STUDIES OF PHOSPHORUS DIGESTIBILITY WITH BROILERS

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I hereby declare that the research in this dissertation is of my own investigation. Where use was made of the work of others it has been duly acknowledged in the text.

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Contents

General Introduction

Sustainability of poultry farming in the world is dependent on both environmental and economic viability. Unfortunately, there are many situations where environmental and financial goals are in direct conflict. An example is in the disposal of poultry excreta, a waste product of industry that is increasing in size at a considerable rate. Whereas this excreta is an inexpensive source of fertilizer on lands (that could certainly benefit from both the organic and inorganic matter contained in the litter). The excess phosphorus (P) above the amount required by plants seeps down the soil profile and contaminates the water table or through runoff gets into surface water bodies which leads to eutrophication and encourage the growth of algae. In countries like France and The Netherlands, there are already laws in place stipulating the maximum amount of P that may be produced from poultry enterprises, which proves the profoundness of this issue. There is an urgent need to reduce the amount of P in the litter to ensure the sustainability of poultry production to meet the protein needs of the fast growing world population.

The large amounts of P in the excreta do not necessarily imply that the birds are fed more than their P requirements because as some of excreta P is the dietary P which the bird is unable to utilize. It is this P that is most likely to be present in the excreta. Attempts to address the problem of excess P in the litter should start with defining more accurately the amount of P present in feed ingredients that can actually be utilized by poultry. The term 'available phosphorus' commonly used in the feed industry refers to the amount of phosphorus in a non-phytate form, incorrectly assumed to be available (i.e. digested and utilized) to the bird. Incorrect assumptions about P digestibility could as easily result in deficiencies as well as excesses in dietary P supplied. A P deficiency, when the amount of P supplied in the diet has been overestimated, will reduce productivity, and hence profitability. An excess P supply, where the nutritionists have underestimated the digestible P content of the feed by underestimating the digestible P content of each ingredient, would lead to excessive use of MCP, which would be costly, and result in excessive excretion of P. All this implies that in order to reduce P in the excreta we must know how much P is digestible in every ingredient.

It is not possible to measure the availability of dietary nutrients to a bird or animal, as this property is as much a function of the individual being fed as of the ingredient itself. At present feed formulation programs used in South Africa and most other countries of the world are based on the assumption that in all plant ingredients 67% of the P exists in the form of phytate, which is undegradable by birds and that the P from animal and inorganic P sources is 100% available. Recent studies have shown that these assumptions are incorrect by showing that the phytate content in plant ingredients varies, that the phytate is degradable to an extent and that P from ingredients of animal and inorganic sources is less than 100% available to the bird. A quantification technique that is objective, accurate and based on how much P can be retained by the bird from each of the ingredients commonly used in poultry diets, is the first step in attempting to reduce excreta P. This would make it possible to provide a more precise quantity of digestible P in poultry diets thereby reducing P excretion to a minimum.

There are many factors affecting the digestibility of P from the ingredients; of major importance among these being the phytate in plant ingredients. The monogastric animals have a very limited ability to degrade phytate because they have a limited activity of the enzyme phytase, which degrades phytate, in their intestines. This renders the phytate P largely inaccessible to the monogastrics, which contributes immensely to the problem of environmental pollution associated with poultry excreta. For many years tibia ash content was favored as a parameter for measuring the availability of P, since P is one of the main components of the bone. Recently, a number of researchers have voiced doubts about the reliability of tibia ash as a parameter for estimating P digestibility, the main reason being the fact that there are many critical factors other than P that influence the content of ash in bone. Many researchers have hailed determining the exact amount of P that is digestible from an ingredient as the most accurate method of determining the availability of P from ingredients.

There is an industrially produced microbial phytase that can be supplemented in diets to improve P digestibility. However, the supplementation of this enzyme in diets needs to be treated with caution because of the variability of the phytate content of ingredients. Therefore, the responses of the ingredients to the enzyme are supposed to vary. The verification of this aspect after the determination of digestible P contents of ingredients is very critical in the attempts to reduce the excreta P content. The determination of digestible

P content of ingredients will be very helpful in measuring the activity of supplemental phytase, in terms of its effectiveness in improving P digestibility of various ingredients. Recently many researchers have observed a concomitant improvement in digestibility of some amino acids with the supplementation of phytase enzyme in diets. This can be an immense benefit to the environment because the amount of nitrogen emitted in the form of foul smelling ammonia from poultry farms will be reduced.

The main objective of this dissertation is to look at the ways of addressing the problem of pollution caused by poultry excreta. This will be pursued by first determining the digestible P content of various ingredients commonly used in poultry diets. Two techniques of determining digestible P, i.e. tibia ash and ileal P digestibility, will also be evaluated for accuracy, basing the judgment on literature information. Secondly, after the determination of digestible P content of ingredients, the effectiveness of phytase in improving both amino acid and P digestibilities of these ingredients will be determined. The effect of dietary calcium content on P digestibility will also be investigated.

Chapter 1

Literature review

1.1 Introduction

The public have growing concerns about potential pollution from improperly applied poultry manure to the land, especially in countries of intensive poultry production like The Netherlands and France (Simons, Versteegh, Jongbloed, Kemme, Slump, Bos, Wolters, Beudeker and Verschoor, 1990). In these countries the nutrients supplied to the land through poultry manure have been observed to exceed the crop requirements for nutrients like Phosphorus (P) and Nitrogen (N). Poultry manure from poultry enterprises is often used to improve land fertility. This is one way of utilizing what would otherwise have been a waste. Poultry manure is richly supplied with N and minerals like P and Ca which are utilized by plants for growth. An excess of these nutrients in the poultry manure is toxic to plants, retards their growth and contaminates the water table (Sloan, Harms, Barnard and Nodstedt, 1995; Patterson and Lorenz, 1996). Soils with high P levels are more likely to contribute to P runoff in soluble or sediment-adsorbed form. In coastal areas high P levels may directly stimulate growth of algae. Algae blocks sunlight for submerged aquatic vegetation and decomposing algae deplete oxygen for aquatic species (Bosch, Zho and Kornegay, 1997). Patterson and Lorenz (1997) observed that about 738.4 g/kg of dietary P is excreted in the feces. Reduction of dietary P can bring about a decrease in fecal P content since the two are highly correlated (Wecke, Liebert, Kohler and Tittman, 1992).

For many years the general assumption has been made that chickens are unable to utilize the phytate-bound P, whereas the remaining inorganic plant P together with P from animal and inorganic P supplements, are readily available (Waldroup, 1999; Wecke *et al.*, 1992). There is also an assumption that in all ingredients of plant origin, phytate-P accounts for 0.67 of the total P (Simons *et al.*, 1990). Recent studies have demonstrated that neither of these assumptions is totally correct (Punna and Roland, 1999; Waldroup, 1999). Broilers have been observed to be capable of using a portion of the phytate-bound P, whereas the availability of inorganic P is less than 100% (Waldroup, 1999). A wide range of phytate-P utilization values have been reported in poultry. Values ranging from 0.6-0.82 from

different feedstuffs have been reported (Punna and Roland, 1999). Most diet formulations are based on the incorrect assumptions mentioned about P utilization from different feedstuffs, which often leads to the unnecessary supplementation of diets with P from inorganic P sources to meet birds' requirements. Apart from the cost involved, supplementation leads to excessive supply of P, and consequently high P contents in the excreta.

