

The interaction between the macrominerals calcium and phosphorous, vitamin D and phytase in broilers

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Abstract

Minerals have a variety of functional characteristics with Ca and P mainly as mineral base in skeletal tissues. Their requirements depend on many factors, such as their availability, the Ca/P-ratio and interactions with vitamin D₃ and its metabolites and phytase. The availability in poultry diets is much more complex for P than for Ca. The recommendation is to maintain the Ca/available P-ratio at about 2/1. The metabolites of vitamin D₃ have a clearly higher P-sparing bio-potency than the vitamin D₃ itself. The nutritional efficacy of microbial phytase is mainly related to its mineral efficacy and much less related to the non-mineral efficacy.

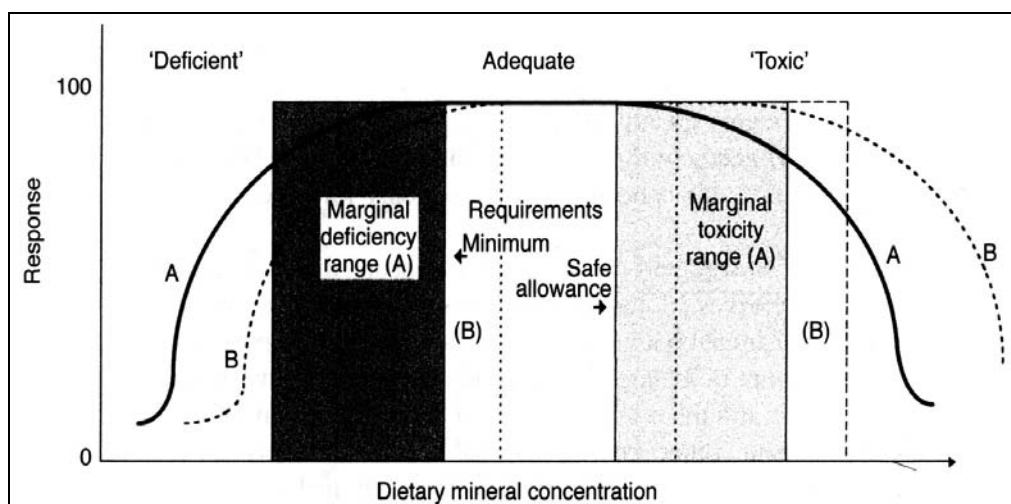
Introduction

All animal tissues and all feeds contain inorganic or mineral elements in widely varying amounts and proportions. During the last century, outstanding advances were made in our understanding of the nutritional significance of minerals from investigations in areas where animals suffered from deficiencies, toxicities or imbalances (figure 1). Twenty two mineral elements are believed to be “essential” for the higher forms of animal life, comprising of 7 macronutrient minerals (e.g. Ca, P, ...) and 15 trace mineral elements. Mineral elements exist in the cells and tissues of the animal body in a variety of functional, chemical combinations and in characteristic concentrations, within quite narrow limits (via homeostatic mechanisms) in order to safeguard the functional and structural integrity of the tissues. Animals have to be supplied with a diet which contains nutrients and minerals in adequate amounts, proper proportions and available form in order to prevent nutritional disorders, which may vary from mild and transient conditions to acute or severe pathological changes.

The functions of the minerals can be classified into 4 types : structural (skeleton), physiological (in body fluids and tissues as electrolytes), catalytic (in enzyme and hormone systems) and regulatory (in cell replication and differentiation). The functions of Ca and P are dominated so quantitatively by their requirements for the mineral base of the skeletal tissues (as hydroxyapatite), providing strength, shape and rigidity, protecting soft tissues and giving attachment to the muscles while simultaneously forming a mobile storage depot. Ca is only present within extra and intra-cellular fluids in low concentrations with, however, an extremely important role in the control of nervous, muscular and hormonal activities. P participates in a wide range of metabolic reactions involving energy transfer and nutrient metabolism as an integral part of the nucleic acids. The hard tissues contain more than 98 and 85% of the body's content of Ca and P, respectively.

It may not be overlooked that responses to a given nutrient greatly depend on the general nutritional and health status. Therefore, this paper will only focus on the main factors and interactions related to Ca, P, vitamin D and phytase in broilers in terms of metabolism, growth and mineralisation.

Figure 1. Schematic dose-response relationship (Underwood & Suttle, 1999)



Calcium & phosphorus

The research on these macrominerals can in fact be related to their requirement and bio-availability, the Ca/P-ratio and all possible interactions with such as vitamin D, acid-base balance,... Because of these mutual interactions it is often difficult to have a general picture of each factorial effect on the zootechnical performance and bone characteristics.

