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Histology Provides the Best Diagnosis of Tibial Growth Plate Defects Caused by Nutrition

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Abstract: Three experiments examined the effects of nutrition on the development of the tibia in broiler chicks. One experiment used 0.05% increments of Nonphytate Phosphorus (NPP) from 0.25 to 0.50% of the diet. Chicks fed 0.35% NPP or less had lower plasma P and lower bone ash than those fed 0.40% or more NPP. Growth plates from P deficient chicks were enlarged. Microscopic examination of the growth plates revealed blood vessel penetration of the hypertrophic zone but failure to remove the chondrocytes. The Ca experiment used 0.1% increments from 0.6 to 1.1% of the diet. These treatments did not affect plasma Ca or bone ash. Chicks fed 1.1 or 1.0% Ca had a low incidence of enlarged growth plates. Diets with 0.9% Ca or less caused more than half of the chicks to have growth plates longer than 2 mm. Most of the enlarged growth plates were classified as Tibial Dyschondroplasia (TD), but some from the 0.6 and 0.7% Ca diets were classified as rickets. In both defects, metaphyseal blood vessels failed to penetrate the growth plate. The accumulated cells in TD had progressed through hypertrophy but had regressed to the size and shape of prehypertrophic chondrocytes. In rickets, the accumulated cells were proliferative. The basal vitamin D diet had 165 ICU kg^{-1}. Vitamin D was increased in five other diets by multiples of 1.81. Dietary vitamin D content had no effect on bone ash or on plasma Ca or P content. If chicks were fed 992 ICU kg^{-1} or less, the proportion of chicks with enlarged growth plates was increased. The defect was classified as TD and appeared to be identical to that resulting from a Ca deficiency. The development of the blood vessels in the growth plate seems to be the most useful criterion for deciding if an enlarged growth plate is due to a Ca or P deficiency.

Key words: Calcium, growth plate, phosphorus, rickets, tibial dyschondroplasia, vitamin D

INTRODUCTION

A small percentage of broilers develop skeletal problems that cause an economic loss estimated at $80 to $120 million per year[1]. Of this amount, a large proportion is due to leg abnormalities. Mortality and morbidity result when broilers are unable to walk well enough to get proper amounts of feed and water. Additional losses occur because parts must be trimmed if birds receive injuries as a result of leg problems. Product is downgraded if bones break when broilers are placed in shackles on the processing line.

Calcium, vitamin D and P are important nutrients for the development of the growth plate in long bones. Deficiencies of these nutrients cause a failure in the maturation and removal of chondrocytes from the growth plate. A P deficiency results in an enlarged growth plate because hypertrophic cells are not removed as rapidly as they are produced[2,3]. A severe Ca deficiency results in an enlarged growth plate because proliferative cells fail to mature to hypertrophic cells for removal[4].

A third abnormality of the growth plate is Tibial Dyschondroplasia (TD). In this condition, most of the chondrocytes proliferate and then hypertrophy[5,6]. Rather than maintaining the hypertrophic state for removal after apoptosis, the chondrocytes shrink to the size and shape characteristic of prehypertrophic cells. An accumulation of these chondrocytes results in TD.

Dietary variables affect the incidence of TD. The National Research Council (1994) lists the requirement for Ca as 1.0%, for nonphytate phosphorus (NPP) as 0.45%, and for vitamin D as 200 ICU per kg for broilers to 21 d of age. Calcium appears to be a prominent nutrient for affecting the incidence of TD. Lowering dietary Ca while maintaining NPP caused a higher incidence of TD[7-9]. Maintaining Ca while increasing NPP also increases incidence of TD[10]. Increasing other anions, such as chloride and sulfate on a milliequivalent basis equal to that of phosphate, also increased TD. Varying dietary vitamin D concentrations alters the incidence and severity of TD[7,9,11,12].