Phosphorus is retained in the body of a bird for various physiological processes. P coefficients of availability for chicken bone mineralization for some feedstuffs have been observed to be above 0.50, whereas for others it is less than 0.20 when compared to monocalcium phosphate (Sauveur, 1989). Determination of the retainable P (digestible P) of all feedstuffs used in poultry diets instead of the assumption that only 0.3 of P from plant ingredients is available can allow for reduction or total elimination of inorganic P supplementation. This will potentially result in a decrease of P in litter. Increasing the utilization of phytate-P also reduces P supplementation and consequently P in the excreta. This can be achieved by inclusion of a phytase enzyme in the diet (Van der Klis and Versteegh, 1996).

Interaction of ingredients in the diets have the potential of reducing the digestibility or retention of P. Fats have been observed to reduce the retention of some minerals in poultry diets (Atteh, Leeson and Julian, 1983). If P in excreta is to be reduced, such interacting factors need also to be investigated.

In this literature review the physiological importance of P, factors leading to high P in excreta, and means of reducing P in excreta, are discussed. Experiments that will be conducted, all of them leading to the reduction of P in the excreta, will also be discussed.

1.2 Phosphorus physiology and biochemistry

1.2.1 Physiological importance of phosphorus

Phosphorus (P) is involved in many important physiological processes in the bodies of animals and birds. Together with calcium (Ca), P forms an integral part of the bone in the form of $CaPO_4$. Since bone growth occurs by deposition of $CaPO_4$, P plays an important

role in such growth. It is logical to say the deficiency of P in the diet can retard bone growth. P plays an important role in energy utilization by virtue of the fact that it is part of Adenosine Triphosphate (ATP). As part of the phospholipids, P is essential for structured components of the cell (Smith, 1990).

Dietary phosphorus is also utilized by hens for egg formation. A portion of this P is incorporated into the yolk and albumen in the form of phospholipids and phosphoproteins. Part of eggshell P exists in a form of calcium phosphate in the cone region (Hossain and Bertechini, 1998a; Powrrie, 1971). Gordon and Roland (1998) observed a significant improvement in eggshell quality when dietary available P was increased from 1 to 3 g/kg because the eggshell weight and egg specific gravity increased from 4.96 g to 5.06 g and from 1.0778 to1.0783, respectively. Different amounts of P in the eggshell have been reported. Powrrie (1971) and Hossain and Bertechini (1998a) discovered 9 g P/kg and 4.4 g P/kg shell, respectively. The yolk and albumen consist of 9.8 g P/kg and 0.2 g P/kg, respectively (Powrrie, 1971). Yolk is comprised of 320 g lipid /kg lipid of which 283 g/kg is phospholipid (Powrrie, 1971).

Phosphorus is indirectly involved in the eggshell formation process. $H_2PO_4^-$ from the blood in the peritubular blood vessels enters the renal tubules in exchange for HCO_3^- which is reabsorbed into the blood and transported to the shell gland. The rate of this exchange increases during eggshell formation, since HCO_3^- forms an integral part of the shell. The $H_2PO_4^-$ excretion increases during active shell formation and less HCO_3^- is excreted (Simkis, 1968). According to this information, low blood P can hamper the eggshell formation process, since there will be less reabsorption of HCO_3^- back into the circulation. This is confirmed by the findings of an experiment by Taylor and Kinkley (1967). It was observed that more urinary P was excreted during laying days than on non-laying days .The uptake of P from the gut was also increased during laying days. It was speculated that the increased P excretion during shell formation was due to the mobilization of skeletal Ca and P for shell formation, since this increase in P excretion was high when a low Ca diet was supplied.

Table 1.1 Calcium and phosphorus requirements of laying pullets and broilers. (NRC, 1994)

Age (weeks)	Inorganic phosphorus (%)	Calcium requirements (%)
Laying pullets		
0-6	0.40	0.90
6-12	0.35	0.80
12-18	0.30	0.80
18+	0.32	2.00
Broilers		
0-3	0.45	1.00
4-6	0.35	0.90
7-8	0.30	0.80

Phosphorus and Ca play a major role in the formation and maintenance of bones. The skeleton accounts for about 0.99 of the calcium and 0.80 of the P in the body (Smith, 1990). Punna and Roland (1999) observed that a diet with 5 g available P/kg diet produced a higher bone mineral content (0.17 vs 0.10 g/ cm^2), bone mineral density (0.24 vs 0.16 g/cm^2) and breaking strength (32.2 vs 22 kg) than a diet containing 1 g available P/kg diet. Graded increases in inorganic P addition to the P deficient diet increased bone ash in a linear fashion (Biehl and Baker, 1997). After three weeks of age, P requirements are greatly reduced since bone formation is almost complete (Table 1.1). This means that after three weeks of age, when a significant amount of feed is consumed, there is little if any need for supplemental P in a typical corn-soya bean meal broiler diet (Waldroup, 1999). The Ca and P combine after absorption in the gut to form $CaHPO_4$ from the combination of Ca^{2+} and HPO_4^{2-}. The $CaHPO_4$ in bone occurs in the form of crystals which are formed by a stepwise addition of ions to a nucleation center (Newman and Newman, 1953).

Body weight gain and feed intake increases linearly as supplemental inorganic P increases up to 4.5 g inorganic P/ kg diet, where they both reach a plateau (Yi, Kornegay, Ravindran and Denbow, 1996). In relative terms, the amount of P needed to maximize various criteria is in the following order: bone calcification > body weight > feed efficiency > mortality. For a number of years, tibia ash or toe ash has been used as the primary determinant of the P requirements of the chick, being rather sensitive to differences in dietary P content or to

differences in biological availability between P sources. The gap between maximum tibia ash and maximum body weight is much narrower today than years past, due to the much more rapid growth of broilers today than in previous years. Though the bulk of the P requirements are related to skeletal development, P is also actively involved in energy metabolism in the body. Therefore, one might expect that the P demands of the modern bird for support of body weight gain should be much greater than in previous years (Waldroup, 1999).

1.2.2 Interaction of phosphorus with other nutrients in the gut

P and Ca interact before and after their absorption from the digestive tract. Excess amounts of either, relative to the other, interferes with the utilization of the other (Smith, 1990). In an experiment conducted by Rao and Roland (1990), low dietary P (3 g total P/kg) resulted in excessive excretion of Ca, when 40 and 60 g Ca/kg diet were supplied. This excessive excretion of Ca was attributed to the decrease in plasma P levels. Low plasma P levels stimulate the synthesis of 1,25-dihydroxy cholecalciferol (1,25-$(OH)_2D_3$) through a different pathway from the one for low plasma Ca. In a situation of low plasma P, an insulin-like growth factor-I, but not parathyroid hormone (PTH), is involved in the stimulation of 1,25-$(OH)_2D_3$ synthesis. The 1,25-$(OH)_2D_3$ synthesized in response to low plasma P, initiates the absorption in the intestines of P and Ca, even though the plasma Ca is not low. However, since the PTH was not responsible for the production of 1,25-$(OH)_2D_3$, the kidneys are not stimulated to prevent the loss of Ca^{2+}, therefore Ca increases in the urine (Rao and Roland,1990) (note: PTH stimulates the reabsorption of Ca in the kidney tubules). Total P of about 6 g/kg diet should always be present in the feed to prevent excessive loss of Ca, which can predispose the bird to kidney abnormalities like uroliths (Rao and Roland, 1990).

