retention between 0 and 10 μg/kg which was not different from either of the other two levels. However, there was an ultraviolet light by 25-(OH)D_3 interaction effect ($p=0.021$) similar to the rickets and bone ash responses.

Experiment 2

Body weight gain, feed efficiency, and serum calcium and phosphorus were not affected in this experiment (Table 7). Phytate phosphorus retention (PP) increased linearly ($p<0.001$) with increasing dietary 25-(OH)D_3 and was significantly higher when 40 μg/kg 25-(OH)D_3 was fed compared to the control birds (Table 7). Phytate phosphorus retention was almost doubled when 70 μg/kg 25-(OH)D_3 was fed. Tibia bone ash increased linearly from 41.8 to 43.9% ($p<0.001$) and birds receiving 25-(OH)D_3 had

significantly higher percent bone ash than birds receiving no supplementation of 25-(OH)D_3 (Table 8). The incidence of TD, TD severity and number 3 scores decreased linearly ($p<0.001$) by dietary 25-(OH)D_3. The same parameters except for severity index # 1 also were decreased quadratically ($p<0.001$). The control group had a higher ($p<0.001$) TD incidence (73%) compared to birds that had been supplemented with 10μg/kg 25-(OH)D_3 (26%). When supplementing at higher levels, (40 and 70 μg/kg of 25-(OH)D_3), a further reduction in TD incidence (5% and 2%, respectively) was observed. Incidence of number 3 scores were parallel to TD incidence with an initial decrease ($p<0.001$) from 59% to 16% with supplementation of 10 μg/kg of 25-(OH)D_3. Similarly, treatment with 40 or 70 μg/kg of 25-(OH)D_3 resulted in less severe lesions, but were not different from each other. Addition of 70 μg/kg of 25-(OH)D_3 to the TD-inducing diet completely eliminated number 3 scores. Overall, severity was decreased ($p<0.001$) at 40 μg/kg using severity index # 1 and at 10 μg/kg with severity index # 2 ($p<0.001$). Severity index 2 mirrored the results of number 3 score incidence. Analysis by the non-linear regression (NLIN) procedure estimated the requirement of 25-(OH)D_3 to reduce TD incidence and severe lesions to be 14.8 μg/kg and 13.5 μg/kg, respectively.

Experiment 3

As in Experiment 2, body weight, feed efficiency and serum calcium and phosphorus is not affected by supplementation of 25-(OH)D_3 (Table 9). Supplementation of 25-(OH)D_3 had an effect on phytate phosphorus retention ($p<0.001$) (Table 9). Birds fed 10 or 40μg/kg 25-(OH)D_3 had significantly higher phytate phosphorus retention (46.0% and 43.1%, respectively) compared to birds fed no 25-(OH)D_3. However, 70

μg/kg significantly reduced phytate P retention to levels similar to controls resulting in a quadratic response ($p<0.001$). There was no treatment effect on percent bone ash in this experiment (Table 10). The incidence of TD was decreased linearly ($p=0.003$) by increasing dietary 25-(OH)D_3 (Table 10). The overall incidence of TD lesions was not as high as in Experiment 2, as TD incidence was 25% for the control group and supplementing 10 μg/kg of 25-(OH)D_3 did not significantly decrease TD incidence. Addition of 70 μg/kg of 25-(OH)D_3 was needed to significantly reduce TD incidence to 5%. However, the effect of feeding 40 μg/kg of 25-(OH)D_3 on TD incidence was not significantly different from 70 μg/kg of 25-(OH)D_3. Severe TD lesions were decreased linearly ($p=0.004$) with increasing 25-(OH)D_3 (Table 10). Similar to Experiment 2, the number 3 scores were eliminated when 70 μg/kg 25-(OH)D_3 was fed and TD severity index # 2 was not different between feeding 40 or 70 μg/kg of 25-(OH)D_3, but was different for severity index # 1 (Table 10). According to the NLIN analysis, the estimated level needed to minimize TD incidence in a normal broiler diet is 65.4 μg/kg.

Experiment 4

As in Experiment 3, body weight and gain: feed, serum calcium and phosphorus and bone ash responses to the various levels of 25-(OH)D_3 were not significant (Tables 11 and 12). Supplementation of 25-(OH)D_3 increased phytate phosphorus retention at $p=0.083$. The addition of 36 μg/kg 25-(OH)D_3 caused an increase of phytate phosphorus retention from 44.2 % to 50.2 % indicating higher phytate degradation at this level. However, higher supplementation levels of 25-(OH)D_3 were not significantly different from control treatment. Supplementation of 25-(OH)D_3 did not significantly affect the

incidence or severity of TD in this experiment (Table 12). In fact, TD incidence was identical at levels of 0 and 72 μg/kg of 25-(OH)D_3. Number 3 scores were actually absent when 18 μg/kg 25(OH)D_3 was fed, but overall no significant effects were observed for TD severity due to low incidence in this experiment (Table 12).

Experiment 5

There was no difference in 20-day body weight, but there was a significant effect on feed efficiency in this experiment (Table 13). The addition of 18 μg/kg 25-(OH)D_3 had significantly lower gain: feed ratio than the addition of 54 μg/kg and higher levels of 25-(OH)D_3, exhibiting a linear effect ($p=0.030$) due to this response. The level of 25-(OH)D_3 had no significant effect on serum concentration of either phosphorus or calcium. In this experiment, phytate phosphorus retention was not affected by dietary 25-(OH)D_3 and phytate phosphorus retention was higher than other experiments when a marginal calcium diet was fed (Table 13). Incidence of TD and severity of birds fed 25-(OH)D_3 were not significantly different from the control group (Table14). Although at 0 μg/kg 25-(OH)D_3 TD incidence was similar to that observed in Experiment 3, there were no significant effects from adding levels up to as high as 90 μg/kg. Dietary supplementation of 25-(OH)D_3 resulted in an increase in bone ash only at 36 μg/kg compared to the negative control but not at higher levels.

Experiment 6

Starter

There were no improvements in body weight, feed efficiency, phytate phosphorus retention or bone ash (Tables 15 and 16) at 17-days of age by supplementing the diet with 40 μg/kg of 25-(OH)D_3. The incidence of TD lesions was the same for the two groups, exhibiting a TD incidence of 12% (Table 16). The severity of TD was too low to observe a significant effect.

Grower

No significant differences were observed at 35-days of age for body weight or gain: feed among broilers fed 0 μg/kg and previously fed 0 μg/kg (control), fed 40 μg/kg for 5 weeks or 0 μg/kg fed after previously fed 40 μg/kg of 25-(OH)D_3 (Table 17). Supplementary 25-(OH)D_3 in the grower diet increased ($p=0.004$) phytate phosphorus retention compared to the control and the non-supplemented group previously fed 40 μg/kg 25-(OH)D_3. No significant differences were observed for leg weakness (Table 17). The removal or supplementation of 25-(OH)D_3 in the grower diet did not influence the incidence or severity of leg weakness of 35-day-old broilers. There were no responses observed for TD incidence or severity in chicks at 35-days of age by the removal of 25-(OH)D_3 from the grower diet (Table 18). The TD incidence was totally absent for the control and for the non-supplemented group previously fed 40 μg/kg. However, complete resorption of the lesion had not occurred in the supplemented group and showed a TD incidence and number 3 score of 7% for both parameters in birds fed 40 μg/kg the entire experiment.

Discussion

The present study shows that ultraviolet light alone can effectively reduce rickets, but not the development of TD in birds fed a vitamin deficient diet and that supplementation of 25-(OH)D_3 to broiler diets in the absence or presence of fluorescent lighting can reduce TD incidence and severity and improve phosphorus utilization in broiler chicks. Experiment 1, due to the high rickets percentage and very low bone ash responses, clearly shows that the filters were effective in blocking ultraviolet light. In the commercial poultry industry, broilers are often raised in large windowless houses and exposure to ultraviolet radiation is limited. Since the mid 1990's, many poultry producers have switched from incandescent lamps to fluorescent bulbs as the source of light in their facilities (Lewis and Morris, 1998). However, fluorescent lamps in battery brooders have been estimated to provide the equivalent of 20 to 40 μg/kg cholecalciferol (Edwards et al., 1994). The cholecalciferol produced in the skin from ultraviolet light in Experiment 1 was equivalent to 10 μg/kg 25-(OH)D_3 obtained orally. The efficiency of utilization of cholecalciferol activity from ultraviolet light may be because the cholecalciferol does not have to be absorbed first in the gut in association with fats before reaching the liver, but can diffuse directly from the skin into the blood stream and be carried quickly to the liver by vitamin D binding protein for 25-(OH)D_3 production (Holick, 1981). Assuming that the biological activity of 25-(OH)D_3 is about 2.5 times that of cholecalciferol (Sunde, 1975; Soares et al., 1978), the addition of 10μg/kg 25-(OH)D_3 to a basal diet containing no cholecalciferol is approximately equivalent to the addition of 27.5 μg/kg (1100 ICU/kg), which has been reported to be adequate vitamin D activity for broiler chicks

(Edwards et al., 1994). Experiment 1 clearly showed that rickets can be significantly reduced with the addition of 10 μg/kg 25-(OH)D_3 and even eliminated provided that ultraviolet light is available. Elliot and Edwards (1997) reported 3% and 0% rickets incidence in birds fed a basal diet (0.90% calcium and 0.46% npP) supplemented with 27.5 μg/kg or 50 μg/kg cholecalciferol, respectively, in the presence of ultraviolet light. In our study, when fluorescent lights were filtered, rickets was eliminated with 70 μg/kg 25-(OH)D_3. Aburto et al. (1998) showed that rickets could be eliminated with supplementation of 40 μg/kg 25-(OH)D_3 in absence of ultraviolet light when the diet contained either low or high vitamin A levels.

Ultraviolet light has also been shown to be very effective in reducing TD incidence and severity and in increasing bone ash as well as overall performance in broiler chicks (Edwards et al., 1994). However, in Experiment 1 of our study the TD incidence was not influenced by addition of 10 μg/kg 25-(OH)D_3 unless ultraviolet light was available which served as an additional source of vitamin D activity to the chick. This was likely due to the growth response to ultraviolet light when vitamin D_3 was deficient. After adequate growth was similarly restored by 10 μg/kg 25-(OH)D_3 when ultraviolet light was blocked, a reduction in TD was only observed by additional 25-(OH)D_3 (70 μg/kg). However, growth was not reduced by dietary vitamin D_3 deficiency when ultraviolet light was present. Therefore, for TD incidence, a response to dietary 25-(OH)D_3 was observed at a lower level (10 μg/kg).

Since ultraviolet radiation was not a factor in Experiment 2, the only source of vitamin D was the cholecalciferol from the basal diet. When 1100 ICU/kg cholecalciferol is present in a diet with high phosphorus and low calcium (TD-inducing) such as in

Experiment 2, vitamin D_3 appears to be inadequate in overriding the TD inducing effect. The possible reason for this is less efficient absorption of cholecalciferol compared to 25-(OH)D_3 (Bar, 1980). The hydroxylation of cholecalciferol or conversion to 25-(OH)D_3 makes the metabolite more polar which changes its absorptive characteristics and increases its water solubility (Blunt et al., 1968). Absorption of cholecalciferol is energy-dependent and is associated with fats while 25-(OH)D_3 absorption involves passive diffusion across the intestinal wall into the blood stream (Calabotta, 1997). In addition, once in the bloodstream, cholecalciferol has a lesser affinity for vitamin D binding protein (DBP) than 25-(OH)D_3, which strongly affects its transport (Haddad and Walgate, 1976). The inefficiency of vitamin D activity in the TD-inducing diet is clearly evident from the high TD incidence of 73% in Experiment 2. Other investigators also reported this type of inefficiency. Edwards and Veltman (1983) had the highest TD incidence when chicks were fed a diet high in phosphorus, low in calcium and containing 1100 ICU/kg cholecalciferol. During the early 1990's Edwards conducted a series of experiments with this TD-inducing diet which contained 1100 ICU/kg cholecalciferol. The investigators reported TD incidences of 42% and 51% at 16-days of age with ultraviolet present (Edwards, 1989), 54% at 16-days of age with ultraviolet present (Edwards, 1990), 94% at 16-days of age in absence of ultraviolet light (Edwards et al., 1992) and 52% at 21 days of age in absence of ultraviolet light (Edwards et al., 1995), in broiler chicks.