This report summarizes the results of titration experiments using dietary Ca, NPP and vitamin D. One objective was to illustrate the growth plate defects caused by these nutrient deficiencies. A second objective was to present an easy way to diagnose the nutrient that caused

Table 1: Composition (%) of the basal diets for the phosphorus, calcium and vitamin D experiments

	Experiment		
Ingredient	P	Ca	Vit D
Corn	55.27	54.72	54.43
Soybean meal	37.54	37.54	37.54
Hydrolyzed fat	5.00	5.00	5.00
Dicalcium phosphate	0.66	1.73	1.73
Limestone	0.78	0.26	0.55
Salt	0.40	0.40	0.40
D,L-methionine	0.20	0.20	0.20
Vitamin premix[1]	0.10	0.10	0.10
Trace mineral premix[2]	0.05	0.05	0.05
Calculated content (%)			
Protein	23.04	23.05	23.04
Lysine	1.23	1.23	1.23
Met + Cys	0.92	0.92	0.92
NPP	0.25	0.45	0.45
Calcium	0.55	0.60	0.70

[1]Provided per kg diet: retinyl palinitate, 6000 IU; cholecalciferol, 300 ICU; D,L-α-tocopherol acetate, 10 IU; menadione sodium bisulfite, 1 mg; thiamin, 1.8 mg; riboflavin, 3.6 mg; niacin, 25, 0 mg; pantothenic acid, 10.0 mg; pyridoxine, 3.5 mg; folacin, 0.5 mg; biotin, 0.15 mg; choline, 500 mg and ethoxyquin, 50 mg. [2]Provided per kg diet: copper, 8mg; iron, 80 mg; manganese, 60 mg; selenium, 0.1 mg and zinc, 40 mg

the growth plate defect. A third objective was to determine the relative ability of these nutrients to cause growth plate defects.

MATERIALS AND METHODS

General: Three chick experiments are reported, one with varying amounts of dietary NPP, another with varying amounts of dietary Ca and a final experiment with varying amounts of vitamin D. All of the chicks came from eggs produced by one broiler breeder flock that was housed in a light-controlled room. When the pullets began egg production, the breeder feed was formulated to contain 600 ICU vitamin D per kg. The flock reached approximately 80% egg production when they were fed 140 g/bird/d. Feed consumption was limited to 140 g/bird/d during the time that eggs were collected for experiments. The feed, a corn-soy diet, was calculated to contain 3.0 Ca, 0.3 NPP and 16% protein.

For the chick experiments, enough of the ingredients was purchased at one time to meet the needs for all experimental diets. The ingredients were stored at –20° C until needed for mixing. Diets for each experiment were prepared from a base mix that was 95% of the final mix and was used for all diets. The other 5% of the diets was used to provide the remaining corn, dicalcium phosphate, limestone or vitamin D premix. After mixing, diets were stored at –20° C until needed.

Phosphorus experiment: Diets were formulated to contain 0.25, 0.30, 0.35, 0.40, 0.45 or 0.50% NPP (Table 1) based on National Research Council[13] composition. The following amounts of dicalcium phosphate and limestone replaced corn to provide NPP as indicated and Ca at 0.85% of the diet; 0.30% NPP, 0.27% dicalcium phosphate and 0.62% limestone; 0.35% NPP, 0.53 dicalcium phosphate and 0.46% limestone; 0.40% NPP, 0.80% dicalcium phosphate and 0.31% limestone; 0.45% NPP, 1.07% dicalcium phosphate and 0.15% limestone; 0.50% NPP, 1.34% dicalcium phosphate. Each diet contained 300 ICU vitamin D per kg. Three replicates of chicks were fed each diet, with replication on successive weeks. Each replicate pen consisted of eight d-old chicks that were randomly allotted to battery brooder pens. The length of the experiment was 19 d. For the first nine d, the incandescent bulb in the brooder pens was used. After that, the only light that was available was from ceiling incandescent lights.