Urolithiasis is an acquired degenerative kidney disease involving renal atrophy, fibrosis and kidney stone (urolith) formation in the collecting ducts and ureters. The condition occurs primarily in caged laying hens, but immature pullets also have been diagnosed as having urolithiasis (Oldroyd and Wideman, 1986). In a study conducted with immature pullets by Wideman, Closser, Roush and Cowess (1985) it was observed that 0%, 12%, 2%, and 14% of hens fed diets with normal Ca (10 g/kg diet): normal available P(6 g/kg diet), high Ca (32.5 g/kg diet):normal available P (6 g/kg diet), normal Ca (10 g/kg

diet):low available P (4 g/kg diet) and high Ca (32.5 g/kg diet):low available P(4 g/kg diet), respectively, were observed to have uroliths. In the same study 1% of the pullets fed diets with high Ca (32.5 g/kg diet):normal available P (6 g/kg diet) developed uroliths compared to 14% of pullets fed a diet with high Ca (32.5 g/kg diet):low available P (4 g/kg diet). No uroliths were observed in the pullets raised on diets with normal Ca:normal available P and normal Ca:low available P. It is evident from the results of this experiment that high Ca (32.5 g/kg diet) leads to uroliths in immature pullets due to the occurrence of excessive kidney excretion of Ca. The effect of excess Ca will accelerate if the diet contains low available P (Wideman *et al.*, 1985), which leads to blocking of collecting tubules and ureters by Ca. Low P levels in the interstitial fluids around the tubules cause this excessive excretion. In the tubules the Ca is reabsorbed into the body by way of being exchanged with P from the interstitial fluids around the tubules. Most chickens affected by uroliths continue to be productive if approximately one-third of their normal kidney mass remains functional, but death result when uroliths obstruct urine drainage from the remaining kidney tissue (Oldroyd and Wideman, 1986). Mortality may be reduced and productivity increased if urolith formation can be prevented. This may be accomplished by modifying the diet to alter the composition of the urine, specifically urinary Ca content.

High dietary Ca and P (22.6 g/kg diet and 8.3 g/kg diet, respectively) increase the intestinal pH and reduce the soluble fraction of minerals, and consequently their availability for absorption also decreases (Yi *et al.*, 1996). This effect will adversely affect the performance of chickens fed on deficient or marginally sufficient concentrations of these minerals, e.g. Cu, Fe, Mg and Zn. This fact is supported by the decrease in shell thickness when the available P level is increased from 2.5 to 4.5 g/kg (Hossain and Bertechini, 1998a). This decrease in shell thickness indicates that the increase in P leads to formation of an insoluble complex containing P and Ca, making Ca less available for shell formation (Hurwitz and Bar, 1971). Van der Klis *et al.* (1997) observed that ileal absorption of P is reduced as Ca content of the diet increases. The P retention was 435 g/kg diet and 344 g/kg diet when Ca content of the diet was 30 g/kg and 40 g/kg, respectively. Van Rensburg and Smith (1992) in their experiment with broilers observed highest P availability when the Ca:P ratio was 2:1.

Dolomitic limestone is often used as a calcium supplement in layer diets. The Magnesium (Mg) content of dolomitic limestone varies from less than 10 g/kg to more than 13 g/kg

(Hess and Britton, 1997). Excess Mg (8 g/kg diet) reduces egg production, decreases feed consumption, decreases body weight, decreases percentage shell and reduces plasma Ca in layers (Hess and Britton, 1997). In broiler chicks, it decreases 28-day body weight, increases mortality and induces leg abnormalities. Excess Mg reduces parathyroid hormone activity and consequently the blood Ca, shell quality and egg production are reduced. High available P (9 g/kg diet) is effective in alleviating all of the above symptoms when there is excess Mg in the diet (Lee and Britton,1980; Hess and Britton, 1997 and Hossain and Bertechini 1998b). This means that the Mg content of the diet should always be monitored since it can influence the P requirements of the bird. Hossain and Bertechini (1998a) observed maximum egg weight with 2.5 g available P/kg and 4.5 g Mn /kg in the diet but 4.5 g available P/kg with 2.00 g Mn /kg significantly reduced feed consumption between 42 and 52 weeks of age in layers.

The amount of available P in the diet should be formulated to cater for production and physiological processes, which involve P. These include bone formation, the ionic exchange between HCO_3^- and $H_2PO_4^-$ in the kidneys during shell formation and formation of different egg components. Caution should be taken not to provide excess P, which would otherwise form insoluble substances with other minerals. The Mg and Ca contents of the diet should also be considered during formulation since they influence, to some extent, the P requirement of the bird.

1.3 Phosphorus in Feedstuffs and Complete Feeds

1.3.1 Feedstuffs

1.3.1.1 Feedstuffs of plant origin

It is estimated that on average 0.67 of the P in ingredients of plant origin exist in a form of phytate-P (i.e. P bound in phytic acid), which has a low availability to poultry (Wailbel, Harms and Damron, 1977; Simons *et al.*, 1990). Recent experiments have shown a great variation of phytate-P content in ingredients of plant origin (Table 1.2 and Table 1.3). Phytate-P, therefore, contributes to the P pollution problems in countries with intensive poultry production because it increases P content of the excreta (Simons *et al.*, 1990).

The phytic acid (inositol-hexaphosphate) has six phosphoric acid groups which are not available to the bird (Gordon and Roland, 1998). It is present in plants as a source of P and cations for use in germination and maturation of plants (Ertl, Young and Royboy, 1998; Royboy, Noaman, Taylor and Pickett, 1991; Kikunaga, Takahashi and Huzisige, 1985). The structure of the phytate shown in Figure 1.1 is a generally accepted structure because this model can best explain many of the physicochemical properties, interactions and nutritional effects. At neutral pH the phosphate groups in phytic acid have either one or two negatively charged oxygen atoms; hence, many cations are able to chelate strongly between two phosphate groups, or weakly with a single phosphate group (Figure 1.2). Phytate has been considered as a nutrient because it contains P. It can also be considered as a toxin because it binds various essential elements and reduces their availability (Sebastian, Touchburn and Chavez, 1998).