The mineral requirements (as a minimal basic with safety margins; expressed on either daily base or unit of product) are affected by: species or breed of animal, maintenance, intensity of production (incl. quality), diet (nutrient density, digestibility, availability and nutritional 'competitive' interactions) and response criteria (growth, bone characteristics,...). There are several published sets of values of the mineral requirements of poultry in order to have a good compromise for optimum growth and bone calcification (NRC, 1984 & 1994; WPSA, 1985). The Ca & P requirements are concerned principally with production as maintenance needs are small. The requirements for Ca and P are generally determined by means of dose-response curves, factorial models (maintenance, body composition, mineral utilisation with a "partial" utilisation for phytate-P) (WPSA, 1985) and rotation schedules (Edwards and Veltmann, 1983; Huyghebaert, 1996). Dose-response trials show that the Ca & P requirements for maximum growth rate are lower than for maximum bone mineralisation. As a consequence most of the research is focused on the impact of these minerals on bone mineralisation since skeletal abnormalities are the major metabolic disorder related to the fast growth rate. Dietary recommendations are in fact based on : kind of dose-response (the optimal requirement incl. margins of safety), need for either a maximum or reasonable bone ash (& quality), retention & excretion (environmental pollution) and return-on-investment.

The bio-availability (as the main factor) of Ca and P depends on a lot of factors, such as : molecular structure, dietary vitamin D concentration, Ca/P-ratio, phytate or oxalate complexes, anion/cation-ratio, Mg, Al, particle size, ... (Huyghebaert et al., 1981 a&b; Huyghebaert, 1996 & 1997; De Groote et al., 1991; Shafey, 1993; De Groote and Huyghebaert, 1997; Applegate et al., 2003). More recently, the research on the Ca & P bioavailability was reviewed by Emfema (Jongbloed et al., 2002).

Much less research is done for Ca (from carbonates, phosphates, animal origin,...) than for P (from phosphates, animal & vegetable origin,...) because Ca is a much cheaper nutrient than P in least-cost diet formulation. Historically, P has been one of the major nutrients limiting crop and animal production worldwide. For that, P has been added to agricultural soils and animal feeds to assure adequate supply of P and optimize production levels. Nowadays, many

soils do not require P additions to attain the economical optimum due to the addition of fertilizers and manures in the past. Intensification of the animal production even led to a situation where the amount of manure P exceeds the P requirement of the local crops. A surplus of P in manure in areas of intensive animal production tends to accumulate in the soils which lead to increased losses of P to surface and ground water resulting in an impaired water quality. Due to this environmental pressure, P-contents of the diets is an increasingly important issue, not only from this environmental point of view but also from a nutritional point of view.

Ca in the commonly used Ca-sources ground limestone and ground oyster shell have the same availability of CaCO_3 as reference, while it appears slightly lower in CaSO_4 and defluorinated phosphate. The Ca in dolomitic-high Mg-limestone is considerably less available. The Ca-availability in mono- and tri-calcium phosphate is 8 % higher in comparison with the reference. It appears that within the normal range of particle size for ground Ca-sources and Ca-phosphates particle size is not an important factor influencing Ca-availability for chicks and broilers.

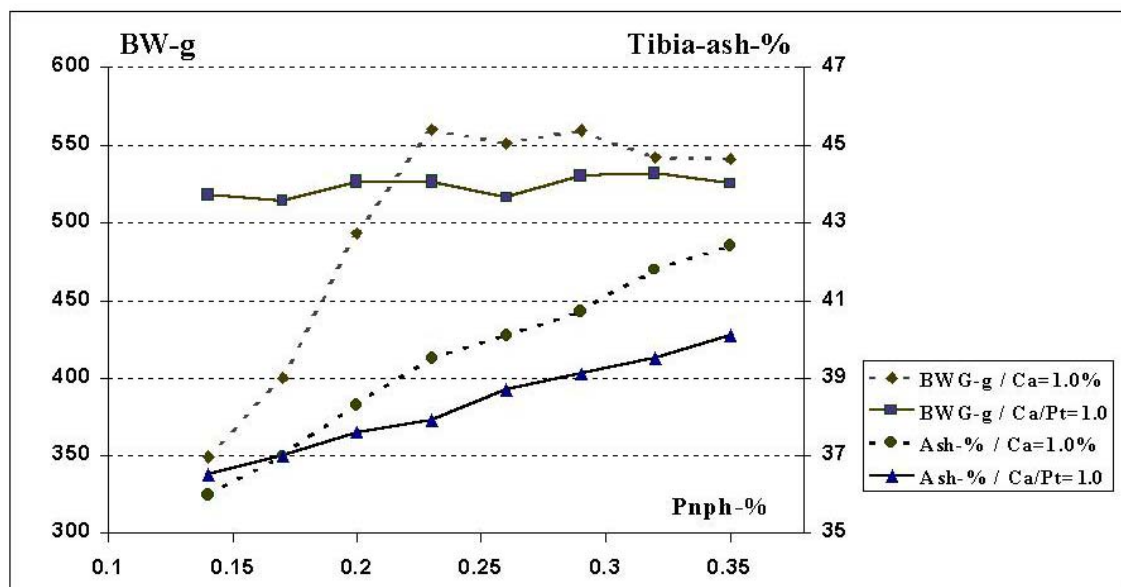
For P, most availability-studies are based on sensitive “bone” response criteria and, more recently, also on true P-absorption and apparent P-retention (Huyghebaert et al., 1980; Ketels and De Groote, 1988). The first category provided only relative values of bioavailability related to a reference source(monoNa-phosphate), while the second category generates absolute values of P-retention or P-absorption, which afterwards can be recalculated to relative values. The water-soluble Na-, K-, NH_4 -, monoCa-phosphates together with the Ca-Mg-Na-phosphate and diCa-phosphate. $2\text{H}_2\text{O}$ have the highest relative bioavailability values for P between 84 and 95%. Mono- and di-Ca-phosphates, diCa-phosphates. $x\text{H}_2\text{O}$, diCa-phosphates (bone) and phosphoric acid are intermediary, with relative values between 80 and 83%. Finally, the P in defluorinated phosphates, diCa-phosphates. $0\text{H}_2\text{O}$, and triCa-phosphates are less available with values between 69 and 79%.