At 19 d of age, each chick was weighed individually. Blood was collected by heart puncture into a heparinized tube. After separation, the plasma was frozen until used for a blood chemistry profile. Chicks were then killed by cervical dislocation. Both tibias were removed and tissue was removed from the bone. The proximal end of the tibia was sectioned longitudinally with a razor blade and the visual appearance was noted. Following observation, the left tibia was placed in 10% formalin for two days, then demineralized in a dilute acid solution of one part 6N acetic acid and one part 20% citric acid for two d. A slide was prepared using standard procedures and the tissue was stained using hematoxylin and eosin (H + E).

The right tibia was used to determine bone mineralization using bones from chicks in only the second and third replicates. The bone was weighed after soft tissue was removed. Bones were then dried at 80°C and weighed again. Fat was extracted in a Soxhlet apparatus using petroleum ether for 16 h. After drying and weighing again, bones were ashed at 550°C overnight.

Calcium experiment: Experimental diets were calculated to contain 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1% Ca. Each increment of dietary Ca was provided by replacing 0.26% corn with limestone (Table 1). All diets were calculated to contain 0.45% NPP and 300 ICU vitamin D per kg. All procedures were identical to those in the P experiment except that each pen had 10 chicks. One additional measurement was the length of the growth plate when the left tibia was sectioned.

Vitamin D experiment: The basal diet was calculated to contain 165 ICU vitamin D per kg, 0.70% calcium and 0.45% NPP. In addition, 300, 545, 992, 1803 and 3278 ICU of vitamin D per kg were fed. All other procedures were the same as for the Ca experiment.

Table 2: Analyzed values for the nutrient variables in the phosphorus, calcium and vitamin D experiments

Phosphorus experiment						
Calculated NPP (%)	0.25	0.30	0.35	0.40	0.45	0.50
Analyzed total P (%)	0.50	0.55	0.61	0.64	0.68	0.72
Calcium experiment						
Calculated Ca (%)	0.60	0.70	0.80	0.90	1.00	1.10
Analyzed Ca (%)	0.68	0.80	0.89	1.01	1.10	1.21
Vitamin D experiment						
Calculated vitamin D (ICU kg^{-1})	3218					
Analyzed vitamin D (ICU kg^{-1})	2885					
Calculated Ca (%)	0.70					
Analyzed Ca (%)	0.81					
Calculated NPP (%)	0.45					
Analyzed total P (%)	0.69					

Table 3: Blood parameters affected by levels of dietary NPP (n=3)

Diet NPP	Blood P	Anion Gap	AST[1]	Bili-rubin	Total Protein
%	mg/dL	mEq/L	U/L	g/dL	mg/dL
0.25	3.38^{d}	29.8^{a}	228^{a}	1.5^{a}	2.9^{a}
0.30	3.79^{cd}	23.4^{b}	178^{b}	1.1^{b}	2.7^{ab}
0.35	4.38^{c}	25.2^{b}	182^{b}	1.1^{b}	2.6^{bc}
0.40	6.36^{b}	26.9^{ab}	149^{c}	0.8^{c}	2.4^{c}
0.45	7.05^{a}	29.9^{a}	160^{bc}	0.9^{c}	2.5^{bc}
0.50	7.01^{a}	26.2^{a}	149^{c}	0.7^{c}	2.5^{bc}
SEM	0.22	1.57	9.0	0.09	0.09
P	0.0001	0.02	0.0003	0.0001	0.01

$^{a-d}$Means in a column with different letters are significantly different ($p<0.05$). [1]Aspartic transaminase

Diet analyses: Diets were analyzed for experimental nutrients (Table 2). Total P content was analyzed by Inductively Coupled Plasma (ICP), Ca was analyzed by Atomic Absorption Analytical Spectrometry and vitamin D content was determined.

Statistical analyses: The design of each experiment was a completely randomized block, with experiments replicated over time. Data were analyzed using SAS[14] hen main effects were significant, means were separated by Least Significant Difference with $p \leq 0.05$.