The addition of 25-(OH)D_3 to the TD-inducing diet containing 1100 ICU/kg cholecalciferol in Experiment 2 confirmed that supplementing this vitamin D metabolite could significantly reduce the development of TD at less than 70 μg/kg. Supplementation

of 10 μg/kg 25-$(OH)D_3$ in Experiment 2 reduced TD incidence and severity by 64% and 72 %, respectively. When Edwards (1989) supplemented 10 μg/kg of 25-$(OH)D_3$ to a similar diet, the author obtained a 28 % TD incidence, which is comparable with Experiment 2 (26% TD incidence). Although TD incidence and severity was reduced at 10μg/kg of 25-$(OH)D_3$, this is still relatively high and may be unacceptable in the commercial broiler industry. The conversion of cholecalciferol to 25-$(OH)D_3$ may be inefficient or the plasma pool of 25-$(OH)D_3$ is too low for a sufficient amount of 1,25-$(OH)_2D_3$ synthesis to impact target tissues at the receptor level. Yarger et al. (1995) observed significantly lower serum 1,25-$(OH)_2D_3$ concentrations in relation to higher 25-$(OH)D_3$ concentrations. As dietary 25-$(OH)D_3$ increased from 69 to 690 μg/kg the serum 1,25-$(OH)_2D_3$ decreased while serum 25-$(OH)D_3$ concentrations increased linearly. In a similar study, Mitchell et al. (1997a) reported that dietary 25-$(OH)D_3$ levels of 20 to 40 μg/kg decreased plasma 1,25-$(OH)_2D_3$. The investigators also reported that plasma 25-$(OH)D_3$ increased linearly with increasing levels of dietary 25-$(OH)D_3$. The effect was especially strong with the high TD incidence (HTD) line of chicks (interaction $p<0.05$). The incidence of TD decreased at 5 μg/kg in the low incidence (LTD) line chicks and was about 20% with increasing dietary levels up to 40 μg/kg. These authors had somewhat higher dietary concentrations of calcium (1.0 %) than the present trial. It is possible that some or a majority of the 1,25-$(OH)_2D_3$ is unbound and is functioning at the cellular level (vitamin D receptor). The technique used to measure plasma 1,25-$(OH)_2D_3$ only measures the bound form, therefore lower concentrations than actual of 1,25-$(OH)_2D_3$ that are bound to DBP are detected in the plasma in this case (Soares, 1995). Meanwhile, plasma 25-$(OH)D_3$ is usually bound to DBP and also has a greater affinity for

the protein including albumin than 1,25-$(OH)_2D_3$ (Vieth et al.,1990). This might explain the linear increase in plasma 25-$(OH)D_3$ in Yarger et al. (1996) and Mitchell et al. (1997a) when dietary 25-$(OH)D_3$ was increased in the diet. Whatever the mechanism, increasing supplementation levels to 40 or 70 μg/kg of 25-$(OH)D_3$ in a TD-inducing diet of Experiment 2 in our study further reduced the TD incidence to 5% and 2%, respectively, without compromising performance. A higher plasma pool of 25-$(OH)D_3$ due to the increased dietary level might have allowed for more free 1,25-$(OH)_2D_3$ at target tissues and greater 1,25-$(OH)_2D_3$ synthesis due to the calcium deficient diet. The net availabilty of 1,25-$(OH)_2D_3$ for TD prevention may be the greatest when 25-$(OH)D_3$ is supplemented between 40 to 70 μg/kg to a calcium deficient diet.

In Experiment 3 the TD incidence and severity were also decreased with 25-$(OH)D_3$, which suggests that 25-$(OH)D_3$ supplementation is effective in reducing TD in both normal broiler diets as well as TD-inducing diets. In Experiment 3 the cholecalciferol was increased to 2200 ICU/kg together with increased calcium concentration (0.85%) and lower non-phytate phosphorus concentration (0.45%). This basal diet alone (control), without 25-$(OH)D_3$ added to the diet, resulted in one-third the incidence of TD compared to Experiment 2. Supplementing 2200 ICU/kg cholecalciferol in combination with higher calcium and lower phosphorus levels alone can reduce TD incidence in Ross x Ross chicks. Roberson (1999) fed a normal broiler (0.95% Ca) diet with vitamin D_3 activity of >800 ICU/kg and reported low TD incidence (24%). This is in contrast to a study conducted by Lofton and Soares (1986), in which TD incidences were rather high at 28 days (40%) after feeding broiler chicks a normal corn-soybean meal diet containing 2000 ICU/kg cholecalciferol. Edwards et al. (1992) also reported

high TD incidence in 16-day old chicks fed a near adequate calcium (95%) diet containing 2000 ICU/kg. It is also of interest to note that supplementing this normal broiler diet in Experiment 3 with 10 μg/kg of 25-(OH)D_3 did not significantly decrease the incidence of the TD lesion or its severity, which is in contrast to Experiment 2. Leach and Lilburn (1992) stated that some other nutritional factors that influence TD incidence may be independent of factors affecting lesion severity and effects may not always be parallel. Incidence and severity of TD in Experiment 3 was not significantly different from the control group until 70 μg/kg 25-(OH)D_3 was fed.

Experiments 4 and 5 demonstrated that supplementing 25-(OH)D_3 as a source of vitamin D activity to reduce TD lesions in modern broiler chicks is not always effective in the Ross x Ross strain. The results showed that TD incidence was consistently down to about 8% when 36 to 54 μg/kg are fed, but was not a statistically significant response even when TD incidence was 22% in the basal group of Experiment 5. This is in contrast to Experiment 3 in which there was a linear decrease in TD incidence and severity at levels of 25-(OH)D_3 up to 70 μg/kg. Based on Experiments 3, 4, and 5, feeding 70 μg/kg of 25-(OH)D_3 is not much different from feeding intermediate levels in the range of 18 to 54 μg/kg. Mitchell et al. (1997a) also had conflicting results with regards to incidence when the two experiments were compared. In one of the experiments, they reported a significant reduction in TD incidence in LTD line of chicks, from 44% to 10% after 25-(OH)D_3 supplementation levels of as low as 5 μg/kg. In another experiment, 5 μg/kg 25-(OH)D_3 only reduced TD incidence from 61% to 38% (LTD line) while supplementation levels between 10 to 40 μg/kg resulted in half the TD incidence observed at 5 μg/kg. Rennie and Whitehead (1996) reported 10% TD incidence in Ross broiler chicks at 3

weeks of age with dietary addition of 75 μg/kg 25-(OH)D_3. Many of the earlier studies have rarely reported TD incidences below 10% with any supplementation level of 25-(OH)D_3 when feeding a normal broiler diet. Although complete prevention has been reported, and birds were fed very high levels, 250 μg/kg 25-(OH)D_3 in combination with 250 μg/kg ascorbic acid for 3 weeks (Rennie and Whitehead, 1996). The only metabolite of vitamin D that seem to be able to absolutely prevent the condition consistently is the active metabolite, 1,25-$(OH)_2D_3$ (Sorenson, 1991; Rennie, 1993) or the synthetic analog 1α-hydroxycholecalciferol (Edwards, 1990).

The TD incidence of the control groups in both experiments reported by Mitchell et al. (1997a) were fairly high compared to our experiments which was very similar in design even when a low TD line was used. The reason for this might possibly be the strain of chicks used in the author's studies. Some broiler strains are more susceptible to TD than others. When a TD-inducing diet was fed to Peterson x Hubbard and Ross x Arbor Acres chicks, the TD incidence and severity was higher, and bone ash lower, in the Peterson x Hubbard for all three experiments (Elliot and Edwards, 1994). In another experiment, Edwards (1984) reported some indication that Ross x Arbor Acres chicks had significantly lower TD incidence compared to Peterson x Hubbard. The Ross strain may have been selected against TD so aggressively over the years that it is fairly resistant to the metabolic disorder, provided that diets are adequate in calcium, phosphorus and cholecalciferol. Genetics plays a significant role in TD incidence and therefore the potency of 25-(OH)D_3 in reducing TD depends to a large extent on the broiler strain.

Chickens with increased incidence of TD had fewer intestinal vitamin D receptors (VDR) than chickens with low incidence (Soares et al., 1990). Comparisons between

HTD and LTD lines developed by Auburn University shows higher bone ash in LTD line chicks and plasma concentrations of 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ lower or equal to HTD line chicks.

When Rennie and Whitehead (1996) fed a wheat based commercial basal diet without 25-$(OH)D_3$ supplementation to Ross chicks, the incidence was fairly high (64%) displaying a TD incidence commonly seen with Peterson parental lines. The reason for the high incidence may be that the basal diet was not a corn-soybean diet, but consisted of wheat-soybean diet. The wheat fibers contain non-fermentable fibers that can increase the bulk of intestinal contents, increasing the rate of passage and enhancing gut motility. This reduces the time for calcium absorption. Furthermore, these non-fermentable fibers stimulate proliferation of microbes, which in turn binds minerals such as calcium making it less available (Groff, 2000). Traditionally, practical starter diets used in broiler studies on TD have been corn-soybean meal based. No published studies compare the effectiveness of corn-soybean meal and wheat-soybean meal diets in alleviating TD. However, Edwards (1985) demonstrated that various soybean meals when fed to broilers can produce different TD incidences in chicks depending on the year and source from which the soybean meal was obtained. A soybean meal from one source consistently produced a TD incidence of 34-69% while a soybean meal from another source produced only 14-18%. Leach and Neisheim (1965) stated that corn and soybean meal diets appear to have some protective properties against TD. After feeding broiler chicks this type of diet Leach and Neisheim (1965) found that it completely prevented the occurrence of the cartilage abnormality. The investigators speculated that there might be an unidentified nutritional factor affecting bone formation in corn and soybean meal diets.

From the results in Experiment 6, it is difficult to know whether 25-(OH)D_3 has any effect on TD incidence at all in the current Ross x Ross broiler chick when fed a normal broiler diet. The speculation that intermediate levels within the range of 18 to 40 μg/kg 25-(OH)D_3 based from the previous experiments were necessary to reduce TD incidence and severity, can be disputed. The low TD incidence of 12% with the control diet suggests that the chicks were fairly resistant to the development of the cartilage abnormality. On the other hand, it is possible that a significant number of the birds that were not examined at 17-days and used for phase 2 had the TD condition at the time and were not accounted for in the TD incidence of phase 1. The lesion in these second phase birds might have spontaneously resorbed by 35-days of age, hence the low incidence in the second phase birds. The majority of the TD lesions of the control group of phase 1 might have been less severe TD scores and therefore more easily resorbed during the grower phase. As birds mature, the vitamin D system becomes more efficient with regards to the conversion of cholecalciferol or 25-(OH)D_3 to 1,25-$(OH)_2D_3$ which is less efficient in young, immature and rapidly growing broilers (Edwards, 1989; Edwards, 1990). It is difficult to see any response by 25-(OH)D_3 on TD during the grower phase since we do not know accurately the birds subclinical condition at 17-days of age. However, no effect was observed on TD in the second phase of Experiment 6. This is in contrast to Zhang et al. (1997) who saw a slight decrease in TD incidence of LTD line birds when fed a grower diet supplemented with 68.9 and 344.5 μg/kg from 4 to 6 weeks of age. Birds fed the control diet in Zhang et al. (1997) had a similar TD incidence at 6 weeks and at 4 weeks of 17.2% and 14.2%, respectively. The control birds in phase 2 of Experiment 6 in our study had virtually no TD incidence at 5 weeks of age. This is in

contrast with Scheideler and Ferket (2000) who reported 17.5% TD incidence at 3 weeks and 20% at 9 weeks in a flock of male Ross x Ross birds fed a normal starter and grower broiler diet, respectively.

Environmental factors might also have played a role in the low TD incidence as well as severity observed in the non-supplemented birds of Experiment 4, 5 and 6. Birds that are exposed to stress during their growth period may have higher incidence of TD. Stressed birds have greater susceptibility to infection as the immune system is suppressed. Toxic stresses which result in malabsorption of nutrients such as the fat-soluble vitamin D_3 (fat-malabsorption) that are essential for bone development and growth can increase TD incidence (Calabotta, 1997). Stress levels are more likely to be higher under commercial conditions. The level of stress that birds experienced during the experimental periods might not have been high enough to induce a strong effect on the development of TD. The type of surface that broiler chicks are raised on also influences the incidence of TD. In the commercial broiler industry, broiler chicks are commonly raised on the floor of various litters depending on the farm and flocks often exhibit 15 to 30% TD incidence (Veltmann and Jensen, 1980). In a study by Veltmann and Jensen (1980), four-week old broiler chicks raised on wood shavings had 39% TD incidence versus 2% incidence in chicks raised on wire floors of battery brooders. In another study, chicks raised on wire floors had 8% TD versus 26% in chicks raised on conventional floor pens. Certain types of mycotoxins have been shown to induce TD up to 85-90% in chicks if the compound is present in feed (Walser et al., 1980) but a major outbreak of mycotoxins in the feed is fairly rare and probably only plays a small factor in increased TD incidence in the modern broiler industry.

Phytate phosphorus retention was clearly improved by ultraviolet light in Experiment 1 as vitamin D activity increased resulting in an increased degradation of phytate phosphorus. The improved retention of phosphorus and possibly calcium probably allowed an increased availability of these minerals contributing to the improved bone ash and reduction of rickets incidence in Experiment 1. Cations such as calcium can chelate to phosphate groups of phytic acid forming insoluble Ca-phytate complexes in the intestine (Nelson and Kirby, 1987). When the phytate is hydrolyzed minerals such as calcium that are part of the molecule are released. Shafey et al. (1990) suggested that cholecalciferol stimulates this hydrolysis resulting in enhanced absorption of both phosphorus and calcium.

The linear increase observed on phytate phosphorus retention by the increasing levels of dietary 25-(OH)D_3 in Experiment 2 confirmed that 25-(OH)D_3 can improve phosphorus availability in chicks. Angel et al. (2001) reported that 70 μg/kg 25-(OH)D_3 had a significant sparing effect (0.035% P spared) on non-phytate phosphorus in Ross chicks fed a diets containing graded levels of non-phytate phosphorus (0.24, 0.32 and 0.40%) and calcium at 0.80%. As with the TD inducing diet of Experiment 2, the retention of phytate phosphorus was also improved in a normal broiler diet in Experiment 3. Recent studies have shown that supplementation of 25-(OH)D_3 to broiler diets improve phytate phosphorus utilization. Edwards (1996) reported that a diet containing 0.67% Ca and 0.33% npP supplemented with 5 μg/kg 25-(OH)D_3 resulted in increased phytate phosphorus retention from 53% to 74%. In our studies, the phytate phosphorus retention was not as high as Edwards (1996) with supplementation of 25-(OH)D_3. This difference is probably due to the lower calcium levels used in the study by Edwards (1996). When

calcium was decreased from 1.0% to 0.5%, phosphorus utilization was increased by 15 % (Mohammed et al., 1991).