Furthermore, compared with Ca, the situation is much more complicated for P because of the important contribution in vegetable feedstuffs ; a major fraction (about 2/3 but with a wide variation) of plant P is present as phytate-P. Phytates are in fact salts of phytic acid, an inositol with 1 to 6 phosphate groups giving inositol-1-phosphate (IP-1) to inositol-6-phosphate (IP-6) (Eeckhout and De Paepe, 1994; Van Der Klis and Versteegh, 1996).

There is a marked disagreement concerning the utilisation of phytate-P by poultry, being partly related to differences in the evaluation system, such as : non-phytic P, available P, digestible P,... Data are, however, not exactly related with each other because of e.g. the presence of endogenous phytase (as important in wheat, triticale, rye, wheat by-products,...), the feedstuff-dependent phytate-bonds and -location (Adeola et al., 2004; Van Der Klis and Versteegh, 1996) and a lot of other factors: age of the bird, bird strain/genotype, dietary fiber and Ca-level,... Whilst about two-thirds of the P in plant products is present with a phytin linkage, the NRC(1984) suggested that approximately 30% of the plant products is present as non-phytin P and may be considered to be available to poultry. On the other hand, Van Der Klis and Versteegh (1996) demonstrated that broilers can use a part of the phytate-P with e.g. very low degradation-values for sunflower and degradation-values up to 50% for most legume seeds and wheat. These balance trials are, however, done at rather low dietary P and Ca-concentrations (Pav.=0.18% and Ca=0.5%). Phytate may form chelations with cations and thereby interfering with the intestinal absorption of these minerals ; as a consequence the Ca-availability in plant product is clearly lower than in inorganic and animal sources. The adverse impact of a high dietary Ca-level on P-retention is more pronounced for phytate-P than for mineral-P (Shafey, 1993; Huyghebaert et al., 1981b). In a study of Van der Klis and Blok (1997), the phytate breakdown decreased from 34 % to 10 % increasing the Ca-content of the diet from 30 g/kg to 40 g/kg.

There are numerous studies on the effect of the Ca/P-ratio on zootechnical performance and bone characteristics with the bone mineral content (ash content and composition) as the most reliable and sensitive measure of the Ca and P status of the chick. The review of Shafey (1993) demonstrated that there is an agreement that excess Ca depress chicken performance but that there is some disagreement concerning the maximum tolerated level of Ca varying from 1.3% (older references) up to 3.0% (more recent references). Within some limits, the Ca homeostasis can be maintained as controlled by three major hormones : parathyroid hormone, calcitonin and 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃, the active metabolite of vitamin D₃) (Hurwitz, 1985). Thereby, the Ca-excess becomes more pronounced especially when P is marginal (e.g. at lower dietary levels of available P). The ideal Ca/P-ratio can change with the dietary level of phytate-P. In general, the recommendation is to maintain the Ca/available P-ratio at about 2/1. However, the concept of an optimal Ca/avP-ratio is influenced by the availability and intake of both elements. The disagreement between authors regarding the relationship between dietary levels of Ca and P tolerance would suggest that there are other factors involved. The dietary Ca/P-ratio influences both the zootechnical performance and bone mineralisation as ash-content and –composition (Huygebaert et al., 1981a&b; Shafey, 1993)(figure 2). A meta-analysis (Tran and Skiba, 2004; Sauvart et al., 2005) of the relevant literature data may provide excellent tendencies e.g. on the impact of the Ca/Pt-ratio on daily weight gain (figure 3 with a maximum at a Ca/Pt-ratio of about 1.4); the present relatively large variation and low R² are mainly due to the unaccurate description of the experimental set-up (as for the dietary levels of the “available”macro-minerals) (Nys et al., 1997). In general, an increase of dietary Ca-level (from 0.45 up to 0.78%) will result in a reduction of the P-absorption (in %-intake) but in an increase of the P-retention (in %-absorption). The efficiency of both Ca and P retention is maximal at the point of intersection between the Ca/P-ratio as absorbed and as retained (Van Der Klis and Versteegh, 1996); this means a Ca/Pdig-ratio of about 2.2.