RESULTS

Phosphorus experiment: Chicks that were fed only 0.25% NPP, 55% of the NRC[13] requirement, developed a severe deficiency. Blood P was reduced (Table 3) by low dietary P and increased to a maximum level when chicks were fed 0.45% NPP. Anion gap, which is the difference between the cations Na and K and the anions Cl and HCO_3, was also affected by dietary P. At 0.25% NPP, chicks had a large anion gap. Chicks fed 0.30 or 0.35% NPP had a decreased anion gap, while more dietary NPP raised the anion gap.

Three other blood indicators increased when dietary NPP was very low (Table 3). They were aspartic transaminase, bilirubin and total protein. All of the indicators reached a plateau with 0.40% dietary NPP. Other important blood minerals that were not affected by treatment, with means for the experiment, were: Ca 11.2 mg/dL; Na, 158.1 mEq/L; K, 5.4 mEq/L and Cl, 117.4 mEq/L.

Table 4: Body weight and bone measurements as affected by dietary NPP (n=3)

Diet NPP	Body Wt	Wet Tibia	Dry bone ÷ wet bone	Fat-free bone ÷ wet bone	Ash
%	g	g			%
0.25	433^{c}	6.22	0.302^{c}	0.224^{c}	0.234^{c}
0.30	515^{b}	6.26	0.324^{bc}	0.257^{c}	0.274^{bc}
0.35	508^{b}	6.35	0.321^{bc}	0.277^{bc}	0.292^{ab}
0.40	587^{a}	6.43	0.352^{a}	0.307^{ab}	2.332^{a}
0.45	573^{ab}	6.44	0.344^{ab}	0.295^{ab}	0.319^{ab}
0.50	620^{a}	6.17	0.350^{a}	0.311^{a}	0.319^{ab}
SEM	31.2	0.76	0.007	0.012	0.015
P	0.02	0.99	0.02	0.03	0.03

$^{a-c}$Means in a column with different letters are significantly different ($p<0.05$)

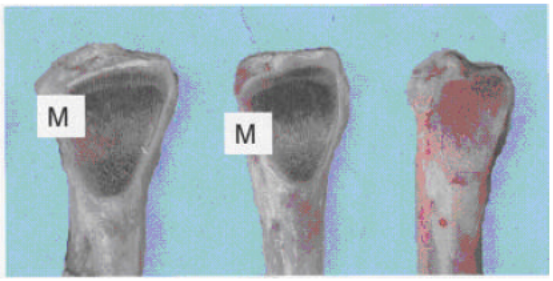

Fig. 1: A sectioned tibia from a chick fed the required amount of NPP, shown on the left, has a thin growth plate that extends to the area of Mineralization (M). When fed a moderately deficient diet, a chick has a longer growth plate (center). The tibia from a chick fed a diet very deficient in NPP, shown on the right, has no distinct zones and fails to flair

Body weight was lower when dietary NPP was below 0.40% (Table 4), but tibia weight was not affected by treatment. During the experiment, several of the chicks fed 0.25% NPP had difficulty walking. The tibia from deficient chicks contained more moisture, as indicated by the dry bone:wet bone ratio. The effect was also evident in bones that were extracted with ether. Percent ash in ether-extracted bones followed a similar pattern.

When tibias were sectioned to examine the growth plate, differences due to P status were evident (Fig. 1). Bones from chicks slightly deficient in NPP had a darker appearance, an enlarged growth plate with less distinct zones and a growth plate that oozed blood. Bones from the most deficient chicks were pale, had no distinct zones and failed to flare, which is a decrease in diameter from the cartilaginous end of the joint to the cortex of the bone.