Many publications have revealed a negative effect of phytate on protein digestion. These papers suggest that the phytate can exert it effect both on the substrate and on the enzymes responsible for protein hydrolysis. The interaction between the phytic acid and proteins is ionic and pH dependent. Phytic acid can form complexes with proteins at both acidic and alkaline pH. At acidic pH (about pH 2) phytic acid is strongly negatively charged while proteins are strongly positively charged, thus phytate–protein complexes can be formed. At high pH (basic pH) a strong protein-phytate interaction is suggested. At this pH level both phytate and protein is negatively charged so that multivalent cations such as Ca are thought to mediate such phytate-protein complexing. The complexes formed have a low solubility (Sebastian *et al.,* 1998). Phytate is known to inhibit a number of digestive enzymes such as pepsin, alpha-amylase and trypsin (Sebastian *et al.,* 1998).

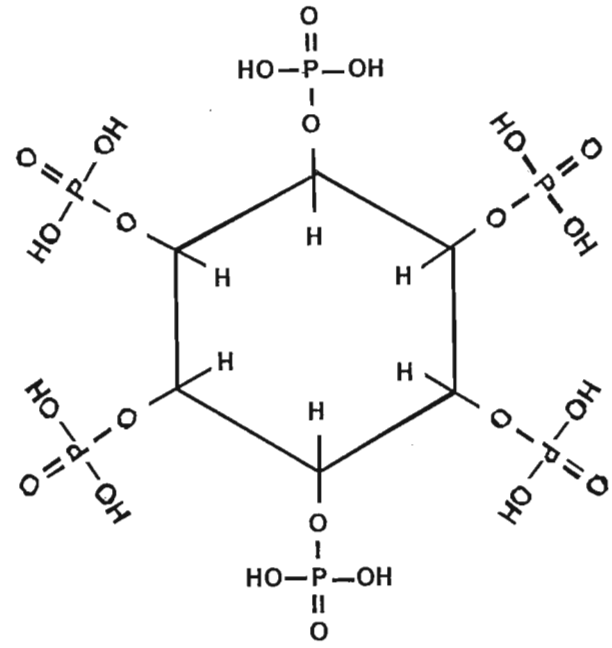

Figure 1.1 Structure of phytic acid (Sebastian et al., 1998)

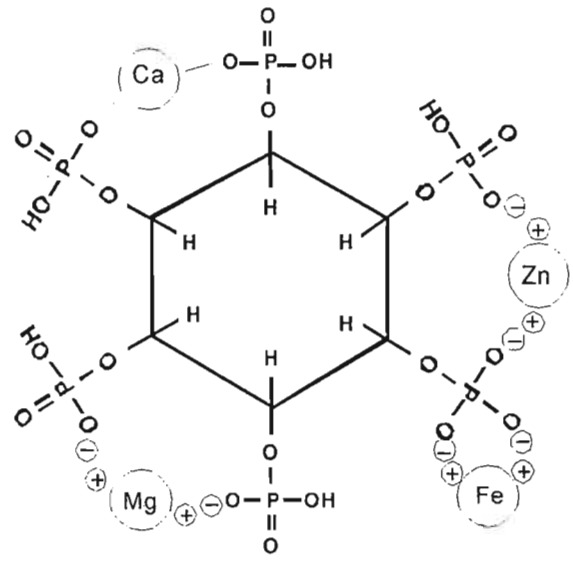

Figure 1.2 Phytic acid chelate at neutral pH (Sebastian et al., 1998)

Knuckles, Kuzinicky and Betschat (1985) investigated the effect of phytate on the hydrolysis of casein and bovine serum albumin (BSA) by pepsin *in vitro*. The phytate significantly decreased pepsin digestion of both casein and BSA. The effect of phytate

level was observed to be linear and independent of digestion time. They suggested that the cause of the decreased digestibility of the two protein sources is the formation of phytate–protein complexes. The proteins in these complexes are less susceptible to enzymatic attack because the sites of action of enzymes in these are covered by the phytate. It was also observed that the decrease in digestion of BSA was smaller compared to that of casein. This difference was attributed to the difference in amino acid profile and/or the difference in the ability of the two proteins to bind phytate. The difference in the effect of phytate on digestibility of different protein sources can be further affirmed by the fact that soluble corn germ proteins do not bind phytate while soybean protein binds strongly to phytate. These two protein sources have a markedly different amino acid profile (Knuckles *et al.*, 1985).

O'Dell and de Boland (1976) investigated the effect of amino acid profile on the binding of phytate by protein sources. Soyabean protein was able to bind phytate stronger than sesame protein. This observation was attributed to the fact that soybean protein is rich in lysine while sesame is rich in arginine. It was also observed that the order of binding strength to phytate was lysine>histidine>arginine when phytate was reacted with serum albumin.

The phytate affects the functioning of various digestive enzymes mainly by its ability to chelate Ca^{2+} ions. These ions are required by these enzymes for their activation and stability (Sebastian *et al.*, 1998). Trypsinogen, the inactive precursor of trypsin, is known to bind Ca^{2+} ions at two sites; one, occurring also in the trypsin, is located in the body of the molecule (Singh and Krikorian, 1982). The second, a weaker binding Ca^{2+} ion, occurs only in trypsinogen and has been postulated to enhance the formation of active trypsin by increasing the rate of hydrolysis of Lys(6)-Ile(7) bond in trypsinogen (Caldwell, 1992). Singh and Krikorian (1982) observed that the trypsin activity in digesting casein decreases as phytate concentration was increased. The inhibitory effect of phytic acid on trypsin activity was attributed to the binding of trypsin Ca. They also suggested that under some circumstances *in vivo* this could well negatively affect the conversion of inactive trypsinogen into trypsin (Singh and Krikorian, 1982). The question naturally arises whether the phytate-based inhibition reported in various studies has any significance in animal nutrition in terms of possible effects on protein availability (Singh and Krokorian, 1982).

Thompson and Serraino (1986) investigated the effect of phytate content on amino acid digestibility *in vivo*. The phytate content of rapeseed was reduced by 50% in one dietary treatment and left unaltered in one treatment; these diets were fed to weanling rats. The true and apparent digestibilities of amino acids were not significantly different between the high and low phytate (phytate content reduced by 50%) diets. Caldwell (1992) suggested possible explanations for the observed absence of effect of phytate on protein digestion in some studies. The first was the effect of the amino acid profile of the test ingredient, i.e. the test ingredients could be low in lysine. Secondly, it was suggested that it could be the ability of the pancreas to increase secretion of digestive proteases in response to proteolytic dysfunction instigated by the phytate. Pancreatic enzyme secretion is stimulated by cholecystokinin (CCK). In many species the release of CCK is under negative feedback control by trypsin.

Recent experiments have shown that the extent of utilization of phytate-P differs between ingredients (Punna and Roland, 1999). Some plant feedstuffs, such as wheat and barley, contain powerful phytases capable of hydrolyzing phytic acid to inorganic P and inositol within the bird's digestive tract (Table 1.2). Eeckhout and Paepe (1994) observed that wheat, barley and wheat byproducts have phytase activities that are well above 500 PU (phytase units)/kg. High variability that was observed in phytase activities of wheat byproducts was suggested to be due to different processing conditions to which the wheat was subjected. The byproducts of wheat exhibited very high phytase activities, which for some was above 4000 PU/kg. Pelleting of fine wheat bran reduced phytase activity from 4601 to 2513 PU/kg. These findings are supported by the observations of Sauveur (1989) who showed that P availability for chicken bone mineralization is above 50% in wheat and barley whereas it is less than 20% in maize and soybean. Wecke *et al.* (1992) observed lowest P utilization in maize/soyabean diets not supplemented with inorganic P. Punna and Roland (1999) observed that chickens fed corn based diets retained less phytate-P than chickens fed wheat-corn-based diets. The greater part of variation in phytate-P utilization exhibited by different feedstuffs can be attributed to variations in phytase activities of these feedstuffs.