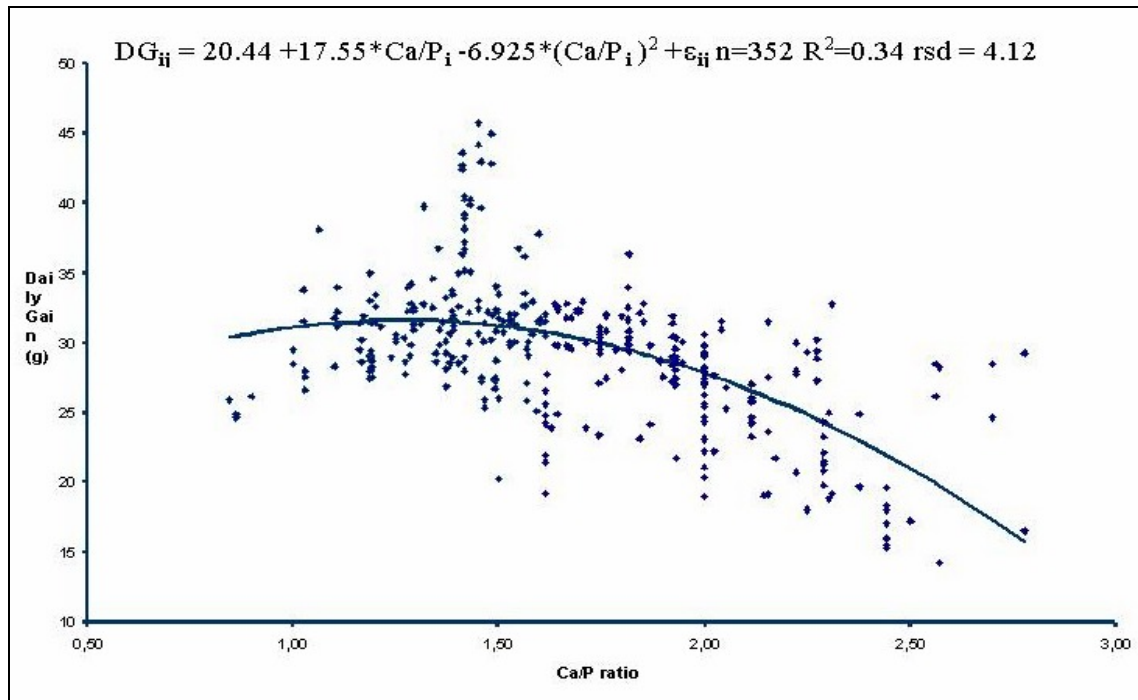
Figure 2. The impact of the dietary Ca-level and Ca/Pt-ratio on body weight and tibia ash-% (Huyghebaert et al., 1981a)



Ca & P are interacting with Na & Cl and acid-base balance in general thereby affecting the zootechnical performance, water intake, excreta moisture and bone mineralisation (deposition of minerals, “TD”-skeletal deformation) (interaction with environmental temperature!).

Mongin and Sauveur (1977) put forth the hypothesis that the metabolic acidosis in chickens fed high dietary chloride levels caused impaired bone mineralisation from alteration of vitamin D metabolism (inhibiting the synthesis of 1,25-(OH)₂-D₃); the “Na+K-Cl”-balance should be at least 220 meq/kg feed. Also the report of Huyghebaert et al. (1981b) confirmed the negative effect of a narrow Na/Cl(=1/3)-ratio on the P-availability in hydrated diCa-phosphate from 100 to 88%.

Figure 3. The impact of Ca/P ratio on daily gain (from 1 to 21/24 days of age) in broilers (Lescoat and Nys, 2005. personal communication/ from 47 publications with 789 observations)