Calcium experiment: Few differences occurred in response to the dietary calcium levels. Body weight was

Table 5: Differences resulting from dietary calcium levels (n=3)

Diet Ca	Body Wt	Creat-inine	Chole-sterol	Anion Gap
% g	mg/dL	mg/dL	mEq/L	
0.6	594	0.23^{b}	131^{b}	26.2^{a}
0.7	562	0.23^{b}	147^{a}	25.7^{a}
0.8	551	0.22^{b}	135^{b}	23.1^{ab}
0.9	558	0.26^{a}	138^{ab}	23.2^{ab}
1.0	634	0.21^{b}	136^{b}	20.5^{b}
1.1	658	0.22^{b}	128^{b}	23.5^{ab}
SEM	35.7	0.009	3.4	1.11
P	0.26	0.03	0.04	0.05

^{ab}Means in a column with different letters are significantly different ($p<0.05$)

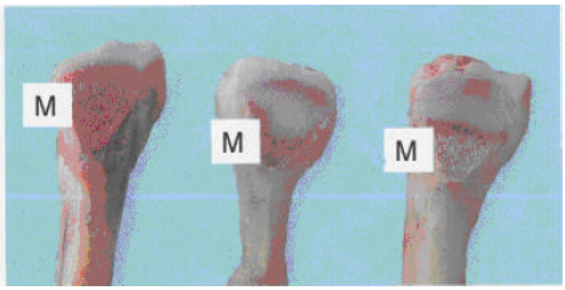

Fig. 2: Sectioned tibias show the effects of calcium nutrition. The bone on the left is from a chick with adequate dietary calcium. Chondrocytes mature rapidly, producing a thin growth plate and are replaced by Mineral (M). Chicks fed a moderately calcium deficient diet tend to develop Tibial Dyschondroplasia (TD) (center). The white cartilage plug is the prominent feature. A pink layer is visible within the cartilage plug and an irregular red edge outlines the distal end of the cartilage plug. When fed more severely deficient calcium diets, some chicks develop rickets (right tibia). Rickets is indicated by a cartilage mass that is slightly gray in appearance, has a proximal pink layer that is less distinct than found in TD and has a smooth red edge that lines the distal end of the cartilage

not affected by dietary Ca from 0.6 to 1.1% (Table 5). Differences in blood chemistry were present for creatinine, cholesterol and anion gap. Means of other important blood indicators that were not affected by dietary Ca were: Ca, 10.5 mg dL^{-1} and P, 5.9 mg dL^{-1}.

Tibia weights were not affected by treatment, averaging 7.00 g per bone. No differences were evident after drying, ether extraction or ashing.

The appearance of tibial growth plates was affected by treatment. Chicks fed 1.1 or 1.0% Ca had growth plates that were usually 2 mm or less in length (Fig. 2). Treatments with 0.9%

Ca or less had an increased number of growth plates longer than 2 mm: 1.1% Ca, 7/30: 1.0% Ca, 4/30; 0.9% Ca,

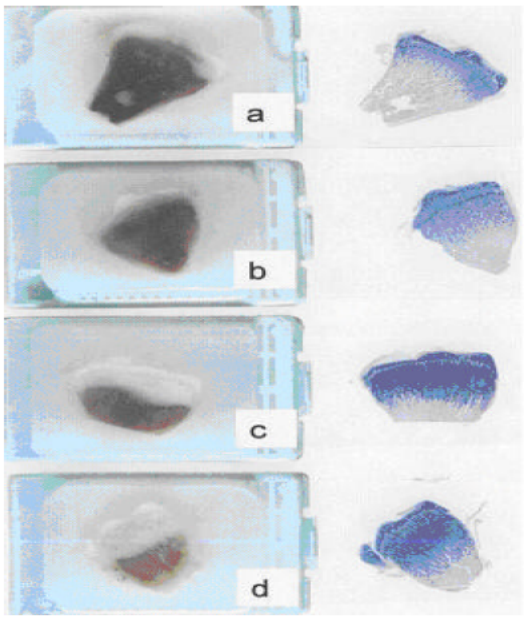

Fig. 3: Sectioned tibias embedded in paraffin blocks are shown after they were fixed with formalin and demineralized. A section of each tibia is shown to the right, after placement on a microscope slide and staining with H and E. Block a shows a normal growth plate, all of it dark except for the articular cartilage. After staining with H and E, a narrow band of chondrocytes stains dark blue. Block b shows the tibia from a NPP deficient chick. It is dark, like the normal tibia. After staining, a thin band is dark blue, followed by a thicker band that is dark blue but porous in appearance. Blocks c and d both have large areas that are white after fixation and demineralization. After staining with H and E, the areas that were white in the blocks stain an intense dark blue