Table 1.2 Total and phytate phosphorus contents and phytase activities of selected plant ingredients and their byproducts (Eeckhout and Paepe, 1994)

Feedstuff	Total P (%)	Phytate P (% of Total P)	Phytase activity (Units/kg)
Wheat	0.33	67	1193
Barley	0.37	60	582
Peas	0.38	45	116
Wheat bran	0.95	76	4601
Wheat bran (pelleted)	1.01	77	2573
Wheat middlings	0.80	66	4381
Wheat feed flour	0.56	70	3350
Malt sprouts	0.60	2	877
Corn distillers	0.90	21	385

Table 1.3 Phytate phosphorus content of selected plant feedstuffs (Eeckhout and Paepe, 1994)

Feedstuff	Phytate Phosphorus (%)
Sorghum	70
Oats	59
Barley	60
Rye	61
Wheat	67
Maize	68

1.3.1.2 Feedstuffs of animal origin

For many years the assumption has been made that P from animal proteins is readily available (Waldroup and Adams, 1994). Recent studies have refuted this assumption by showing that P from these ingredients is actually less available than assumed, but still remains superior to plant sources (Table 1.4) (Waldroup, 1999). This is supported by findings of Wecke *et al.* (1992) that P utilization is superior for fish meal compared to soyabean meal. The P in casein and in isolated soyabean meal protein is estimated to be 48% and 3% available, respectively (Harrold, Slanger, Haugse and Johnson, 1983)

Table 1.4 Total and available phosphorus contents of selected ingredients (Waldroup, 1999)

Feedstuff	Total P(%)	Available P (% of total P)
Bone meal	7.6	59.0
Fish meal	2.2	74.0
Meat meal	2.9	65.0
Meat and Bone meal	6.0	66.0
Dicalcium phosphate	19.7	55.0
Monocalcium Phosphate	22.6	84.0

Animal protein supplements, such as meat and bone meal and poultry byproduct meal, are utilized by the poultry industry both for their high quality protein and for their P content. Any reduction in P availability in these feedstuffs can seriously affect their value in poultry feeds. Because of the continued problems with leg disorders in boilers, reduction in P provided by animal protein sources may further curtail usage of these supplements (Waldroup and Adams, 1994).

Waldroup and Adams (1994) conducted an experiment to verify the concerns about the reduction of the availability of P from animal protein sources. There was no difference in P availability when using bone formation as the criterion between combined poultry byproduct meal or combined meat and bone meal samples, compared with the reference standard, which was monocalcium phosphate. The relative biological availability of P from

fishmeal, poultry byproducts meal and meat and bone meal were 102%, 101% and 102%, respectively. High variability of available P content in animal byproducts has been reported (Table 1.4). This is a major problem associated with the use of animal byproducts, especially when dietary excesses are to be limited (Waldroup, 1999).

Use of average values for P content of animal byproducts can result in considerable overestimation (resulting in potential P deficiencies) or underestimation (resulting in increased excretion) of the dietary P level when these values are used for feed formulations. Because of the economics of P nutrition, it does not seem feasible to avoid totally the use of animal byproducts. It does appear that a more stringent quality control program must be implemented so that the actual mineral content of each shipment is known and verified prior to feed manufacturing, so that the ingredient matrix can be adjusted accordingly (Waldroup and Adams, 1994; Waldroup, 1999).

1.3.1.3 Inorganic phosphorus sources

The most commonly used inorganic P sources in poultry diets are the calcium phosphates. These phosphates are produced by reacting phosphoric acid with limestone to produce mixtures of mono- and dicalcium phosphates (MCP and DCP). The composition of these mixtures is determined by the quantity of limestone that is reacted with phosphoric acid (Waldroup, 1999). It was previously assumed that all P from inorganic sources is available but recent studies have shown that this is not the case (Table 1.4). Waldroup (1999) observed that the P digestibility in turkeys utilizing MCP and DCP is 519 and 306g/kg diet, respectively. Because supplemental phosphates generally provide approximately 60% of the non-phytate P needs of the chick, small variations in their bioavailabilities may impact significantly on the feacal P content (Waldroup, 1999). The P retention percentage of a diet decreases as the inorganic P content of the diet increases (Table 1.5). This decrease in P retention is significant when the inorganic P content of the diet is increased from 4.5-5.4 g P/kg diet (Table 1.5)

Table 1.5 Effect of inorganic Phosphorus on total Phosphorus retention (Yi et al., 1996)

Inorganic P (% of the diet)	P retention (% of the dietary P)
0.27	58.5
0.36	57.9
0.45	57.2
0.54	47.1

1.3.2 Complete Feeds

1.3.2.1 Effects of dietary fat on phosphorus retention

Fats are widely used in poultry feeds as a concentrated source of energy, with the aim of promoting rapid growth (Table 1.6). Fatty acids from fats are capable of dissociating in solution to form soaps in the presence of metals (Atteh *et al.,* 1983). Many experiments have shown a decrease in retention of some minerals as the fat content of feeds increase.

Table 1.6 Effect of fat supplementation on body weight gain (Atteh *et al.*, 1983)

Supplemental Fat (%)	Body weight gain (g)
0	469.6^{a}
3	481.2^{a}
6	478.2^{a}
9	513.7^{b}

Similar letters show non-significant differences between values and different letters show significant differences, e.g. $^{a, b}$

Atteh *et al.* (1983) observed a significant reduction in bone Ca content as fat inclusion levels in the diet increased from 0 to 9% (Tables 1.7). This reduction was not observed in the contents of other minerals, i.e. magnesium (Mg), P, zinc (Zn) and manganese (Mn). In contrast to the observations by Atteh *et al.* (1983), Whitehead, Dewar and Downie (1970) observed a decrease in the retention of Mg and Zn and also of Ca and iron (Fe) as fat content of the diet increased above 5%. The reduction in Ca retention with fat

supplementation suggests that a substantial formation of insoluble Ca soaps resulted, which were not absorbed (Atteh *et al.*, 1983). There was a less drastic effect of a blend of animal and vegetable (A-V) blend on bone metabolism compared with corn oil. This suggests that, in addition to fat level, fat source has an effect on the retention of minerals due to differences in fatty acid profile of different fat sources. The reduction of percentage retention of minerals as fat inclusion levels increase is not related to the rate of passage of digesta through the digestive tract.