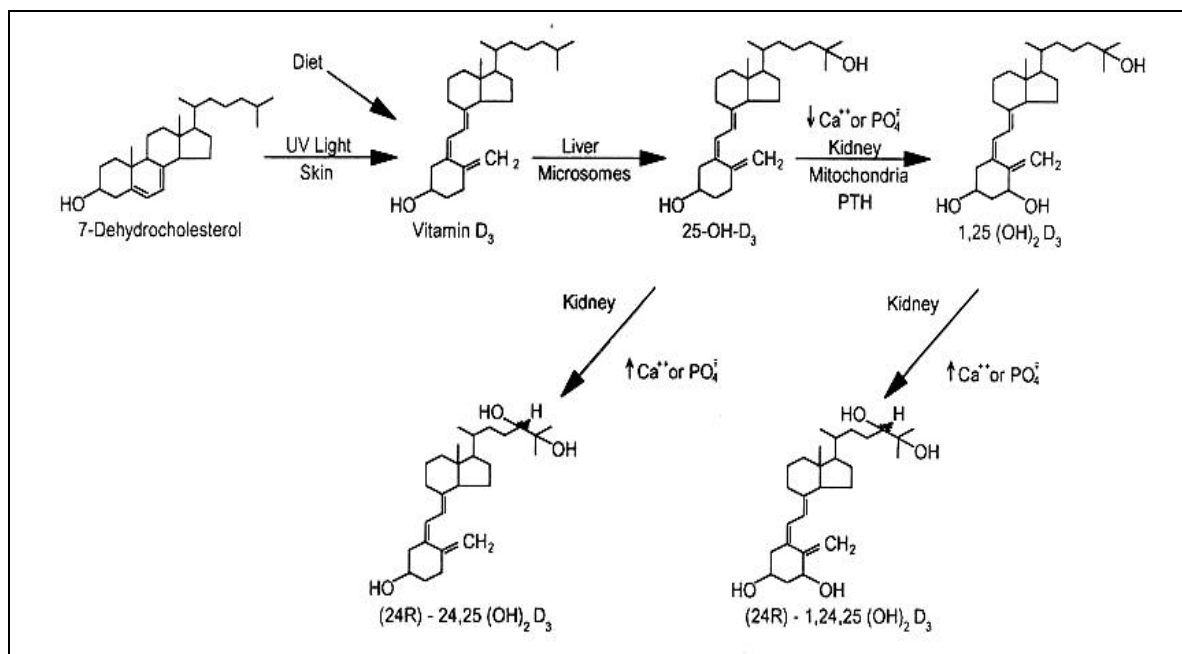
Vitamin D and metabolites

Vitamin D refers to a group of closely related compounds that poses antirachitic activity, such as : ergocalciferol (vit. D₂) and cholecalciferol (vit. D₃). The absorption of vit. D₃ (being supplied into the diet) depends greatly on intestinal conditions (e.g. the emulsification). Vit. D₃ is converted to 25-OH-D₃ (in the liver), which in turn is converted (1) at low blood Ca- & P-levels to 1,25-(OH)₂-D₃ (stimulated by parathyroid hormone “PTH” vs calcitonin) or (2) at normal blood Ca- & P-levels) into one of two other metabolites 24,25-(OH)₂-D₃ or 1,24,25-(OH)₃-D₃ (in the kidney) (figure 4)(Ammenuddin et al., 1985). The half-life times of vit. D₃ & 25-OH-D₃ are similar (resp. 25 & 20 days) with on the other hand a rapid clearance of about 6 hours for 1,25-(OH)₂-D₃!

Vitamin D₃ (1,25-(OH)₂-D₃) controls the levels of blood Ca & P through specific mechanisms in the intestine, kidney (secretion/reabsorption) and bone (deposition/mobilisation) (DeLuca, 1979). The mechanism of action of these metabolites may be (1) directly through additional uptake of liberated phosphate ions (P_i), up-regulation of endogenous intestinal phytase activity, increase in apparent ileal hydrolysis of phytate-P (providing an additional P from phytate, or (2) indirectly through improving the rapid phase (transcaltachia) and slower phase (mediated through Ca-binding proteins) calcium uptake

from the small intestine. Calcium at pH of the small intestinal digesta can chelate to the phytin molecule and dramatically reduce the phytin molecules' solubility. By removing a portion of calcium from this equation, the phytin molecule is more soluble and accessible to the hydrolytic actions of endogenous (intestinal) or exogenous phytases. Secondly, the catalytic actions of some phytases are inhibited by high concentrations of P_i (Wodzinski and Ullah, 1996); a vitamin D_3 -mediated translocation of P_i from the intestine into the blood (Wasserman and Taylor, 1973) may be assisting the hydrolytic action of phytase by reducing

Figure 4. Metabolism of vitamin D & metabolites (Applegate and Angel, 2004)



the inhibitory effect of P_i . In comparison with vit. D_3 (as reference=100%), the relative biological effectiveness varied from 100-400 for 25-OH- D_3 and from 200-1500 for 1,25-(OH)₂- D_3 (Applegate and Angel, 2002 & 2004; Soares et al., 1978; Soares et al., 1995). Part of this differential can be explained by differences in intestinal absorption, being related to molecular polarity. “Relative” bio-potency also depends on : the particular response characteristic measured (tibia ash, bone strength, plasma-Ca, Ca-absorption, cortical bone quality, tibial dyschondroplasia incidence,...), dietary concentration and ratio of Ca and P, genotype, age,... (Fritts and Waldroup, 2003; Whitehead et al., 2004; Applegate et al., 2003). Table 1 provides a summary of these bio-potency values for broilers (as sparing effect of P for the major part related to phytate-P). However, there are indications that 25-OH D_3 may need to be used in conjunction with vitamin D_3 , at least to obtain specific responses (Applegate and Angel, 2004). Moreover, metabolites are not only characterized by their specific metabolic activity but also by their safety : e.g. 25-OH D_3 has a much wider margin of safety (10-fold margin between effective dose and toxic dose) than 1,25-(OH)₂- D_3 .

The vit. D_3 requirement and suggested commercial specification are resp. 200-1000 (depending on Ca & P deficiencies/imbances, type of response parameter, dietary levels of vitamin A and C,...) and 3000 IU/kg in broilers (1 IU=0.025 μ g) (Whitehead and Portsmouth, 1989). For example, Edwards (1999) reported the vitamin D_3 requirement of broilers for growth to be 275 IU/kg, for bone ash to be 503 IU/kg, for plasma $[Ca^{2+}]$ to be 552 IU/kg, and for rickets prevention to be 904 IU/kg. Historically, the poultry industry has supplemented

vitamin D₃ at concentrations well above what is typically reported as the requirement by the National Research Council (1994), because of past variability in analyses and as “insurance factors” to prevent field incidences of leg abnormalities in the case of meat birds (rickets and tibial dyschondroplasia).

Table 1. Efficacy of different vitamin D₃ metabolites on phytate-phosphorus and phosphorus utilization in broilers (Applegate and Angel, 2004).