When Hubbard X Peterson chicks were fed diets containing 2200 ICU/kg cholecalciferol and 0.33% phytate phosphorus (0.51% total P) with various levels of Ca (0.4 or 0.88%), supplementation of 210 μg/kg 25-(OH)D_3 improved ileal hydrolysis of phytate phosphorus from 42.9% to 64 % (Applegate et al., 2000). In our study, the supplementation of 25-(OH)D_3 improved phytate phosphorus retention slightly, but significantly at 36 μg/kg in Experiment 4 from 44.2 to 50.2% but not in Experiment 5. However, the phytate phosphorus retention overall in Experiment 5 (average 51.9%) was generally higher than any of the experiments when a marginal calcium diet was fed. Analyzed total phosphorus was slightly lower for Experiment 5 (0.67%). Higher P_i (inorganic phosphorus) are able to inhibit the catalytic action of certain phytases (Woodzinsky and Ullah, 1996). Perhaps 25-(OH)D_3 facilitates the activity of phytase in the hydrolysis of phytic acid indirectly by transporting P_i away from the intestinal mucosa into the blood, hence decreasing the inhibitory effect of P_i. The phytate phosphorus retention in Experiment 6 (average 41%) for the starter group was also lower than Experiment 5, resulting in no improvement by 40 μg/kg 25-(OH)D_3 supplementation. The basal diet had a higher analyzed calcium level of 0.94%, which possibly decreased hydrolysis of phytate.

Low bone ash (25.2%) when birds were fed a diet with no added cholecalciferol in Experiment 1 is in agreement with Edwards et al. (1994) who reported 29% under similar conditions in 16-day old birds. Exposing birds to ultraviolet light gave almost an identical response in bone ash (41.6%) compared to Edwards et al. (1994) (42.1%)

whereas Elliot and Edwards (1997) observed 37% at 16-days of age. Addition of 10 μg/kg 25-(OH)D_3 in our study resulted in similar bone ash (41.2%) produced by ultraviolet light alone. Sunde (1976) reported that 10 μg/kg 25-(OH)D_3 did not result in maximum bone ash at 3 weeks in turkey poults (42.9%) but that it was near the maximum obtained with 25 μg/kg of cholecalciferol (46.7%). Boris et al. (1977) indicated that 25-(OH)D_3 is twice as potent as cholecalciferol in maintaining maximum bone ash.

Experiment 2 resulted in 41.8 % bone ash with 1100 ICU/kg cholecalciferol in the control diet which confirmed the findings of Boris et al. (1977). However, Edwards et al.(1996) stated that 1,250 ICU/kg is not adequate to produce maximum bone ash. Experiments 4 and 5 had a fairly consistent bone ash percentage of about an average of 43-44% with 2200 ICU/kg cholecalciferol with or without 25-(OH)D_3 supplementation. However, Experiment 3 had the highest and most consistent bone ash with an average of 45%. Although maximum bone ash was obtained with 36 μg/kg 25-(OH)D_3 in Experiment 5, a study by Mitchell et al. (1997a) achieved this at 10 and 20 μg/kg 25-(OH)D_3. In Experiments 4 and 5 of the present study, 36 μg/kg 25-(OH)D_3 appears to give the best results overall when considering bone ash %, the virtual absence of severe TD lesions and consistent gain: feed response.

The extremely low 17-day body weight (254 g) observed for chicks consuming the vitamin D deficient diet in pens with filtered lights of Experiment 1 was expected. Normally, slower growth rates result in lower TD incidence and severity (Edwards, 1987; Roberson et al., 1993). In a study by Edwards et al.(1994), 16-day body weight was about 350 g compared to about 400 g when adequate vitamin D activity was provided. Once ultraviolet light was available to the chicks bodyweight tended to increase with 25-

(OH)D_3 supplementation, but was not significantly different. Many of the earlier studies (McNutt and Haussler, 1973; Cantor and Bacon, 1978: Yarger et al., 1995) suggested that adding 25-(OH)D_3 to the diet for broiler chicks improved body weight gain and feed efficiency compared to cholecalciferol. However, there were no responses observed for body weight gain with 25-(OH)D_3 supplementation in any of our experiments after Experiment 1. Body weights averaged between 400 to 500 grams for 17-day old birds (Experiment 2 and 6) and 600 and 700 grams in 20-day old birds (Experiment 3, 4 and 5), which is consistent with earlier studies conducted with birds of the Ross strain that were of similar design (Rennie and Whitehead, 1996; Roberson, 1999). In Experiment 2 Ross x Arbor Acre chicks were used, but the strain difference does not seem to have affected body weight gain.

Feed efficiency was restored with ultraviolet light in Experiment 1 and supplementation of 25-(OH)D_3 up to 70 µg/kg in presence or absence of light did not give any beneficial results. If the diet is adequate in cholecalciferol (1100 ICU/kg) body weight and feed efficiency will normally not respond to supplemental 25-(OH)D_3 (Edwards, 1989). This was confirmed with all our experiments except for Experiment 5 which had significantly lower gain: feed at 36 µg/kg 25-(OH)D_3 but a ratio was still an efficiency considered normal (0.729) for 20-day old broilers.

In Experiment 1, the low body weights were also accompanied by abnormally low serum calcium (4.52 mg/dl), which largely contributed to the poor mineralization reflected in the low bone ash percentage. These chicks were severely deficient in vitamin D and as a result calcium absorption was inevitably impaired. Edwards et al.(1994) showed that chicks fed a diet with no cholecalciferol in the absence of ultraviolet light

also had serum calcium levels of 7.7 mg/dl compared to about 11.0 mg/dl in chicks given ultraviolet light or adequate vitamin D_3. Mitchell et al. (1997a) reported increased plasma calcium with as low as 5 μg/kg cholecalciferol. Serum calcium and serum phosphorus were fairly constant across all experiments and were not affected by 25-$(OH)D_3$ supplementation. Calcium ion concentrations in the circulating blood is necessary for blood clotting and inter-neural signal transmission and its metabolic role is vital to life and therefore extracellular calcium (ECF $[Ca^{2+}]$) must be sustained. The skeleton serves as a calcium reserve from which ECF can aquire calcium during deficiency and excess calcium is simply excreted in the feces (Heaney, 2002). This is why serum calcium will not be heavily influenced by supplemental 25-$(OH)D_3$. However, prolonged severe calcium or vitamin D deficiency will lower calcium in the blood as was observed in Experiment 1 and the result of this is reflected in the bone mass or ash content.

A possible explanation for the relatively higher serum phosphorus concentrations in Experiment 1 could be that it came from the dissolution of amorphous calcium phosphate, which is found in large amounts in young bone. The amorphous calcium phosphate has lower calcium to phosphorus ratio than pure hydroxyapatite, the latter that is found more commonly in mature animals. Moreover, the TD-inducing diet is high in phosphorus relative to calcium, which possibly increased serum phosphorus. Although, the phosphorus absorption is not as efficient in the vitamin D deficient status, a low absorption of phosphorus will suppress PTH levels, signaling to the kidney to increase phosphorus reabsorption and decrease phosphate excretion. Phosphorus levels in the body are highly regulated by the renal reabsorption and excretion process, whereas calcium levels rely primarily on PTH to stimulate 1,25-$(OH)_2D_3$ production from 25-

$(OH)D_3$ and less on renal reabsorption and excretion (it is presumed that 99% of all filtered calcium is automatically absorbed by the kidney (Deluca 1982)). The latter metabolite, which is probably already present in very low concentrations in the pool due to the vitamin D deficient status inevitably, limits 1,25-$(OH)_2D_3$ production and subsequently its participation in improving intestinal absorption of either mineral.

Despite the large amount of new information that has been published on TD during the past decade the etiology of TD is still not clear. The implications of feeding different metabolites of cholecalciferol such as 25-$(OH)D_3$ have been the most identifiable solutions in combating the TD lesions. The responses observed with 25-$(OH)D_3$ on TD development in our study and earlier nutritional studies are variable. Studies on other suggested beneficial effects of dietary 25-$(OH)D_3$ such as growth stimulation have also been inconsistent, but to a lesser extent, and appears to be affected by age, strain and genetics. The role of 25-$(OH)D_3$ in phosphorus utilization has also received more attention in recent years and a relationship between 25-$(OH)D_3$ and phytate degradation has been established although the mechanism in achieving this is also unclear. There is evidence that shows that 1,25-$(OH)_2D_3$ is a more superior metabolite in preventing TD (Edwards, 1989, 1990; Rennie et al., 1993) and increasing phytate phosphorus retention (Edwards, 1993) in broiler chicks. The advantage of 1,25-$(OH)_2D_3$ is its ability to interact with receptors at target tissues which is due to its hydroxylation at position 1 on its carbon skeleton. The 1,25-$(OH)_2D_3$ metabolite binds to the VDR with high affinity and specificity. The 25-$(OH)D_3$ metabolite is only able to compete for the receptor when concentrations are three-fold that of 1,25-$(OH)_2D_3$ (Soares, 1995). The

potency of 1,25-$(OH)_2D_3$ allows for a very small margin of safety from toxicity which is why the commercial use of 1,25-$(OH)_2D_3$ is tightly regulated.

CHAPTER 3

SUMMARY, CONCLUSION AND IMPLICATION

Skeletal deformities and leg weakness have become one of the major problems to the poultry industry. Skeletal problems likely cause the broiler industry close to 120 million and the turkey industry 40 million dollars in losses every year (Sullivan, 1994). Tibial dyschondroplasia (TD) is a common skeletal abnormality found in young rapidly growing meat-type poultry (ducks, broiler chickens and turkeys) and is influenced by nutrition as well as genetics (Riddel, 1981; Edwards, 1984). Feeding 25-(OH)D_3 provides growth stimulation in addition to TD prevention and the metabolite can improve phosphorus utilization in broiler chicks. The objective of this study was to study the effects of 25-hydroxycholcalciferol on the incidence and severity of TD and phosphorus utilization in broiler chicks and to estimate the level needed to reduce TD and improve phosphorus utilization when chicks are fed a normal broiler diet. Based on the previous studies we hypothesized that dietary supplementation of 25-(OH)D_3 will reduce the incidence of TD in broilers at a lower level than the manufacturer's recommendations of 69 μg/kg regardless of whether the birds are fed a normal broiler diet or TD inducing diet.

In conclusion, the low TD incidence observed with 25-(OH)D_3 supplementation in our studies is partly contributed by the type of broiler strain that was used. The dietary calcium concentration of about 0.90% seems to be a threshold level for Ross x Ross chicks in our studies, a point at which TD incidence will not be reduced with any level of 25-(OH)D_3 supplementation. This threshold appears to be different in other meat-type

strains. The addition of 40 to 70 μg/kg clearly reduced and minimized TD when birds were affected by a low or marginal calcium diet. Lower level of 25-(OH)D_3 is much more effective when calcium is deficient (TD-inducing). Supplementation with 25-(OH)D_3 does not seem to improve performance in normal starter or grower broiler diets. Lower levels of 25-(OH)D_3 can increase phytate phosphorus in broiler starter diets. The inclusion of 25-(OH)D_3 in the grower diet appears beneficial as it improved phytate phosphorus retention.

Supplementation of 25-(OH)D_3 to normal starter and grower diets may have important practical effects in the commercial poultry industry as it can increase phosphorus utilization. The 25-(OH)D_3 metabolite increases phytate phosphorus retention in the gut of the birds. Excessive levels of phosphorus in the manure is of great environmental concern in areas of intensive animal agriculture production which poultry growers and integrators are faced with. The majority of the producers in the commercial poultry industry who supplement 25-(OH)D_3 in their operations remove it from the diets in the grower phase (personal communication, Marty Allison, 2002). This is likely due to a cost factor as the price per pound (lb) per ton of feed (69μg) of 25-(OH)D_3 is $1.65. The inclusion of 25-(OH)D_3 in the grower diet may potentially reduce some of the excessive phosphorus that is excreted in the manure. In addition, with regards to 25-(OH)D_3 supplementation and TD prevention, Ross birds fed 0.90-1.0% Ca do not appear to need Hy-D to prevent TD, but may be important for bone health for companies formulating at 0.85%.

Table 2. Summary of Experiments

Experiment 1

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
			(ug)	(ICU)
1	1	No	0	0
2	1	Yes	0	0
3	2	No	10	0
4	2	Yes	10	0
5	3	No	70	0
6	3	Yes	70	0

Experiment 2

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
			(ug)	(ICU)
1	1	No	0	1100
2	2	No	10	1100
3	3	No	40	1100
4	4	No	70	1100

Experiment 3

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
			(ug)	(ICU)
1	1	No	0	2200
2	2	No	10	2200
3	3	No	40	2200
4	4	No	70	2200

Experiment 4

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
			(ug)	(ICU)
1	1	No	0	2200
2	2	No	18	2200
3	3	No	36	2200
4	4	No	54	2200
5	5	No	72	2200
6	6	No	90	2200

Table 3. Summary of Experiments

Experiment 5

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
			(ug)	(ICU)
1	1	No	0	2200
2	2	No	18	2200
3	3	No	36	2200
4	4	No	54	2200
5	5	No	72	2200
6	6	No	90	2200

Experiment 6
Phase 1

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
	Starter		(ug)	(ICU)
1	1	No	0	2200
2	2	No	40	2200

Experiment 6
Phase 2

Group 1	Diet	UV-Light	25-(OH)D3	Cholecalciferol
	Grower		(ug)	(ICU)
1	1	No	0	2200
2	2	No	40	2200
3	3	No	0	2200

Table 4. Summary of results (compared to control)

	25-(OH)D_3 (μg/kg)	UV-light	Body Weight	Gain: Feed	Serum Ca	Serum P	Bone Ash	TD incidence	TD severity	%PP retention
Experiment 1	0	yes	*↑	↑	↑	—	↑	—	—	↑
	10	no	↑	↑	↑	↓	↑	—	—	↑
	10	yes	↑	↑	↑	↓	↑	↓	↓	↑
	70	no	*↑↑	↑	↑	↓	↑↑	↓	↓	↑
	70	yes	↑↑	↑	↑	—	↑	↓	↓	↑
Experiment 2	10	N/A	—	—	—	—	↑	↓	↓	—
	40	N/A	—	—	—	—	↑	↓↓	↓↓	↑
	70	N/A	—	—	—	—	↑	↓↓	↓↓	↑
Experiment 3	10	N/A	—	—	—	—	—	—	—	↑
	40	N/A	—	—	—	—	—	—	—	↑
	70	N/A	—	—	—	—	—	↓	↓	—
Experiment 4	18	N/A	—	—	—	—	—	—	—	—
	36	N/A	—	—	—	—	—	—	—	↑
	54	N/A	—	—	—	—	—	—	—	—
	72	N/A	—	—	—	—	—	—	—	—
	90	N/A	—	—	—	—	—	—	—	—
Experiment 5	18	N/A	—	↓	—	—	—	—	—	—
	36	N/A	—	—	—	—	—	—	—	—
	54	N/A	—	—	—	—	—	—	—	—
	72	N/A	—	—	—	—	—	—	—	—
	90	N/A	—	—	—	—	—	—	—	—
Experiment 6 II	40-40	N/A	—	—	—	—	—	—	—	↑

*↑↑ or ↓↓ represents significantly higher or lower values compared to ↑ or ↓, respectively, within each experiment.