17/30; 0.8% Ca, 17/29; 0.7% Ca, 22/30 and 0.6% Ca, 22/30. From the 0.9% and 0.8% Ca treatments, all of the enlarged growth plates were grossly classified as TD. From the 0.7 and 0.6% Ca treatments, most of the growth plates were classified as TD, but several were classified as rickets.

Table 6: Body weight and blood chemistry changes caused by various levels of dietary vitamin D (n=3)

Vitamin D	Body Wt	K	Na
ICU kg^{-1}	g		mEq/L
mEq/L			
165	663	5.43^{ab}	154.0^{ab}
300	621	5.52^{a}	153.9^{ab}
545	635	4.91^{c}	153.0^{b}
992	669	4.93^{c}	154.0^{ab}
1803	678	5.10^{bc}	155.9^{ab}
3278	700	5.05^{bc}	156.8^{a}
SEM	27.3	0.13	1.1
P	0.40	0.02	0.03

$^{a-c}$Means in a column with different letters are significantly different

(p<0.05)

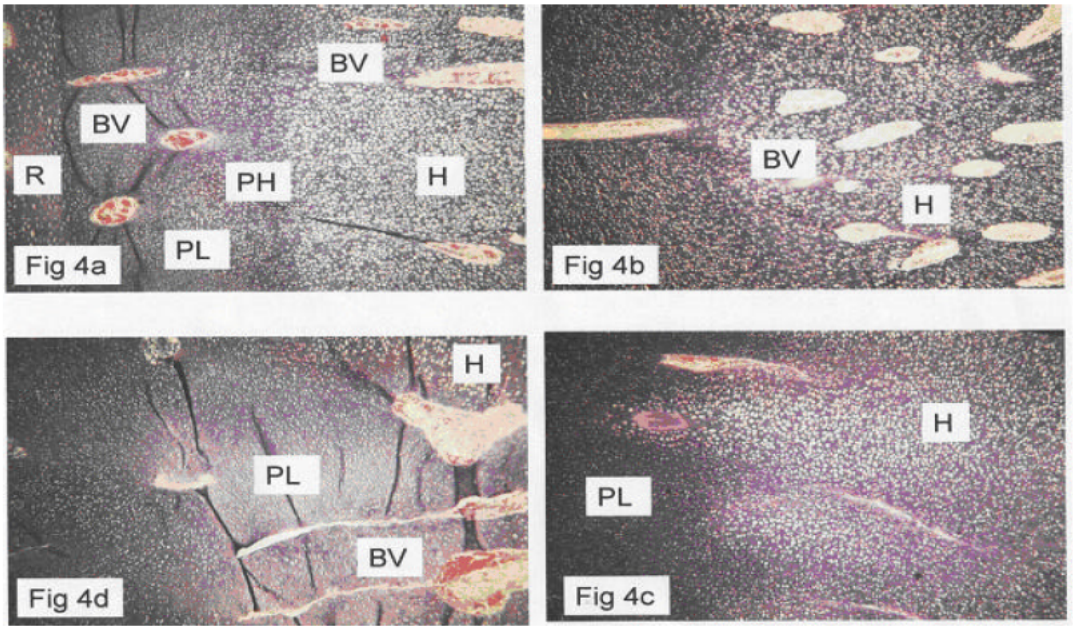

Fig. 4: Photographs from the slides shown in Fig. 3. Fig 4a shows a normal growth plate where BV is blood vessels, H is zone of hypertrophy, PH is zone of prehypertrophy, PL is zone of proliferation and R is resting zone. The growth plate is thin enough to view all of these features in one field (40x). Figure 4b is the tibia from a NPP deficient chick. BV are more prominent in H and appear to weave through H. Figure 4c shows the tibia from a Ca deficient chick with TD. H appears to take place in most chondrocytes, but they eventually regress in size. BV are absent from H. Figure 4d shows rickets. Chondrocytes that accumulate are mostly PL, though some H are visible. BV are absent from the PL and those at the border of the PL are enlarged or blunt