Age	Metabolite	Suppl. Amount	Phosphorus spared
1-9 d	1,25-(OH) ₂ -D ₃	5 µg/kg	0.057 %
...	1α-OH-D ₃	5 µg/kg	0.025 to 0.03 %
...	1,25-(OH) ₂ -D ₃	5 µg/kg	0.038 to 0.056 %
...	25-OH-D ₃	5 µg/kg	No consistent change
0-21 d	1,25-(OH) ₂ -D ₃	5 µg/kg	0.03 to 0.059 (at typical starter P-levels)
8-20 d	1α-OH-D ₃	20 µg/kg	~ 0.06 %
7-21 d	25-OH	210 µg/kg	0.065%
8-22 d	25-OH	210 µg/kg	No change
12-21 d	25-OH	70 µg/kg	0.035 %
11-20 d	25-OH	35 µg/kg	0.03 %

Phytate-P and phytase

Most poultry diets are composed of plant-based ingredients with a major fraction of the P present as phytic acid in the form of myo-inositol hexaphosphates. The role of phytate is to store P for use by the developing embryo. Phytate is characterized by its low P-availability as well as its chelating capacity with other nutrients thereby adversely interfering with their availability. Indeed, phytate can potentially bind other nutrients such as cations (positively charged minerals like calcium, zinc, copper,...), amino acids and starch, which reduces the digestion and absorption of these nutrients. Calcium is a special issue with regards to phytate and phytase. Calcium shows the lowest binding affinity to phytate (Vohra et al., 1965), but is present at the highest level in poultry diets.

Phytate also has some other less known properties. Phytate disturbs the activity of endogenous digestive enzymes like pepsin and trypsin under gastrointestinal conditions. Paradoxically, the ability of phytate to chelate minerals has been reported to have some protective actions too such as accumulation of heavy metals (Pallauf and Rimbach, 1997). Phytates also seem to act as a natural antioxidant and are anticarcinogenic (Zhou and Erdman, 1995). The mechanism explaining this protective effect is the complexing of Fe by phytate which reduces the Fe-catalysed production of free radicals and lipid peroxidation.

The amount of phytate-P in the diet of the birds depends on a lot of factors and is quite variable (Eeckhout and De Paepe, 1994). Next to the plant species, there is the stage of maturity, degree of processing, the soil type, the fertilization level,... Especially cereals, cereal by-products and oil seeds contain large amounts of phytate. Concentrations also differ according to the part of the plant considered. In wheat, phytate is concentrated in the aleurone layer of the seeds, whereas in oil seeds, it is distributed throughout the kernel. Thus the

phytate level and dispersion within the plant are not static across the different plant species with consequences for the potential degree of 'non' mineral binding.

There are numerous studies demonstrating the favourable effect of phytase on the phytate-P availability. Phytases are phosphatase enzymes that are able to catalyze the hydrolysis of phosphate ester bonds forming inorganic phosphate and lower phosphoric esters of myo-inositol. Nelson et al. (1971) were the first to show that when phytase from *Aspergillus ficuum* was incorporated in the diet of broilers, a marked improvement of the utilization of phytate P was realised. In other words, phytase releases phosphorus from the phytate, making it available for monogastric animals thereby reducing environmental excretion. However, only since the 90-ies microbial phytases are used in commercial pig and poultry diets.

It is important however to remember that there are four possible sources of phosphatases which can be found in the gastro-intestinal tract (GIT) of an animal. First of all, the plant phytases which are formed in the seeds during germination to release phosphorus for the growing embryo. The levels can vary from 0-6000 FTU/kg. Wheat and rye show the highest phytase activities, barley a moderate activity and corn or oats a negligible activity. Secondly, membrane-bound phytase has been found in the small-intestinal mucosa of many animals. In the case of poultry, only at low Ca-levels (<0.6 %) and low P_{av}-levels (0.2 %) however, birds seem to be able to utilize a small part of the phytate P (Van der Klis and Blok, 1997). Thirdly, phytase is produced by the microflora in the GIT (rather limited in birds). Finally, an exogenous 'of microbial origine' phytase can be added to the diet.

There are currently two primary classes of phytase enzymes: a 3-phytase and a 6-phytase. The 3-phytase initiates the dephosphorylation of phytin at the 3-position on the inositol ring, the 6-phytase at the 6-position. Natuphos® e.g. is a 3-phytase produced by *Aspergillus niger* var. *van tieghem* (formerly known as *A. ficuum*), Ronozyme™ P a 6-phytase produced by *Peniophora lycii*. In the literature, there is some disagreement in their efficacy. In a study of Juin et al. (2001) no difference in the use of either phytase sources was reported. This was confirmed by the results of Pos et al. (2003) and Payne et al. (2005). However, Sands et al. (2003) indicated a greater response from an 3-phytase product compared with the 6-phytase product. The newest phytases (6-phytase : Phyzyme XP) are derived from *Escherichia coli* (Augspurger et al., 2003; Dilger et al., 2004; Onyango et al., 2005). In the study of Augspurger et al. (2003), it was found that the use of the latter phytase has an advantage compared to the commercial phytases (Natuphos and Ronozyme) in young broilers.