Figure. 4. Effects of dietary 25-(OH)D_3 with or without UV-light on 16-day body weight. (Experiment 1).

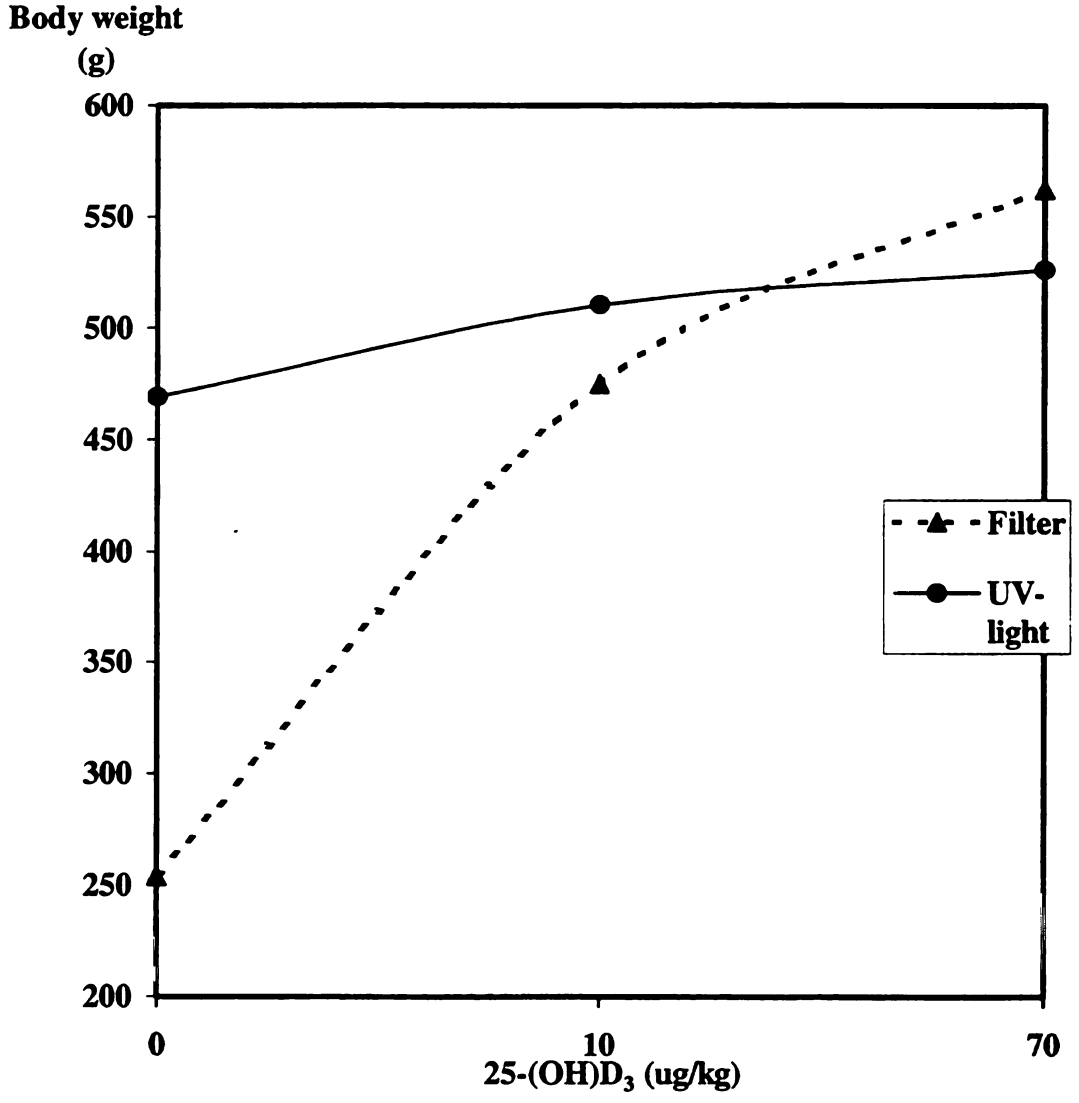

Figure 5. Effects of dietary 25-(OH)D_3 with or without UV-light on rickets incidence at 16 days of age (Experiment 1).

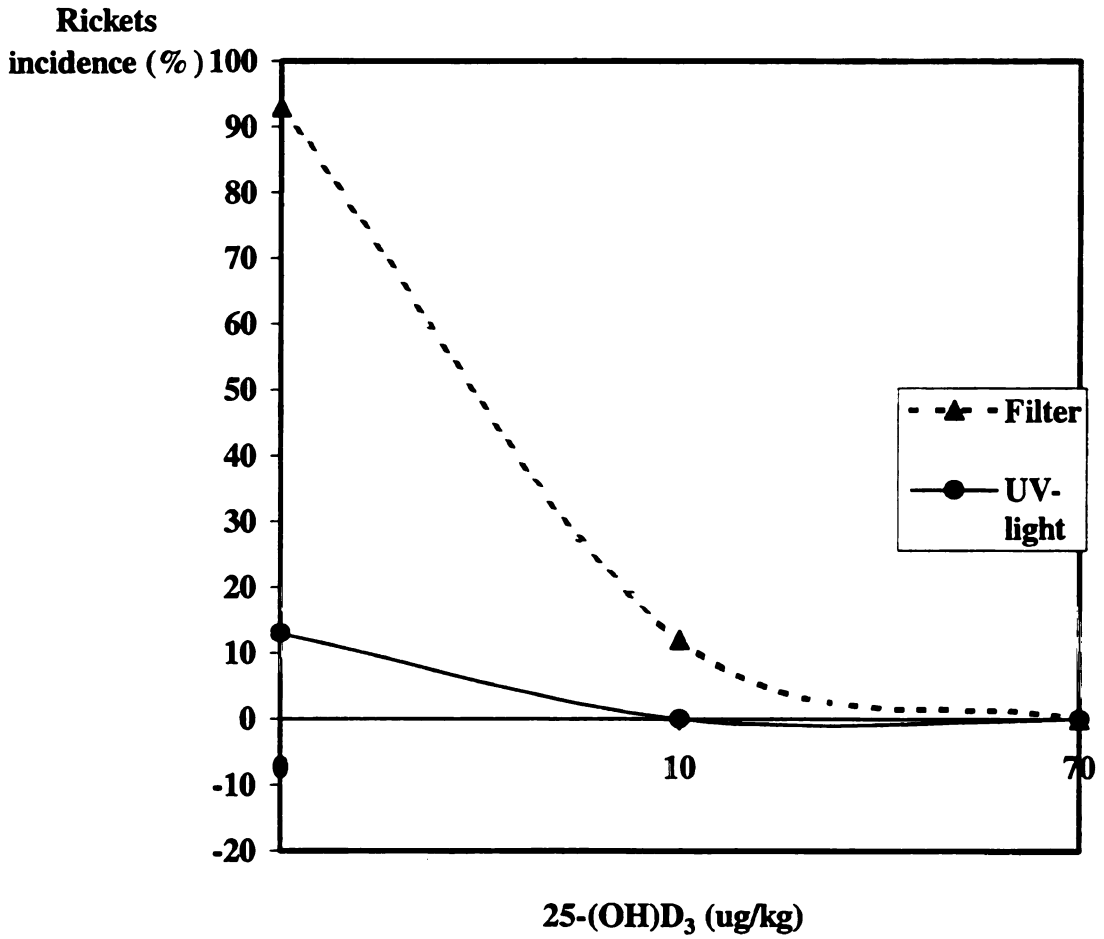

Figure 6. Effects of dietary 25-(OH)D_3 with or without UV-light on TD incidence at 16 days of age (Experiment 1).

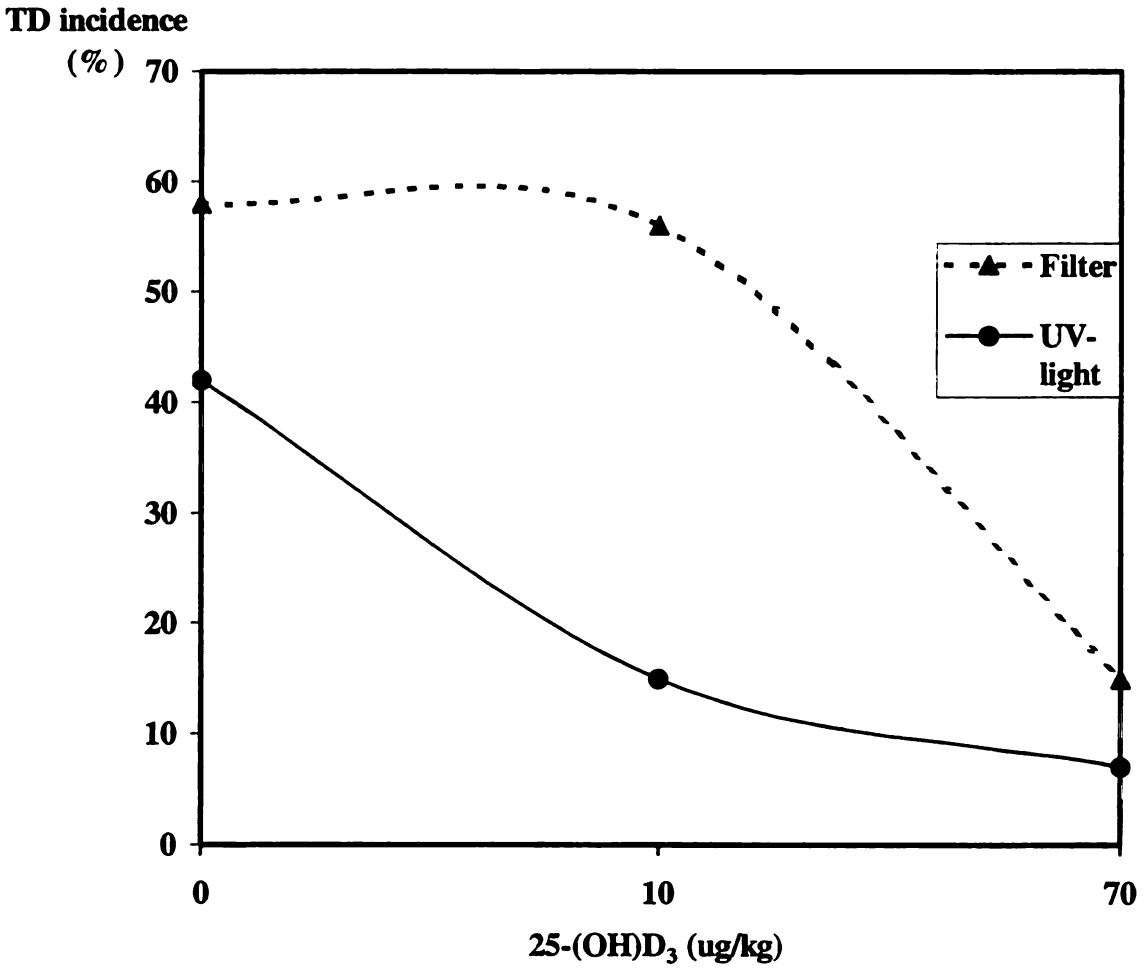

Figure 7. Effects of dietary 25-(OH)D_3 with or without UV-light on TD severity (N3%) at 16 days of age (Experiment 1).