Maetos, Sell and Eastwood (1982) observed that the rate of passage of food through the digestive tract decreased as the fat content of the diet increased. The lower effect of A-V blend fat on bone metabolism relative to corn oil can be attributed to a higher proportion of oleic acid, the soaps of which have been shown to be well utilized (Atteh *et al.*, 1983). In contrast, corn oil contains predominantly linoleic acid, the soaps of which are less well utilized. This fact is supported by Whitehead *et al.* (1970) who suggested that some chemical processes might have occurred in the gut, which resulted in the reduction of absorption of minerals, probably the formation of soaps (Table 1.8).

It is expected that a significant reduction in bone Ca content would be accompanied by a significant decrease in bone P content, since both minerals are closely related in bone formation. But the bone P was not significantly reduced in the study by Atteh *et al.* (1983) (Table 1.8). The product of the reaction between the component fatty acids and P is probably soluble and readily absorbed, explaining the non-significant change in bone P content (Atteh
et al., 1983). Since there seem to be contrasting observations on the effect of fat on P retention, further investigation of this aspect is necessary for the objective of reducing P in the excreta. It is thus possible that chicks consuming P at their previously determined minimum requirements may become deficient if fats are added due to poor retention of P (Atteh *et al.*, 1983).

Table 1.7 Effect of fat supplementation on bone mineral retention percentage (Whitehead *et al.*, 1970)

Supplemental Fat (%)	Ca	Fe	Mg	Zn
0	30.31	44.40	40.36	34.27
Maize oil 5%	27.95	38.22	30.10	27.09
Maize oil 10%	24.47	33.12	25.51	27.67
Maize oil 15%	24.95	30.32	27.15	23.07
Palmitic 10%	12.09	11.31	18.08	19.87
Tallow 10%	22.22	21.66	22.03	24.61
Lard 10%	14.49	18.64	19.87	20.67

Table 1.8 Effect of fat supplementation on mineral composition (%) of the bone (Atteh *et al.*, 1983)

Fat (%)	Bone Ash (%)	Ca	P	Mg	Zn	Mn
0	45.1	32.4	15.2	0.58	35.4	10.3
3	45.8	29.7	14.7	0.57	39.1	10.5
6	45.7	30.0	15.3	0.61	38.0	9.6
9	45.9	30.1	15.0	0.67	37.1	10.7

1.3.2.2 Factors affecting dietary phytate phosphorus utilization

Interaction of phytic acid with other nutrients in the diet can further decrease the utilization of phytate-P from ingredients of plant origin. This has been observed in various experiments mainly with Ca and inorganic P contents of the diets. Punna and Roland (1999) observed that broilers consuming a P deficient (1 g available P/kg diet) diet utilized significantly more phytate-P than birds consuming a P adequate (5 g/kg diet) diet. Birds on

the P deficient diet utilized 655 g phytate-P/kg diet, while those on an adequate P diet utilized only 195 g phytate-P/kg diet. Birds on P adequate diets were not pressed to utilize their P sources from phytate molecules. It was also observed in this trial that an increase in dietary Ca content reduced the utilization of phytate-P. To enhance the utilization of phytate-P, the Ca supplied in the form of dicalcium phosphate should not be replaced in the P deficient experimental diets. Not replacing Ca more closely simulates P deficiency conditions occurring in the industry (Punna and Roland, 1999).

1.4 Reducing phosphorus content of excreta

1.4.1 Phytase

Phytic acid chelates mineral ions in the ingredients of plant origin (Biehl and Baker, 1997). These ions, including P, are less available for utilization by the birds. Microorganisms and fungi produce an enzyme 3-phytase, which removes orthophosphate from the third position of the phytate. Phytases from both these sources can successively remove the remaining orthophosphates, resulting in intermediates ranging from free myo-inositol to mono- to tetra-phosphates of inositol (Lynch, 1996). This makes P more available to the bird, and consequently less P is excreted. Maenzi and Classen (1998) reported a 0 and 8 g/kg diet hydrolysis of phytate P from corn and wheat-based diets, respectively, which makes the P contained in these ingredients more available for absorption in the gut. The endogenous phytase present in the avian duodenum is relatively inefficient in releasing inorganic P from the phytate complex present in feeds based on plant ingredients (Biehl and Baker, 1997). However, exogenous phytase addition is very efficacious in enhancing the utilization of phytate P. Supplemental phytase could prove beneficial in improving the availability of P to the bird and reducing excreta P, therefore preventing the pollution of water tables.

Supplemental phytase can improve the performance of birds fed a diet deficient in P, to be equivalent to that of birds whose diet has been supplemented with monocalcium phosphate (4.5 g available P/kg diet) (Simons *et al.*, 1990; Lynch, 1996; Gordon and Roland, 1997; Van der Klis, Versteegh and Kies, 1997; Carlos and Edwards, 1998, Maenzi and Classen, 1998). Gordon and Roland (1997) observed that 1 g monocalcium phosphate/kg of the feed is equivalent to 280 IU of phytase /kg of feed. Van der Klis *et al.* (1997) observed 250

phytase units /kg diet to be equivalent to 1.3 g P from monocalcium phosphate. Broz, Oldale, Perrin-Voltz, Rychen, Schulze and Nunes (1994) observed that 500 Phytase Units (PU)/kg increased weight gain by 131 g/kg, feed intake by the same margin, tibia ash percentage from 527 to 544 g/kg bone weight, and P in excreta was reduced by 10.1%. The increase in P retention is accompanied by an increase in retention of Ca (Simons *et al.*, 1990). Gordon and Roland (1998) observed that supplementation of 300 phytase units/kg to a diet containing 28 g Ca/kg diet and 1 g inorganic P/kg diet increased shell weight from 4.89 g to 5.17 g/egg and the egg production of the unsupplemented group decreased from 78% to 49% while the production was maintained at 78% by the supplemented group when inorganic P was reduced from 3 to 1 g/kg diet. Simons *et al.* (1990) observed an increase of 60% in P availability and a reduction of 50% in excreta P when 2000 PU/kg was supplemented. Yi *et al.* (1996) observed that supplemental phytase of 350, 700 and 1040 PU/kg of corn-soya diet increased body weight gain by 11 to 22%, feed intake by 6 to 25%, toe ash percentage by 44 to 188 g/kg toe weight and P retention by 30 to 250 g/kg diet. In this experiment it was calculated that the P excreted could be reduced by phytase supplementation from 55 to 33 kg of phosphate per year in 350 broilers, therefore reducing the possibility of environmental pollution by P. Phytase also improves feed conversion efficiency (Table 1.9) (Simons *et al.*, 1990). When Nahashon, Nakacie and Mirosh (1994) supplemented a layer diet with lactobacillus and cane molasses, the improvement in layer parameters were the same as those of prepared phytase. They suggested that the observed decrease in gut pH with supplementation could have aided the solubility and consequently the absorption of P in the gut. The acid environment facilitates the ionization of minerals, whereas a basic environment complexes the minerals with OH^-.