The distinction was based on the border between the chondrocytes and the mineralized area. Those that had an irregular border were listed as TD, but those that had a straight border were listed as rickets.

Vitamin D experiment: Vitamin D levels in the experiment did not affect chick weight or any of the bone measurements, including ash. Only the Na and K concentrations, of the blood parameters, were altered by dietary treatment. Checks with growth plates longer than 2 mm were as follows: 165 ICU kg^{-1}, 13/30; 300 ICU kg^{-1}, 14/30; 545 ICU kg^{-1}, 10/30; 992 ICU kg^{-1}, 9/30; 1803 ICU kg^{-1}, 1/30; 3278 ICU kg^{-1}, 2/30. Of the growth plates 2 mm or longer, all had the appearance of TD (Table 6).

Histology: Samples of the tibia embedded in paraffin as shown in Fig. 3. Block a had a narrow growth plate and was dark in appearance. After sectioned and stained with H + E, a narrow band of chondrocytes stained purple. Block b was dark and after staining, had a longer, but porous, purple appearance. It was from a chick deficient in NPP. Block c had been classified as TD. When embedded in paraffin, it had a large white growth plate with an irregular border. Staining left a large area with a dense purple color. Block d had been classified as rickets. When stained, the white area in the block became a dense purple color.

Figure 4 shows photographs of the various growth plates using 40x magnification. With the chicks used in this research, all of the stages of chondrocytes in the growth plate of a normal tibia could be seen in one field (Fig. 4a). Chondrocytes in the Resting zone (R), zone of proliferation (PL), zone of prehypertrophy (PH) and Hypertrophy (H) are visible. Blood Vessels (BV) from the epiphysis and metaphysis are labeled. Those from the metaphysis were pointed and generally parallel to the plane of the section. A photograph of the growth plate from a chick deficient in NPP is in Figure 4b. Hypertrophy of the chondrocytes appeared to be normal, but failure to remove the chondrocytes resulted in a lengthened cartilage area. Blood vessels extend the length of the zone of H. They approached the zone of PH more closely than occurs in a normal growth plate. Their appearance suggests that blood vessels weave up and down relative

to the plane of the section. Figure 4c is the photograph of a growth plate with TD. Blood vessels are absent from the zone of H. Most of the chondrocytes from PL go through H but then decrease in size when they remain in the growth plate for a longer time than they should. Fig. 4d shows a growth plate with rickets. The enlarged part of the growth plate contains PL chondrocytes. Blood vessels from the metaphysis are blunted and concentrated along the border between the chondrocytes and mineralized area, similar to their condition in TD. An area is visible that contains H chondrocytes. The zone of PL appeared to shrink during the slide preparation, resulting in dark-staining lines.

DISCUSSION

Establishing the cause of a growth plate defect is often difficult. Results from the present research provide information that helps distinguish between a problem with Ca or P in the growth plate. These broilers had no abnormality at 19 d of age if the growth plate was 2 mm or less. When the growth plate was enlarged, observation of the sectioned tibia provided additional information. If the enlarged area was white with a distinct reddish border, the problem was caused by a deficiency of Ca or vitamin D (Fig. 2 and 4). Whether the excess cartilage was due to an accumulation of PL stage chondrocytes, which is rickets, or to an accumulation of further differentiated chondrocytes, which is TD, could only be determined accurately by microscopic examination. Our results suggest that TD results from a mild Ca deficiency in the growth plate, while rickets results from a more severe Ca deficiency. The presence of both conditions in chicks from one experimental treatment has been noted previously[4,15].