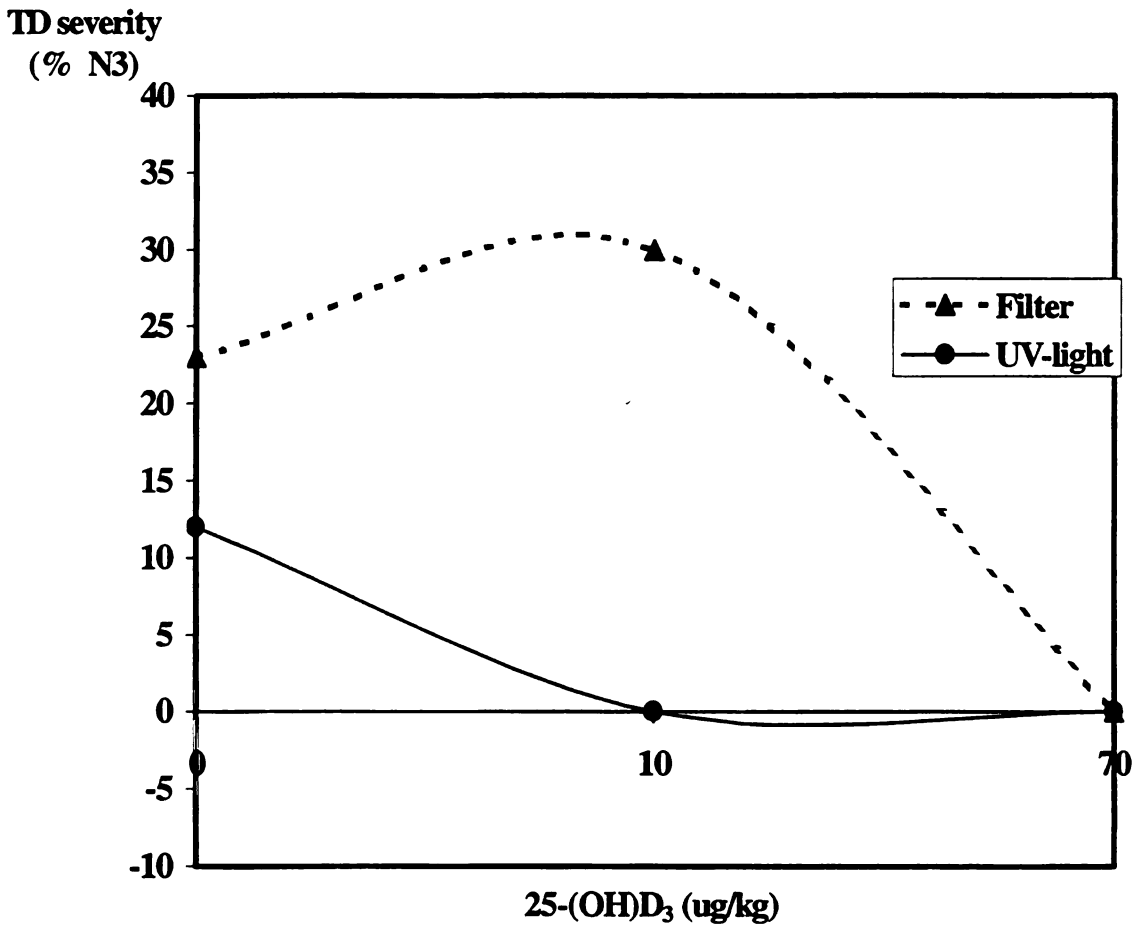

Figure 8. Effects of dietary 25-(OH)D_3 on TD incidence at 17 days of age (Experiment 2).

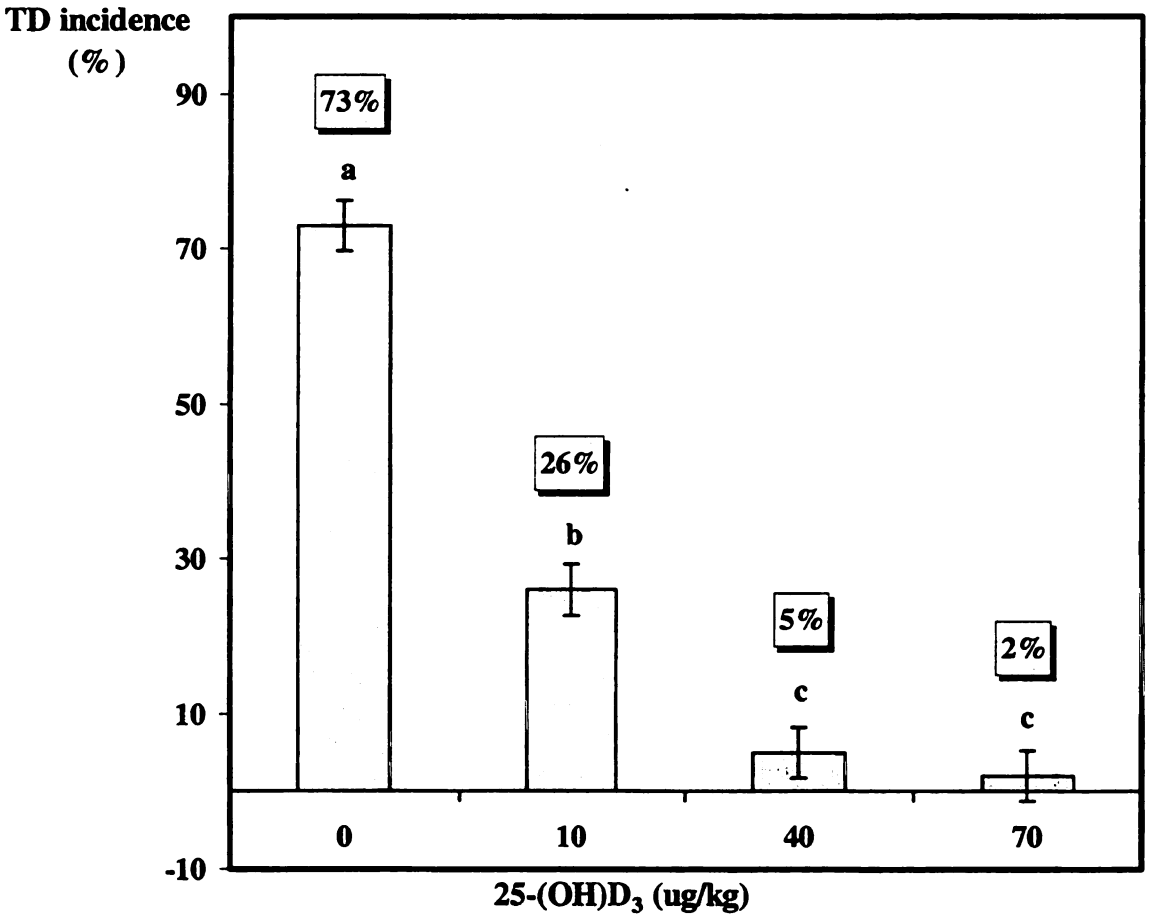

Figure 9. Effects of dietary 25-(OH)D_3 on TD severity (%N3) at 17 days of age (Experiment 2).

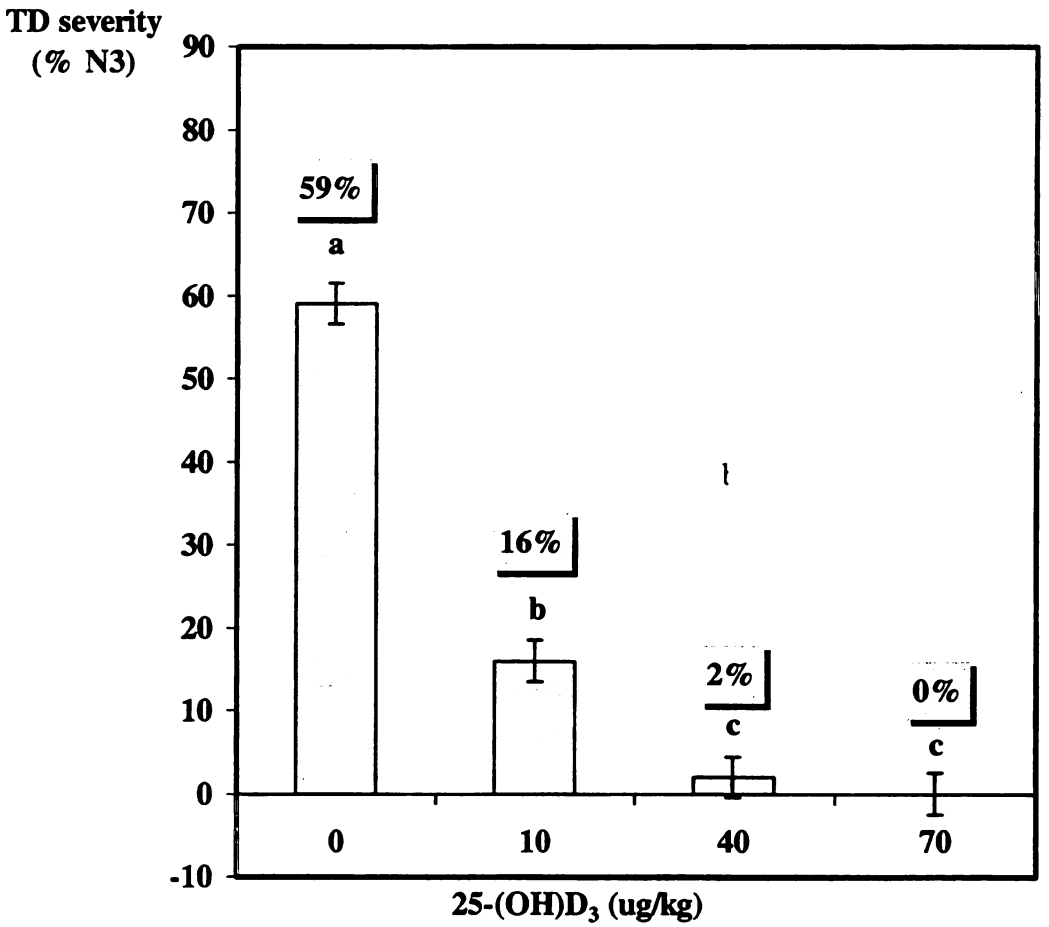

Figure 10. Effects of dietary 25-(OH)D_3 on % phytate phosphorus (PP) retention at 17 days of age (Experiment 2).

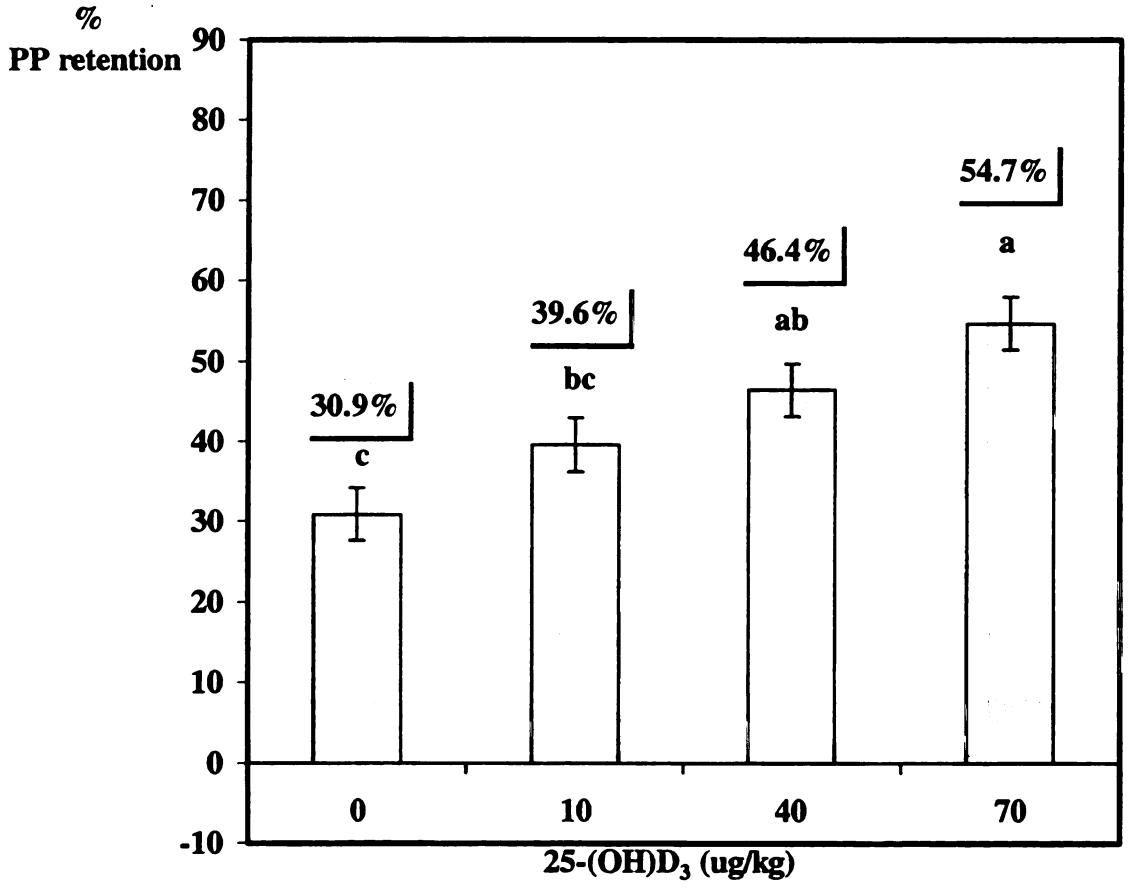

Figure 11. Effects of dietary 25-(OH)D_3 on TD incidence at 20 days of age (Experiment 3).