Enzyme activity is expressed in activity units (FTU) where 1 FTU is defined as the quantity of enzyme that liberates 1 µmol inorganic P per min from an excess of sodium phytate at pH 5.5 and 37 °C. The degree of 'optimal' activity of the phytase depends on : moisture content, 'moderate' temperature (incl. thermostability during feed processing), intestinal viscosity and pH-range. Next to the fact that phytate forms insoluble precipitates with cations at higher pH-values (starting from pH 4), there is also the effect of pH on the phytase activity. The optimal pH for wheat phytase activity is 5.5 (Bos, 1990). As pH becomes lower, wheat phytase activity decreases rapidly. This would mean that main activity of the wheat phytase is concentrated in the crop, but not in the gizzard. However, microbial phytases are also active at very low pH values as those in the gizzard of the bird (Simons et al., 1990). On the other hand, as phytases are enzymes, they are subsequently susceptible to normal protein-degrading digestion processes. Thus, no phytase activity is expected in the intestinal tract of the animals (Jongbloed et al., 1992; Yi and Kornegay, 1996). But according to Kumar et al. (2003) phytase from *E. coli* shows a clearly higher resistance to protease attack than the phytases from *Aspergillus niger* and *Peniophora lycii*, which in his turn is an important factor for a higher bio-efficacy (Augspurger et al., 2003).

The value of microbial phytase in releasing phytate-bound P and improving P-availability of plant ingredients for poultry is well documented (Nelson, 1976; Simons et al., 1990;

Huyghebaert, 1981a&b; Yi et al., 1996). Reported improvements in P availability are generally in the range of 20-45 % (Selle et al., 2000). In order for poultry producers to use microbial phytase efficiently, accurate equivalency values of phytase for P should be known. The phosphorus equivalency value of a phytase is by definition the amount of inorganic phosphorus that can be removed from the diet by a given phytase unit of activity. For direct comparison of the equivalency value of the phytase for phosphorus and digestible or available (relative) P, equivalency values must be adjusted by the estimated digestibility or bioavailability (relative) of the inorganic P-sources that phytase replaces. According to Radcliffe (2001), a wide range of P-equivalency values of phytase were found in their studies, ranging from 0.21 to 1.07 g of P from inorganic P for poultry fed 500 U of phytase per kilogram of diet. In a study of Augspurger et al. (2003) bioavailable release values of 0.32 and 0.28 g P (KH₂PO₄) for 500 FTU/kg diet for Natuphos and Ronozyme, respectively, were found. In two studies of Huyghebaert (1996, 1997) average P-equivalency values of microbial 500 FTU phytase (Natuphos 5000) of respectively 1.23 (DCP.2H₂O) and 0.82 g available P (mono-calcium phosphate) were found. The quite wide ranges of equivalency values might be an indication for the variable impact of many factors, like: dietary P-content, calcium level, type of 'feedstuff'-diet, age of animals,... Also the response criteria (growth performance, tibia ash) has his influence on the results. In a study of Yan et al. (2001), in the presence of 800 units of phytase per kg, 0.24, 0.151 and 0.109 % non-phytate phosphorus were needed to optimize tibia ash, body weight gain and feed conversion, respectively. Against, 0.33, 0.186 and 0.163 % in the absence of phytase. A study of Rosen (2001, 2002) using a multifactorial analysis on a dataset of 296 controlled experiments with phytase, showed statistically significant influences of negative control performance, duration of feeding, year of test, phytase dose, dietary P-content, caging, above-average mortality, cereal base, anticoccidial use and fat supplements on microbial phytase activities. The author found that the liveweight equivalence value measured between 4 and 3 g P/kg is almost double that for application between 7 and 6 g P/kg. The modelling programme indicated also that the feed conversion effect is less in maize compared with wheat-based diets (Wu et al., 2003) and improved by the use of anticoccidials. Moreover, due to genetic selection, improvement of feeds and management techniques, the effects of phytase on feed conversion have been reduced over the years. Higher dosages of phytase (3-5 times the dosage used to reduce P-excretion) can improve sub-standard broiler performances. According to a study of Ravindran et al. (2001), the response in weight gain to added phytase (Natuphos®) reached a plateau at 500 FTU/kg diet (quadratic effect, $p < 0.001$). However, phytase supplementation had no effect on gain per feed to 250 FTU/kg diet and then increased quadratically with further additions to 1000 FTU/kg. Payne and Southern (2003) found linearly increased daily gain and feed intake figures when using 300, 500 or 750 FTU of Natuphos/kg of diet.

The responses are further complicated by the effect of phytase on the utilisation of other elements as minerals, amino acids, metabolisable energy,... Indeed, at the same time phytase releases P, there is a potential increase of the retention of some other (positively-charged) minerals (Sebastian et al., 1996a&b; Zanini and Sazzad, 1999). Phytase supplementation (600 FTU/kg) of a low-P diet increased the relative retention of P, Ca, Cu and Zn by 12.5, 12.2, 19.3 and 15.7 %, respectively, in male broilers (Sebastian et al., 1996a). There are however no current matrix recommendations for 'trace' minerals other than P and Ca for any phytase.

Several studies have demonstrated improvements in growth performance of birds following phytase supplementation of adequate-P diets, which could partly be explained by an enhanced protein utilisation (Namkung and Leeson, 1999; Ravindran et al., 2000; Ravindran et al., 2001; Wu et al., 2003). In a study of Ravindran et al. (2001) the lysine equivalency value was calculated to be: 500 FTU phytase/kg diet = 0.074 % lysine. Other researchers could not indicate any positive effect on CP or amino acid utilisation (Peter et al., 2000; Peter and

Baker, 2001; Adeola and Sands, 2003). In general, there can be concluded that there is considerable variation in the reported amino acid responses to phytase supplementation. This again can be related with the complex factors of influence which probably include the source and concentration of the phytate (and dispersion and accessibility) and protein in the diet (e.g. in so far protein and amino acids are limiting), the digestibility of the protein component, Ca, P and vitamin D levels, the phytase inclusion rate and animal factors (species, genetics, sex,...) (Adeola and Sands, 2003). A general rule, however, seems to be that poorly-digestible feedstuffs (containing sufficient phytate-P) are more responsive to phytase addition (Selle et al., 2000).