Chicks with a moderate NPP deficiency had a lengthened growth plate that was dark In appearance (Fig. 1). Growth plates from chicks with a more severe NPP deficiency had little definition of the zones. For these, observation of the growth plate in the paraffin blocks provided additional information (Fig. 3). Normal growth plates and P deficient growth plates were dark, except for articular cartilage. The dark color was probably due to the effect of formalin and dilute acid on red blood cells throughout the section. In contrast, lack of blood vessels due to a Ca deficiency supplied no red blood cells to the enlarged growth plate, resulting in a white color. A final decision about a normal growth plate or the cause of a defective growth plate could be made based on the stained section and microscopic examination.

A P deficiency causes a more severe effect on a broiler chick than an equally limiting Ca content. The diet with 0.25% NPP supplied 55% of the required NPP and the diet with 0.30% NPP supplied 67% of the required NPP. Both of these caused significant decreases in body weight, plasma P and bone ash (Table 3 and 4). The 0.6% Ca diet, which supplied 60% of the required Ca, affected none of these criteria. Our results suggest that the tibial growth plate should also be examined when establishing requirements for Ca, P and vitamin D. This might be done by using a radiographic examination[16]. Enlarged growth plates could be detected by this procedure, but the cause of the defect could only be determined by microscopic examination.

When TD is diagnosed in broilers, our results suggest that Ca should receive primary attention. With vitamin D at 300 ICU kg^{-1}, more than half of the chicks developed TD when fed 0.9% Ca by calculation and 1.01% by analysis. A small increase of 0.1% Ca greatly reduced the incidence of TD, so the effect of a slight Ca deficiency appears to be magnified in the tibial growth plates. A corrective action is to increase the dietary Ca by 0.1%. Conditions other than dietary Ca can affect Ca available to the body. Vitamin D affects the amount of Ca available to the body and increasing amounts reduced growth plate defects[15]. The amount of NPP in the diet affects Ca available to the body. Excess NPP should be reduced to the required amount, or an alternative solution is to increase the dietary Ca to an appropriate amount to counteract the excess NPP. Dietary phytase may also have a negative effect on Ca status if the NPP resulting from phytase addition is underestimated, thus inducing a Ca deficiency.

Our experiments were not designed to determine if the effects of Ca and NPP were direct or if they caused their effects by altering acid-base conditions. The most simple equation for Dietary Cation Anion Balance (DCAB) uses only milliequivalents of monovalent ions:

$$DCAB = mEq\ Na + mEq\ K - mEq\ Cl$$

Anion gap also includes HCO_3:

$$\text{Anion gap} = mEq\ Na + mEq\ K - mEq\ Cl - mEq\ HCO_3$$

A more complex equation has been proposed that includes monovalent and divalent ions[17]. Their equation used one equivalent for molar concentrations of Na, K and Cl, 1.5 equivalent for molar concentration of Ca, 1.75 for P (presumably NPP) and two for Mg and SO_4. If acid-base balance is important in the growth plate, the more complex equations incorporate environmental conditions and additional dietary constituents as means of altering acid-base balance in the body.

In summary, this research illustrated the three growth plate defects caused by deficiencies of phosphorus,

calcium and vitamin D. Photographs showed the growth plates when removed from the chicken, during the steps needed for preparation of microscopic slides and then when magnified by 40x. A conclusive interpretation of the reason for enlarged growth plates was only possible with magnification. Phosphorus deficiency caused an enlarged zone of hypertrophy with normal to excessive blood vessel penetration, slight Ca deficiency caused an enlarged zone of cells that resembled pre-hypertrophic chondrocytes with no blood vessel penetration and a more severe Ca deficiency resulted in an accumulation of proliferative chondrocytes with no blood vessel penetration. Our results suggest that TD is caused by a mild Ca deficiency in the developing chondrocyte.

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