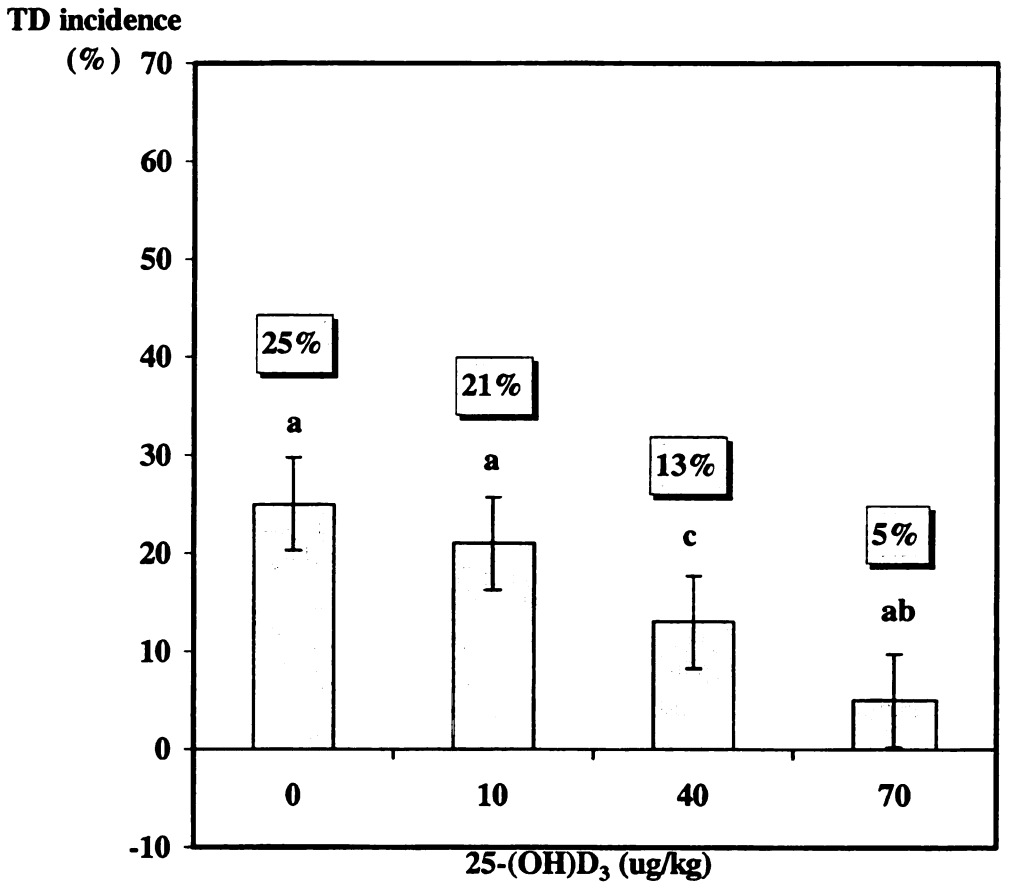

Figure 12. Effects of dietary 25-(OH)D_3 on TD severity (%N3) at 20 days of age (Experiment 3).

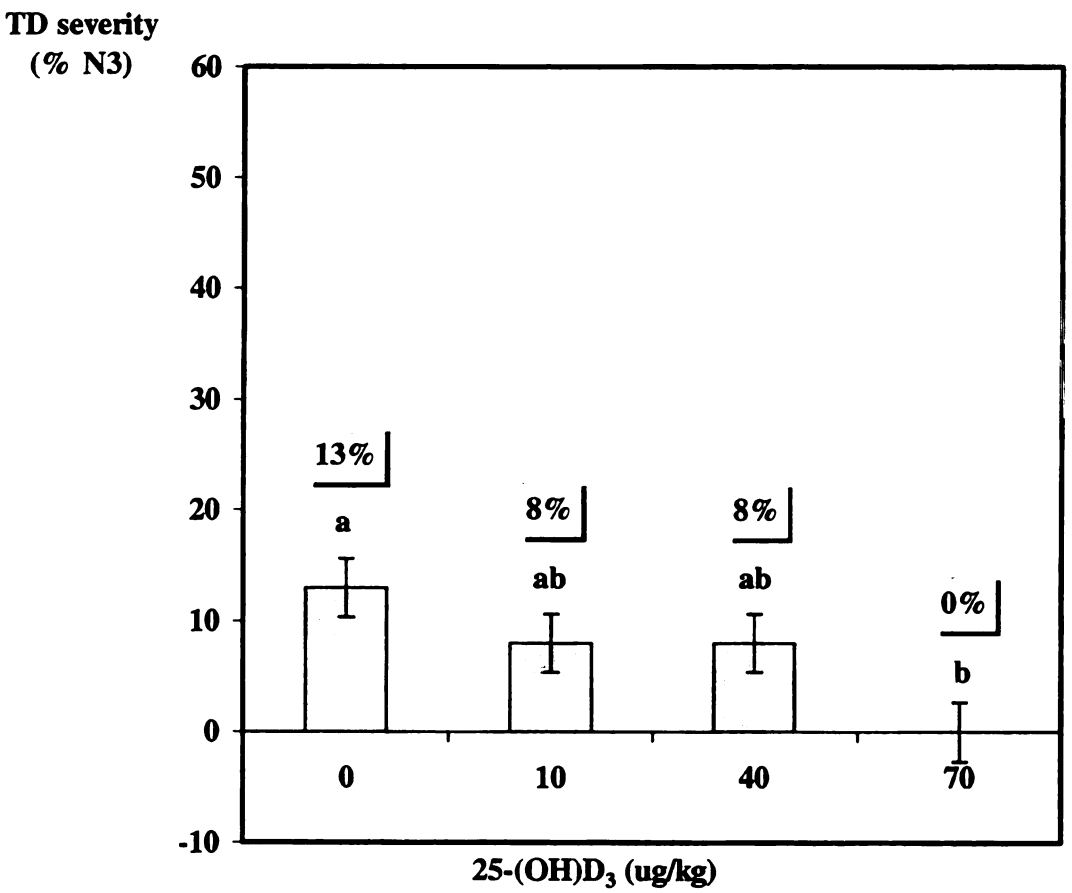

Figure 13. Effects of dietary 25-(OH)D_3 on % phytate phosphorus retention (PP) at 20 days of age (Experiment 3).

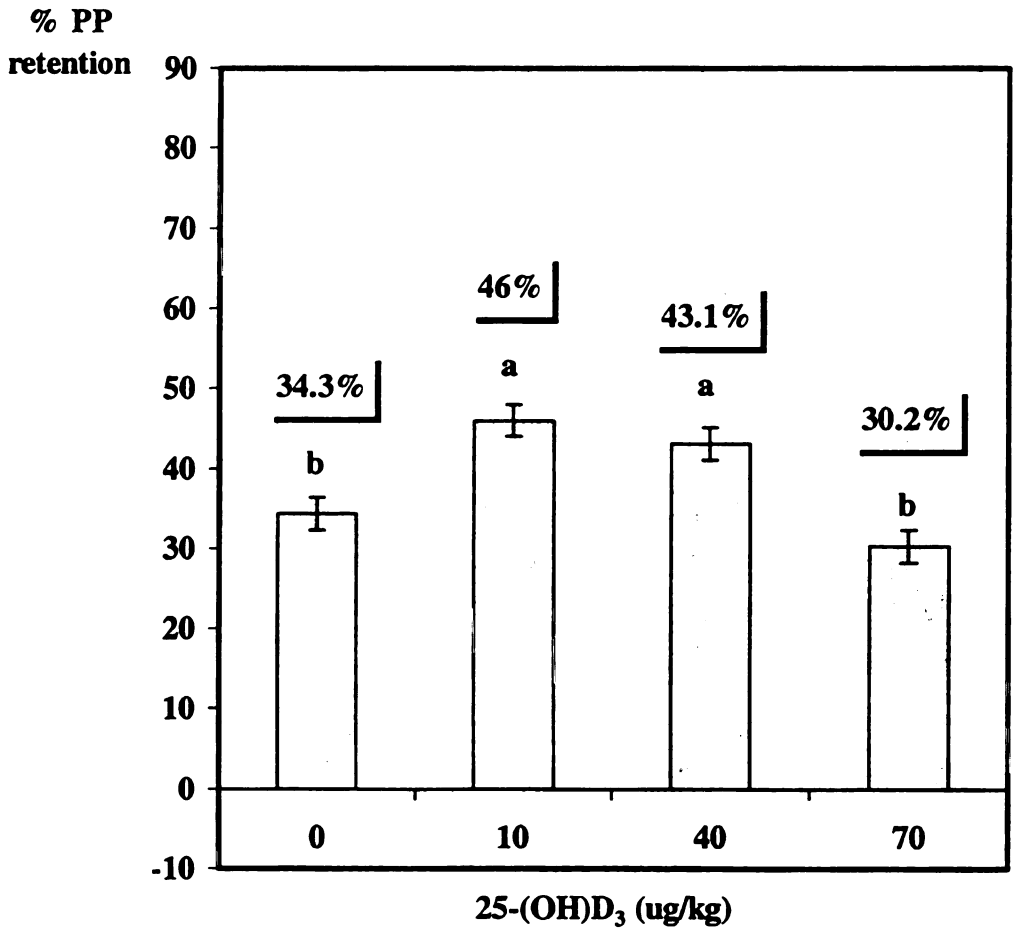

Figure 14. Effects of dietary 25-(OH)D_3 on TD incidence at 20 days of age (Experiment 4).

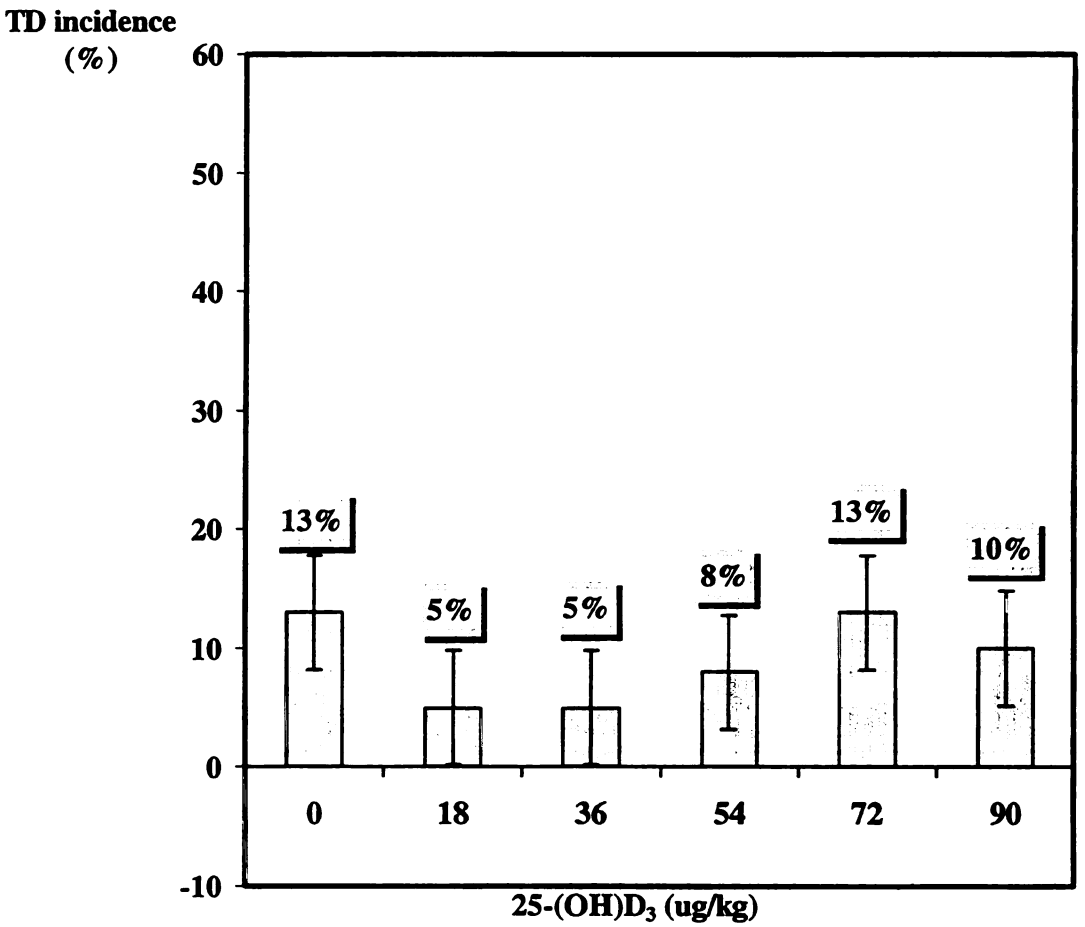

Figure 16. Effects of dietary 25-(OH)D_3 on TD incidence at 20 days of age (Experiment 5).

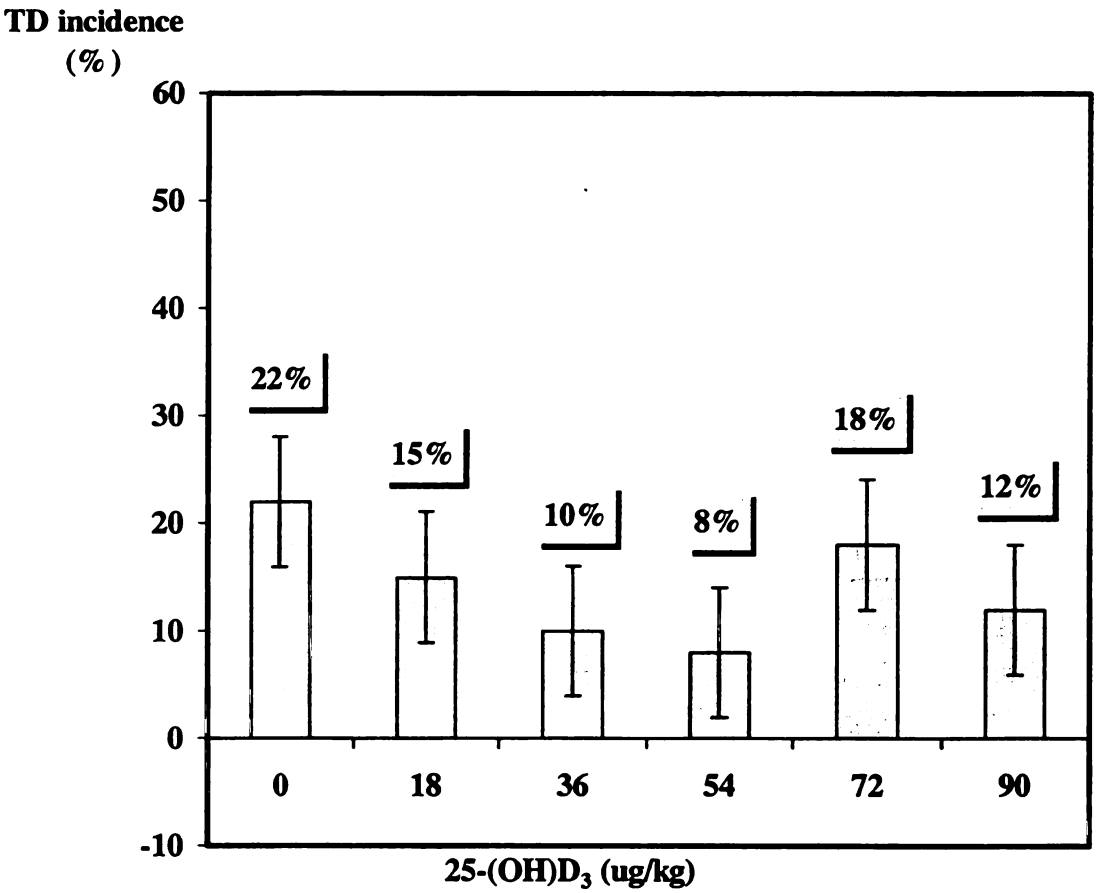

Figure 17. Effects of dietary 25-(OH)D_3 on TD incidence at 17 days of age (Experiment 6, Phase I).