Also the enhancement of metabolisable energy of poultry diets after supplementation with phytase has been documented (Namkung and Leeson, 1999; Camden et al., 2001; Ravindran et al., 2001; Wu et al., 2003). In a study of Ravindran et al. (2000), supplemental phytase increased AME values from 13.36 to 13.54 MJ/kg dry matter in low non-phytate phosphorus diets and from 12.66 to 13.38 MJ/kg dry matter in adequate non-phytate phosphorus diets. In Table 2 the 'non' mineral matrix values of Natuphos 5000 for broilers are summarized; thereby the effective nutrient contribution at e.g; 500 FTU/kg feed may not be overlooked!

High dietary levels of both Ca and P or imbalances of the dietary Ca/P-ratio have been reported to inhibit the bioefficacy of phytase (Huyghebaert et al., 1992a; Sebastian et al., 1996b). In the trial of Huyghebaert et al. (1992a), increasing the Ca-content of the diet from 0.74 to 1 %, had a negative effect on the P-availability (%) of about 4 %-units. However, the improvement due to supplementation of phytase (250 or 500 FTU/kg), was not changed by Ca-concentration. Also tibia ash-% deteriorated significantly with a higher Ca/P-ratio (Huyghebaert et al., 1992b). Indeed, excess Ca precipitates phytate, forming insoluble calcium-phytate complexes that are resistant for phytase hydrolysis. Moreover, excess Ca may increase the pH of the intestinal content, with adverse effects on phytase activity and solubility and absorption of minerals. Dietary Ca:P_{tot.}-ratios between 1.1:1 to 1.4:1 appear critical to the efficient use of supplement phytase (Qian et al., 1997).

Table 2. Natuphos 5000 'non' mineral matrix values for boilers (BASF-brochure, 2002).

Nutrient	One kg Natuphos®5000 (= 5*10 ⁶ units) is equivalent to	Contribution per kg feed at a dosage of 500 U
Phosphorus*	11500 g	1.15 g
Calcium	10000 g	1.0 g
Lysine**	1200 g	0.12 g
Methionine**	100 g	0.01 g
Cystine**	300 g	0.03 g
Threonine**	1300 g	0.13 g
Tryptophan**	300 g	0.03 g
Isoleucine**	1200 g	0.12 g
Crude protein**	22500 g	2.25 g
ME	530000 kcal/2215 MJ	53 kcal

*based on DCP, **apparent ileal digestible

On the other hand, citric acid has been shown to improve phytate P utilization in broilers (Boling-Frankenbach et al., 2001; Snow et al., 2004). The mode of action of citric acid may be associated with its calcium-complexing property and attributed to a reduction of the inhibitory effect of calcium on phytate hydrolysis (Adeola and Sands, 2003). Also vitamin D analogues have been shown to increase phytate P use in chickens (Edwards, 1993; Biehl et al., 1995; Qian, et al., 1997; Snow et al., 2004). An additive effect between the use of phytase and 1,25(OH)₂ D₃ was found by Biehl et al. (1995), Mitchell and Edwards (1996) and Snow et al.

(2004). Mitchell and Edwards (1996) hypothesized that the mechanism of action of 1,25(OH)₂D₃ may be through additional uptake of liberated phosphate ions and possibly through up-regulation of endogenous intestinal phytase activity. The latter could, however, not be confirmed by research of Biehl and Baker (1997) and Applegate et al. (2000). It was therefore hypothesized by Applegate and Angel (2002) that the mode of action of 25(OH)₂D₃ and the other metabolites in improving phosphorus utilization occurs indirectly through improving the rapid phase and slower phase of calcium uptake from the small intestine. By removing a portion of calcium, the phytin molecule is more soluble and accessible to the hydrolytic actions of phytases. Secondly, these authors suggested that 25(OH)₂D₃ may be assisting the hydrolytic action of phytase by reducing the inhibitory effect of phosphorus ions from which the translocation into the blood from intestinal mucosa is dependent upon 25(OH)₂D₃. Substantial controversy exists around the effect of phytase on the proportion of water-soluble P in broiler litter. It has been reported that the proportion water-soluble P to total P-content increases when phytase is used (Delaune et al. cited by Applegate and Angel, 2003; Miles et al., 2003). However, other researchers could not confirm this finding (McMurtry et al., 2002; Newman et al., 2002; Applegate and Angel, 2003; Waldroup and Fritts, 2003; Perry, 2004). Indeed, results and especially concentrations of water-soluble P-contents of broiler litter in literature are quite variable between trials. Moreover, it seems that phosphorus contents of the diets and microbial activity in the manure after excretion have a major effect on the concentration of soluble P-contents on the one hand and the ratio water-soluble to total P on the other hand. More research is needed to fully understand the different factors of influence concerning the soluble P-content of broiler litter.

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