All response parameters (tibia ash, weight gain, feed intake and feed conversion efficiency) show a quadratic response to phytase supplementation (Yi *et al.*, 1996). This means that the level of inclusion of phytase should be where maximum profit can be obtained from that diet. Bosch *et al.* (1997) investigated the unclear aspect of economic benefits of phytase. They observed that the costs incurred by buying phytase are met by the fact that phytase reduces the cost of monocalcium phosphate or dicalcium supplementation from which there may be no benefit.

Table 1.9 The effect of microbial phytase on the apparent availability of total phosphorus, P in manure and performance of broilers* (Simons *et al.*, 1990)

P_i (g/kg)	Ca (g/kg)	Added Phytase (U/kg)	P availability (%)	Ca availability (%)	P In Manure (g/kg)	Feed conversion ratio
4.5	6.0	0.0	49.8^{a}	47.2^{a}	2.7^{a}	1.69^{a}
6.0	7.5	0.0	45.6^{b}	48.9^{a}	3.8^{b}	1.48^{b}
7.5	9.0	0.0	44.6^{b}	46.9^{a}	4.9^{c}	1.38^{b}
4.5	6.0	250.0	56.5^{c}	57.1^{b}	2.3^{d}	1.46^{b}
4.5	6.0	500.0	59.6^{cd}	59.3^{bc}	2.1^{de}	1.40^{b}
4.5	6.0	750.0	59.5^{cd}	60.3^{bc}	2.1^{de}	1.37^{b}
4.5	6.0	1000.0	62.5^{de}	64.3^{bc}	2.0^{e}	1.38^{b}
4.5	6.0	1500.0	64.5^{e}	68.1^{d}	1.9^{e}	1.34^{b}

Different letters signify a significant difference in terms of response for that particular treatment, e.g. a, b, c, d, e.

They also observed that the phytase supplementation increased the value of the manure by reducing its P content. From these facts it was concluded that phytase supplementation is economically viable.

1.4.2 Supplying P to meet physiological requirements

Due to the problem of environmental pollution caused by the N and P contents of poultry excreta, and the expenses attached to dietary P supplementation, ways and means of reducing P content of the diet without compromising performance have been considered. Keshavarz (1998a and b) conducted an experiment with 30-week old pullets in an attempt to reduce dietary P by increasing the P content to adequate levels only during times of the day when there is a physiological need for it. It was assumed that Ca and P intake increase on shell forming days as compared to days on which shell formation does not occur. In this study the birds were fed diets containing 4 and 2 g available P/kg diet, the other group was fed 4 g available P/kg diet during the morning (i.e. 05:00 to 13:00) and 2 g available P/kg diet in the afternoon (13:00 to 20:00) while the third group was fed the P in an inverse of the latter. The hypothesis was that decreasing the P content in the afternoon would be

beneficial in terms of shell thickness. The production traits of eggs were not affected by dietary treatment except for the lower body weight of birds fed 2 g available P /kg diet continuously. The results of this experiment suggest that the available P content of the diet can be decreased to an extent to reduce P levels in the excreta without affecting production.

1.4.3 Plant genetic approach

Ideally, reducing the phytate content of ingredients of plant origin will lead to the increased availability of P and other ions chelated by the phytate for utilization by the birds. Breeding of grain varieties with low phytate content can reduce or eliminate the costs of inorganic P supplementation and also the amount of P excreted by birds (Ertl *et al.*, 1998). There are two main drawbacks with this approach, one being the high correlation of phytate and total P (r=0.99), the other being the high correlation of phytate and protein (Raboy *et al.*, 1991 and Ertl *et al.*, 1998). Breeding programs designed to reduce phytic acid in corn would likely reduce total P in the corn because of the relation of the two. This is not a desirable outcome from a nutritional point of view. Corn carrying defective kernel mutations have shown that embryo mutations have reduced phytic P but not total P, providing evidence that the relationship between the two forms of P could be broken (Ertl *et al.*, 1998). These mutants showed a reduction of between 50 and 60% in phytic acid with no decrease in total P and 75% of P was in the form of non-phytate P in seeds (Ertl *et al.*, 1998 and Waldroup, 1999). Raboy, Dickenson and Neuffer (1990) observed a concomitant increase in inorganic P whenever phytic acid content was reduced in maize mutants.

If the P content of the excreta is to be reduced using the breeding approach, the goal should be to reduce that fraction of the seed total P represented by phytate P, while maintaining seed total P (Raboy *et al.*, 1991). A positive correlation between grain phytic acid and total protein also would be important in breeding programs aimed at either increasing protein or reducing phytic acid (Raboy *et al.*, 1991).

1.4.4 Vitamin D

Recent research suggests that inclusion of Vitamin D (Vit D) isomers in the diet may be a factor in reducing P excretion (Wasserman and Taylor, 1973). These are 1-alpha-OHD_3,

1,25-$(OH)_2D_3$ and 25-OHD_3. The 1- alpha-OHD_3, the precursor of 1,25-$(OH)_2D_3$ (the bioactive form of Vit D) does not require the kidneys, but only the liver, for conversion to 1,25-$(OH)_2D_3$ (Van der Klis *et al.*, 1997). There are two suggested modes of action of these isomers, one being that they enhance the intestinal phytase activity of the birds (Biehl and Baker, 1997 and Waldroup, 1999). Biehl and Baker (1997) suggested that these isomers might be transported back to the gut via biliary flow. The second mode of action is that they somehow make the phytate complex more vulnerable to attack by phytase.

Biehl and Baker (1997) observed that 15 g^{-6} 1-alpha-OHD_3 /kg diet reduced P excreted from 0.89 to 0.59%. The retention of P was 600 g/kg diet in chickens fed 14 g non-phytate P/ kg diet and 440 g/kg diet in those fed 2.1 g non-phytate P/kg diet. Van der Klis *et al* (1997) observed that supplementation 15 g^{-6} 1-alpha- /kg increased weight gains by 27%, tibia ash concentration by 380 g/kg bone weight and total weight of tibia ash by 720 g/kg bone weight. These Vit D isomers are comparable, in terms of efficacy, to the other means of reducing P in excreta. When 15 to 20 g^{-6} 1-alpha- OHD_3/kg is included in the diet, this supplementation produce similar results to 1 g inorganic P/kg diet supplementation or 1200 units/kg of supplemental phytase.

1.4.5 Fermentation

The fermentation process that distillers grains undergo seems to increase the P availability (Sauveur, 1989; Eeckhout and Paepe, 1994), presumably as a result of partial phytate hydrolysis. Phytic acid seems to be totally hydrolyzed during processing of malt sprouts, which have an average phytase activity higher than barley (Eeckhout and Paepe, 1994). Bioavailability of P in high moisture ensiled maize is three to four times higher than that of P in dry maize (Eeckhout and Paepe, 1994).

The ammonia emitted from poultry farms is the source of the unpleasant odor experienced by people living near these farms. The phosphates in the litter act as a buffer to resist the increase in pH by combining with NH_4^+ ions. The resultant NH_4^+ phosphate ions combine with Ca ions to form a solid that reduces the availability of NH_3 to volatization. This negates the benefits of reducing P in the excreta (Ferguson, Gates, Taraba, Cantor, Pescator, Straus, Ford and Burnhams, 1998).