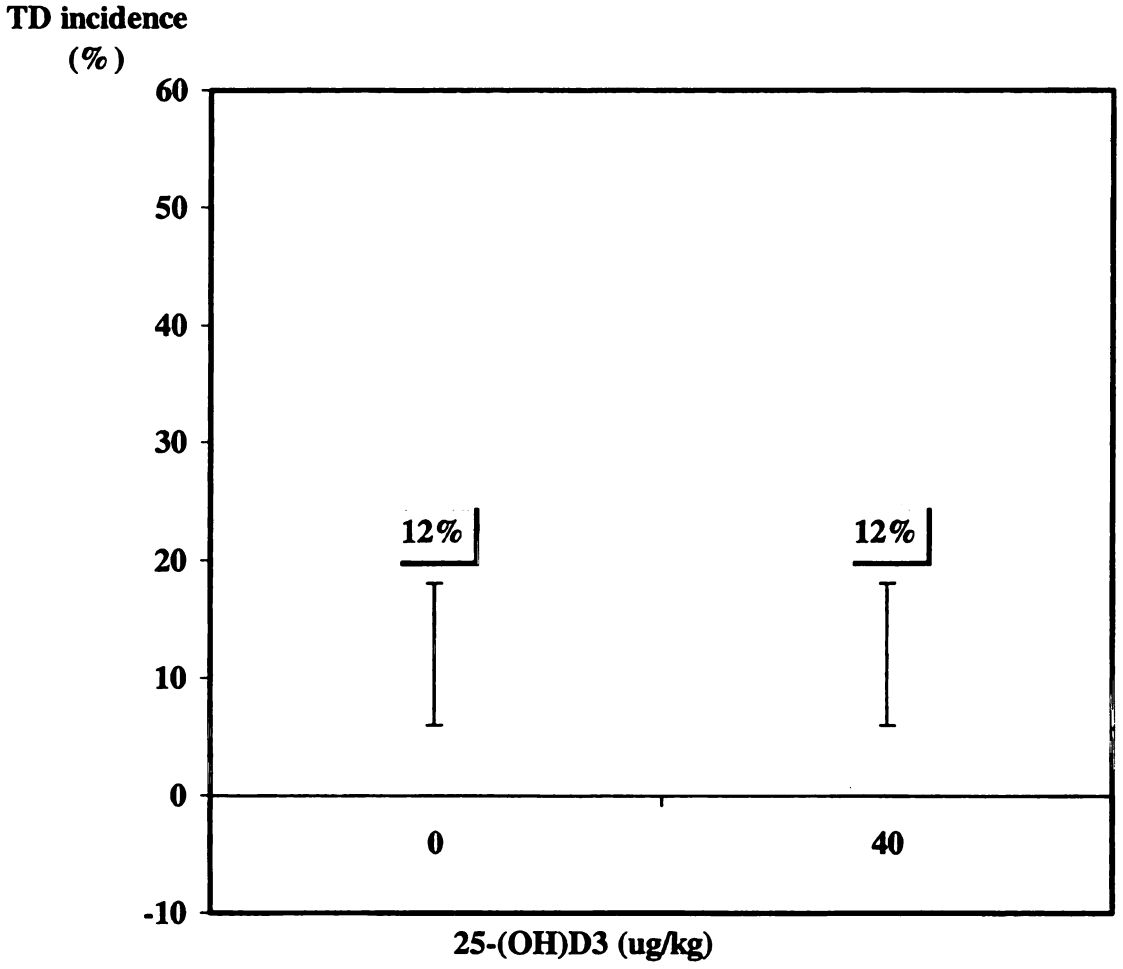

Figure 18. Effects of dietary 25-(OH)D_3 on % phytate phosphorus retention at 35 days of age (Experiment 6, Phase II).

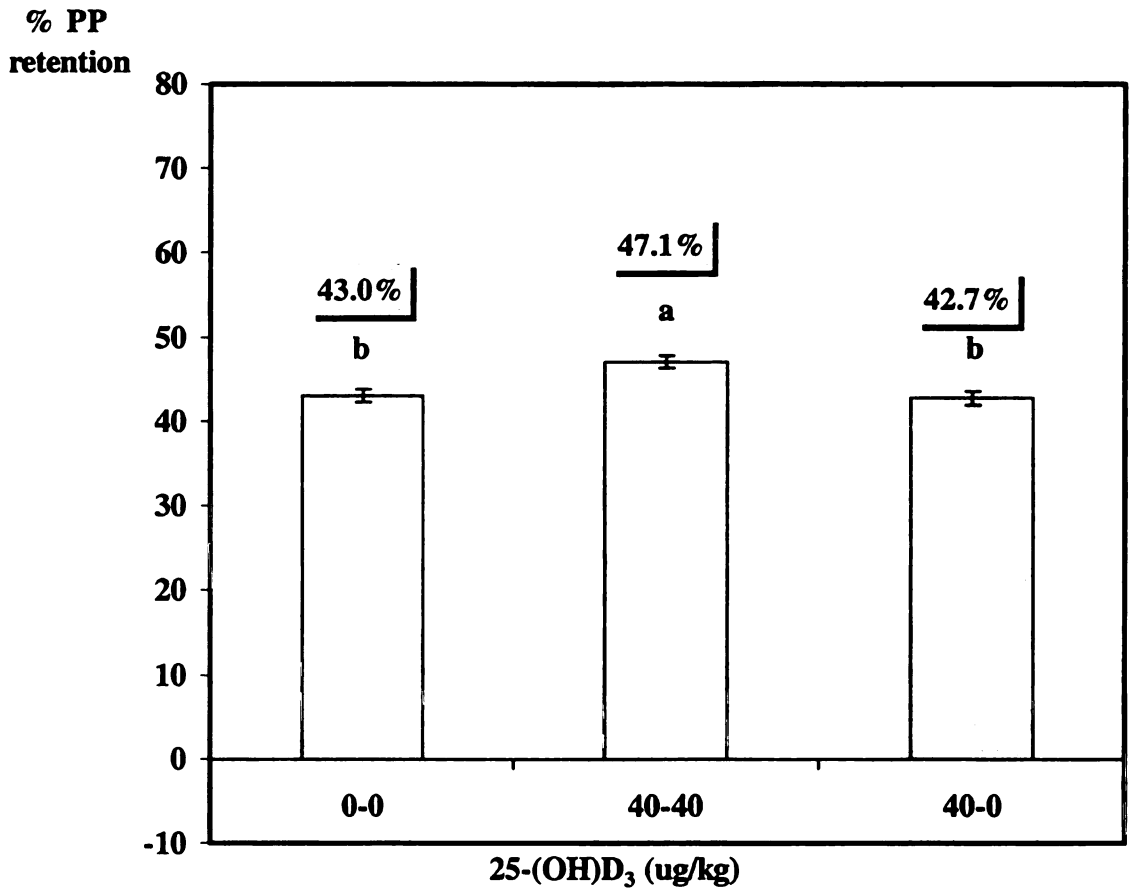

Table 5. Effects of ultraviolet light and 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, and phytate phosphorus retention in 16-day Ross X Ross broiler chicks fed a Vitamin D_3 deficient diet, Experiment 1

Treatments				Serum Concentrations		
25-(OH)D_3	UV light	17-d BW	Gain:feed	Calcium	Phosphorus	Phytate P Retention
(µg/kg)		(g)		mg/dl		(%)
0	no	254[c]	0.545[b]	4.52[b]	9.07[a]	32.9[b]
0	yes	469[b]	0.680[a]	8.23[a]	7.88[b]	56.4[a]
10	no	475[b]	0.696[a]	8.26[a]	7.92[b]	58.0[a]
10	yes	510[ab]	0.673[a]	8.86[a]	7.75[b]	60.5[a]
70	no	562[a]	0.711[a]	8.00[a]	7.61[b]	53.2[a]
70	yes	526[ab]	0.713[a]	7.94[a]	8.56[ab]	51.6[a]
$\bar{x}$		466	0.670	7.64	8.13	52.1
Pooled SE		19	0.019	0.93	0.86	3.1
Main effect means						
25-(OH)D_3						
0		361[c]	0.613[b]	6.37[b]	8.47	44.6[b]
10		492[b]	0.684[a]	8.52[a]	7.85	59.3[a]
70		544[a]	0.712[a]	6.97[ab]	7.14	52.4[ab]
Light						
No		430[b]	0.650[b]	6.26[b]	7.56	48.0[b]
yes		502[a]	0.689[a]	8.30[a]	8.09	56.2[a]
ANOVA						
Source of Variation	df	Probabilities				
25-(OH)D_3	2	<0.001	<0.001	0.095	0.319	0.013
Light	1	<0.001	0.027	0.016	0.496	0.034
25-(OH)D_3 x Light	2	<0.001	<0.001	0.133	0.077	0.021
Regression of 25-(OH)D_3						
Linear		0.002	0.008	0.870	0.181	0.598
Quadratic		0.014	0.076	0.065	0.634	0.030

[a-c] Means in a column with no common superscript differ significantly (P<.05)

Table 6. Effects of ultraviolet light and 25-hydroxycholecalciferol on rickets incidence, development of tibial dyschondroplasia and percentage bone ash in 16-day Ross X Ross broiler chicks fed a Vitamin D_3 deficient diet, Experiment 1.

Treatments		Rickets	Tibial Dyschondroplasia				
25-(OH)D_3	UV light	RI	Incidence	Severity Score 1	Severity Score 2	N3	Bone ash
(µg/kg)		(%)	(%)			(%)	(%)
0	no	93[a]	58[a]	2.19[ab]	1.28[a]	23[ab]	25.2[d]
0	yes	13[b]	42[a]	1.96[ab]	0.78[b]	12[bc]	41.6[c]
10	no	12[b]	56[a]	2.39[a]	1.33[a]	30[a]	41.2[bc]
10	yes	0[b]	15[b]	1.75[ab]	0.23[c]	0[c]	42.5[ab]
70	no	0[b]	15[b]	1.38[bc]	0.20[c]	0[c]	43.1[a]
70	yes	0[b]	7[b]	0.75[c]	0.07[c]	0[c]	42.4[ab]
$\bar{x}$		20	32	1.74	0.65	11	39.3
Pooled SE		4	6	0.29	0.17	5	0.5
Main effects means							
25-(OH)D_3							
0		53[a]	50[a]	2.07[a]	1.03[a]	18[a]	33[b]
10		6[b]	36[b]	2.07[a]	0.78[a]	15[a]	42[a]
70		0[b]	11[c]	1.06[b]	0.14[b]	0[b]	42[a]
Light							
yes		35[a]	43[a]	1.98	0.94[a]	18[a]	36[b]
no		4[b]	21[b]	1.49	0.36[b]	4[b]	42[a]
ANOVA							
Source of Variation	df	Probabilities					
25-(OH)D_3	2	<0.001	<0.001	<0.001	<0.001	0.003	<0.001
Light	1	<0.001	<0.001	0.054	<0.001	0.002	<0.001
25-(OH)D_3 x Light	2	<0.001	0.031	0.737	0.028	0.017	<0.001
Linear		0.016	<0.001	<0.001	<0.001	0.005	0.019
Quadratic		0.008	0.463	0.313	0.858	0.771	0.006

[a-d] Means in a column with no common superscript differ significantly (P<.05)

Table 7. Effects of 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, and phytate phosphorus retention in 17-day Ross X Arbor Acres broiler chicks fed a Vitamin D_3 deficient diet, Experiment 2.

Treatments			Serum Concentrations		
25-(OH)D_3	17-d[1] BW	Gain:feed[1]	Calcium[1]	Phosphorus[1]	Phytate P Retention[1]
(μg/kg)	(g)		mg/dl		(%)
0	415	0.712	10.91	7.71	30.9[c]
10	457	0.727	12.00	7.64	39.6[bc]
40	430	0.692	11.11	6.29	46.4[ab]
70	438	0.698	12.16	6.80	54.7[a]
$\bar{x}$	435	0.007	11.55	7.11	42.9
Pooled SE	13	0.018	0.55	0.58	3.3
Source of Variation			Probabilities		
25-(OH)D_3	0.190	0.532	0.306	0.279	<0.001
Linear	0.761	0.292	0.337	0.138	<0.001
Quadratic	0.620	0.685	0.668	0.259	0.505

[a-c] Means in a column with no common superscript differ significantly (P<.05)
[1]Mean from the analysis of variance

Table 8. Effects of 25-hydroxycholecalciferol on incidence and severity of tibial dyschondroplasia and percentage bone ash in 17-day Ross X Arbor Acres broiler chicks fed a Vitamin D_3 deficient diet, Experiment 2.

Treatment	Tibial Dyschondroplasia				
25-(OH)D_3	Incidence[1]	Severity Score 1[2]	Severity Score 2[3]	Number 3 Score[4]	Bone ash[1]
(μg/kg)	(%)			(%)	(%)
0	73[a]	2.79[a]	2.03[a]	59[a]	41.8[b]
10	26[b]	2.50[a]	0.65[b]	16[b]	43.2[a]
40	5[c]	0.66[b]	0.09[c]	2[c]	43.3[a]
70	2[c]	0.33[b]	0.03[c]	0[c]	43.9[a]
$\bar{x}$	27	1.57	0.70	19	43.1
Pooled SE	4	0.31	0.07	2	0.3
Source of Variation	Probabilities				
25-(OH)D_3	<0.001	<0.001	<0.001	<0.001	<0.001
Linear	<0.001	<0.001	<0.001	<0.001	<0.001
Quadratic	<0.001	0.107	<0.001	<0.001	0.282

[a-c] Means in a column with no common superscript differ significantly (P<.05).
[1]Mean from the analysis of variance.
[2]Total scores ranged from 0 to 3 per pen and increased with increasing severity of TD/total number of birds scored with TD per pen.
[3]Total number of birds scored with TD per pen/ total number of birds with any score from 0 to 3.
[4]Total number of birds scored 3 (severe lesions)/ total number of birds examined at the end of experiment.

Table 9. Effects of 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, and phytate phosphorus retention in 20-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 3.

Treatments			Serum Concentrations		
25-(OH)D_3	20-d[1] BW	Gain:feed[1]	Calcium[1]	Phosphorus[1]	Phytate P[1] Retention
(μg)	(g)		mg/dl		(%)
0	608	0.750	10.38	8.07	34.3[b]
10	617	0.739	10.45	7.47	46.0[a]
40	617	0.737	9.54	8.09	43.1[a]
70	594	0.737	10.44	7.41	30.2[b]
$\bar{x}$	609	0.734	10.20	7.76	38.4
Pooled SE	23	0.017	0.67	0.32	2.03
Source of Variation			Probabilities		
25-(OH)D_3	0.885	0.944	0.725	0.302	<0.001
Linear	0.609	0.653	0.835	0.444	0.083
Quadratic	0.537	0.749	0.332	0.511	<0.001

[a-b] Means in a column with no common superscript differ significantly (P<.05)
[1] Mean from the analysis of variance

Table 10. Effects of 25-hydroxycholecalciferol on incidence and severity of tibial dyschondroplasia and percentage bone ash in 20-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 3.

Treatments	Tibial Dyschondroplasia				
25-(OH)D_3	Incidence[1]	Severity Score 1[2]	Severity Score 2[3]	Number 3 Score[4]	Bone ash[1]
(μg/kg)	(%)			(%)	(%)
0	25[a]	2.47[a]	0.58[a]	13[a]	45.2
10	21[a]	1.71[ab]	0.45[a]	8[ab]	45.0
40	13[ab]	2.58[a]	0.32[ab]	8[ab]	45.1
70	5[b]	1.00[b]	0.09[b]	0[b]	45.3
$\bar{x}$	16	1.94	0.36	7	45.2
Pooled SE	5	0.34	0.32	3	0.51
Source of Variation	Probabilities				
25-(OH)D_3	0.038	0.011	0.019	0.019	0.970
Linear	0.003	0.059	<0.001	0.004	0.756
Quadratic	0.860	0.101	0.931	0.579	0.736

[a-b] Means in a column with no common superscript differ significantly (P<.05).
[1]Mean from the analysis of variance.
[2]Total scores ranged from 0 to 3 per pen and increased with increasing severity of TD/total number of birds scored with TD per pen.
[3]Total number of birds scored with TD per pen/ total number of birds with any score from 0 to 3 per pen.
[4]Total number of birds scored 3 (severe lesions)/ total number of birds examined at the end of experiment.

Table 11. Effects of 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, and phytate phosphorus retention in 20-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 4.

Treatments			Serum Concentrations		
25-(OH)D_3	20-d[1] BW	Gain:feed[1]	Calcium[1]	Phosphorus[1]	Phytate P Retention[1]
(μg/kg)	(g)		mg/dl		(%)
0	652	0.701	10.63	7.74	44.2[b]
18	675	0.700	10.86	9.14	45.9[ab]
36	651	0.692	10.81	8.04	50.2[a]
54	675	0.703	10.65	8.30	45.0[b]
72	694	0.745	10.56	7.22	45.6[ab]
90	704	0.712	9.95	7.80	45.9[ab]
$\bar{x}$	675	0.709	10.58	8.04	46.1
Pooled SE	27	0.019	0.29	0.5	1.3
Source of Variation			Probabilities		
25-(OH)D_3	0.695	0.443	0.599	0.271	0.083
Linear	0.177	0.039	0.270	0.419	0.687
Quadratic	0.418	0.427	0.268	0.278	0.086

[a-d] Means in a column with no common superscript differ significantly (P<.05)
[1] Mean from the analysis of variance

Table 12. Effects of 25-hydroxycholecalciferol on incidence and severity of tibial dyschondroplasia and percentage bone ash in 20-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 4.

	Tibial Dyschondroplasia				
$25\text{-(OH)}D_3$	Incidence[1]	Severity Score 1[1]	Severity Score 2[2]	Number 3 Score[3]	Bone ash[1]
(µg/kg)	(%)			(%)	(%)
0	13	1.33	0.33	10	43.1
18	5	0.75	0.08	0	43.6
36	5	1.25	0.07	2	43.4
54	8	1.13	0.19	3	43.6
72	13	1.75	0.28	5	43.4
90	10	2.00	0.20	3	43.7
$\bar{x}$	9	1.37	0.19	4	43.4
Pooled SE	5	0.63	0.11	3	0.37
Source of Variation	Probabilities				
$25\text{-(OH)}D_3$	0.758	0.771	0.537	0.358	0.887
Linear	0.744	0.642	0.996	0.592	0.955
Quadratic	0.715	0.928	0.709	0.602	0.124

[1]Mean from the analysis of variance.
[2]Total scores ranged from 0 to 3 per pen and increased with increasing severity of TD/total number of birds scored with TD per pen.
[3]Total number of birds scored with TD per pen/ total number of birds with any score from 0 to 3 per pen.
[4]Total number of birds scored 3 (severe lesions)/ total number of birds examined at the end of experiment.

Table 13. Effects of 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, and phytate phosphorus retention in 20-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 5.

Treatments			Serum Concentrations		
25-(OH)D_3	20-d[1] BW	Gain:feed[1]	Calcium[1]	Phosphorus[1]	Phytate P[1] Retention
(μg/kg)	(g)		mg/dl		(%)
0	688	0.759[ab]	9.47	7.06	50.7
18	673	0.729[b]	9.82	7.63	55.8
36	708	0.741[ab]	9.75	7.43	51.2
54	672	0.763[a]	10.42	7.62	51.5
72	697	0.772[a]	9.67	7.69	49.4
90	681	0.772[a]	10.72	7.65	52.6
$\bar{x}$	687	0.748	9.98	7.51	51.9
Pooled SE	28	0.01	0.47	0.43	2.3
Source of Variation			Probabilities		
25-(OH)D_3	0.946	0.038	0.419	0.898	0.500
Linear	0.988	0.030	0.104	0.327	0.631
Quadratic	0.853	0.172	0.860	0.600	0.860

[a-b] Means in a column with no common superscript differ significantly (P<.05)
[1] Mean from the analysis of variance

Table 14. Effects of 25-hydroxycholecalciferol on incidence and severity of tibial dyschondroplasia and percentage bone ash in 20-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 5.

Treatments	Tibial Dyschondroplasia				
25-(OH)D_3	Incidence[1]	Severity Score 1[1]	Severity Score 2[2]	Number 3 Score[3]	Bone ash[1]
(μg/kg)	(%)			(%)	(%)
0	22	1.63	0.35	3	43.3[b]
18	15	1.08	0.23	3	43.3[b]
36	10	0.50	0.20	3	44.2[a]
54	8	1.75	0.17	2	43.6[ab]
72	18	1.96	0.34	5	43.7[ab]
90	12	1.75	0.27	7	43.8[ab]
$\bar{x}$	14	1.45	0.26	4	43.7
Pooled SE	6	0.49	0.11	3	0.23
Source of Variation	Probabilities				
25-(OH)D_3	0.567	0.333	0.841	0.658	0.133
Linear	0.369	0.298	0.921	0.124	0.212
Quadratic	0.215	0.306	0.324	0.323	0.214

[a-b] Means in a column with no common superscript differ significantly (P<.05).

[1] Total scores ranged from 0 to 3 per pen and increased with increasing severity of TD/total number of birds scored with TD per pen.

[2] Total number of birds scored with TD per pen/ total number of birds with any score from 0 to 3.

[3] Total number of birds scored 3 (severe lesions)/ total number of birds examined at the end of experiment.

Table 15. Effects of 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, and phytate phosphorus retention in 17-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 6.

Treatments				
25-(OH)D_3		17-d[1] BW	Gain:feed[1]	Phytate P[1] Retention
	(μg/kg)	(g)		(%)
Starter	0	505	0.772	42.3
Starter	40	496	0.784	40.3
	$\overline{x}$	501	0.778	41.3
Pooled SE		11	0.008	1.8
Source of Variation		Probabilities		
25-(OH)D_3		0.609	0.355	0.371

[1] Mean from the analysis of variance
[2]SEM from the analysis of variance

Table 16. Effects of 25-hydroxycholecalciferol on incidence and severity of tibial dyschondroplasia and percentage bone ash in 17-day Ross X Ross broiler chicks fed a normal broiler starter diet, Experiment 6.

Treatments		Tibial Dyschondroplasia				
25-(OH)D_3	Incidence[1]	Severity Score 1[3]	Severity Score 2[4]	Number 3 Score[5]	Bone ash[1]
(µg/kg)	(%)			(%)	(%)
Starter 0	12	1.38	0.22	2	44.2
Starter 40	12	1.19	0.24	5	43.7
$\overline{x}$	12	1.29	0.23	4	43.0
Pooled SE	3	0.36	0.08	3	0.2
Source of Variation	Probabilities				
25-(OH)D_3	0.997	0.704	0.880	0.555	0.187

[a-c] Means in a column with no common superscript differ significantly (P<.05).
[1] Mean from the analysis of variance.
[2] SEM from the analysis of variance.
[3] Total scores ranged from 0 to 3 per pen and increased with increasing severity of TD/total number of birds scored with TD per pen.
[4] Total number of birds scored with TD per pen/ total number of birds with any score from 0 to 3.
[5] Total number of birds scored 3 (severe lesions)/ total number of birds examined at the end of experiment.

Table 17. Effects of 25-hydroxycholecalciferol on performance, serum calcium and phosphorus, phytate phosphorus retention and incidence and severity of leg-weakness in 35-day Ross X Ross broiler chicks fed a normal broiler grower diet, Experiment 6.

Treatments				Leg weakness		
25-(OH)D_3	35-d[1] BW	Gain:feed[1]	Phytate P[1] Retention	Incidence[1]	Severity Score[3]	Number 3 Score[4]
(μg/kg)	(g)		(%)	(%)		(%)
Starter 0-0 Grower	1857	0.624	43.0[b]	39	1.04	18
Starter 40-40 Grower	1847	0.631	47.1[a]	54	1.14	14
Starter 40-0 Grower	1809	0.621	42.7[b]	37	1.07	15
$\bar{x}$	1838	0.625	44.3	43	1.08	16
Pooled SE	30	0.011	0.7	9	0.25	7
Source of Variation	Probabilities					
25-(OH)D_3	0.526	0.464	0.004	0.401	0.953	0.930

[a-b] Means in a column with no common superscript differ significantly (P<.05).
[1] Mean from the analysis of variance.
[2] SEM from the analysis of variance.
[3] Total scores ranged from 0 to 3 per pen and increased with increasing severity of leg weakness/total number of birds scored with leg weakness per pen.
[4] Total number of birds scored 3 (severe leg weakness)/ total number of birds examined at the end of experiment.

Table 18. Effects of 25-hydroxycholecalciferol on incidence and severity of tibial dyschondroplasia and percentage bone ash in 35-day Ross X Ross broiler chicks fed a normal broiler grower diet, Experiment 6.

Treatments	Tibial Dyschondroplasia				
25-(OH)D_3	Incidence[1]	Severity Score 1[3]	Severity Score 2[4]	Number 3 Score[5]	Bone ash[1]
(μg/kg)	(%)			(%)	(%)
Starter 0-0 Grower	0	0	0	0	43.4
Starter 40-40 Grower	7	0.75	0.22	7	43.6
Starter 40-0 Grower	0	0	0	0	43.4
$\bar{x}$	2	0.25	0.08	2	43.5
Pooled SE	4	0.43	0.06	4	0.3
Source of Variation	Probabilities				
25-(OH)D_3	0.405	0.405	0.405	0.405	0.846

[1] Mean from the analysis of variance.
[2] SEM from the analysis of variance.
[3] Total scores ranged from 0 to 3 per pen and increased with increasing severity of TD/total number of birds scored with TD per pen.
[4] Total number of birds scored with TD per pen/ total number of birds with any score from 0 to 3.
[5] Total number of birds scored 3 (severe lesions)/ total number of birds examined at the end of experiment.

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