1.5 Discussion

The importance of P in poultry production is unquestionable. The problem of excess P in poultry litter originates from the oversupply of P in poultry rations. Recently a considerable amount of research has been focused on reducing the P content of the excreta due to the pollution hazard posed by high litter P. The emission of ammonia from the litter has to be considered when attempts to reduce P are made. The available P concept as a measure of how much P will be utilized in a diet needs to be reviewed. The limitations and inadequacies of the available P values were discussed in this review. With the available P it is assumed that ingredients of plant origin have one-third available P out of their total P, the remaining two-thirds being held up in phytate. This has been observed not to hold true for all these ingredients, since there is a wide variation in their phytate contents. The P from inorganic and animal sources is also not totally available as has always been assumed. The production of feed phosphates has undergone continual improvement; therefore, examination of the most recent studies would be the most informative with regard to the availability of P in these sources.

As dietary P levels increase from deficient to excessive, fecal P levels increase gradually until the point at which tibia ash is at maximum, and then increases steeply. Regardless of whether one employs phytase enzymes, genetically modified corn, or any other means of improving P utilization, the biologically available P content of the diet must not exceed the amount needed by the chick to maximize performance (Waldroup, 1999). This finding calls for an investigation of an accurate measure of P that can be utilized from the diet that will bring about a reduction in excreta P content without compromising production.

The concept of 'available' P is a meaningless term; it is not the amount of P available to the bird, nor is it an accurate estimate of the digestibility of the P in the ingredient or feed. Consequently, it is not worth considering this term any longer. Instead, the digestible P content of ingredients should be known (measured and used) in order to ensure that the correct amount of P is included in the feed. In order to measure digestible P there are two techniques available, i.e. the tibia ash content and the ileal digestible P, and these will be compared.

Phytase enzymes are capable of increasing the digestible P content of ingredients high in phytate P, and the extent to which this enzyme can act in this way can be measured only once the digestibility of P in feedstuffs is known. The measurement of the activity (or efficacy) of phytase enzyme in improving digestibility of P will be measured whilst determining the digestible P content of feedstuffs. Because dietary Ca levels influences P digestibility, a further aspect of the present study will be to measure this relationship.

Chapter 2

Determination of digestible phosphorus content of common feedstuffs used in poultry diets

2.1.1 Introduction

For many years the general assumption has been that broilers and laying hens are unable to utilize phytate-bound phosphorus (P) from ingredients of plant origin, whereas the remaining P, together with P from animal protein sources and inorganic P supplements, are readily available (Waldroup, 1999). Recent studies have disputed this assumption. These studies have shown that broilers are capable of utilizing a portion of the phytate-bound P and that the availability of P from animal and inorganic sources is often less than 100% (Waldroup and Adams, 1994; van der Klis and Versteegh, 1996; Wiltenburg, 1997 and Waldroup, 1999). A P quantification technique that is based on digestibility of the P in raw material, instead of the assumption that inorganic P is 100% available and phytate-P is not available, would be very useful if the excreta P content from poultry is to be reduced (Wiltenburg, 1997) and such a technique has now been developed (van der Klis and Block, 1997).

Phosphorus is a structural component of various body tissues and, as part of adenosine triphosphate, it is involved in energy utilization (van der Klis, 1993b). This means that there are maintenance and production (i.e. growth in broilers and egg formation in layers) requirements of the birds for P (Wiltenburg, 1997). The P that is absorbed by the intestines of the bird is used mainly to meet these requirements. The determination of digestible P content of the feedstuffs used in poultry diets would ensure that the amount of P supplied in formulated diets is the amount that will actually be digested by the bird. Formulating the diets on the basis of digestible P will, assuming an unchanged net requirement, improve the P absorption and thereby reduce the gross P requirement of the bird (van der Klis, 1993b). As the dietary P concentration is generally based on the previous assumption of P availability from the various ingredient sources, this new quantification technique would

result in a more accurate measure of the P in feed ingredients with a consequent reduction of the P in the excreta (van der Klis, 1993a).

The growing broiler is a suitable type of bird to be used for the determination of digestible P content of ingredients on three accounts. The first is that they grow fast, so they have high nutrient requirements. The second is that adult cocks are non-productive, so the values determined with these birds would reflect only maintenance values. The third reason is that laying hens tend to have a diurnal pattern in mineral absorption, and there could be a risk that egg laying would cease due to the experimental treatment (van der Klis, 1993b).

Two techniques have commonly been used in measuring the P content of feed ingredient available to the bird. The first, tibia ash percentage, has been used based on the fact that P is, together with Ca, the main component of the bone (Morrissey and Wasserman, 1971; Harold *et al.*, 1983; Shafey McDonald and Dingle, 1991 and Keshavarz, 2000). Bone growth occurs by laying down $CaPO_4$ crystals to form bone matrix (Newman and Newman, 1953). The second technique makes use of ileal digestibility, which is a more accurate measure of digestibility than making use of tibia ash measurements. It takes into account the fact that P absorption takes place in the digestive tract, in the duodenum and the proximal jejunum. Therefore, whatever P found in the ileum is the undigested P (van der Klis, 1993a). The tibia ash technique is inaccurate on account that bone formation is not only dependent on P absorption from the digestive tract, but also on other factors like the presence of sufficient Ca for bone formation and on the acid-base equilibrium (van der Klis, 1993a). Both these techniques were used in the study reported here, the objective being to measure the digestible P content of (a number of) feed ingredients commonly used in the feed industry in South Africa.

2.1.2 Materials and methods

In this Chapter two experiments were combined into one, since the materials and methods for both experiments were the same in all respects except for the test ingredients used.

2.1.2.1 Pre-trial period

Sexed day-old Cobb broiler strain chicks were allocated, ten to a cage, to 48 wire cages for experiment 1 and to 96 wire cages, the sexes being kept separate. The birds were fed a commercial starter diet for 10 days. Water was made available to the birds through nipple drinkers. Water and feed were provided *ad libitum*. The birds were on a 23L : 1D lighting programme and the temperature were started at 32°C and were reduced by 0.5°C every second day. The starter diet was removed three hours before the introduction of the experimental diets, to purge the digestive tract of P residual from the previous diet.

2.1.2.2 Trial period

Both experiments commenced when the birds were 10 days old and the birds remained on the experimental diets for 10 days. During this period water and feed were provided *ad libitum*, the temperature was maintained at 26°C and the birds were on a 23L : 1D lighting programme.

2.1.2.3 Diets

A basal diet (Table2.1) that met all the nutrient requirements of the chick, except for P, was formulated because a substitution technique was to be used to ensure that all feeds would contain the same Ca content, and that the content of P in each diet could be adjusted without altering the proportions of other nutrients in the diets. This diet was mixed without including filler or limestone, these being added in the proportions required as dictated by the P and Ca contents of the ingredients to be tested. For the determination of ileal digestible P, an acid insoluble ash (celite) was included as an indigestible marker at a level of 30 g/kg to ensure that it would be sufficient for accuracy in analytical detection of this marker.

2.1.2.4 Standard curve

Two standard curves, one for each of the respective experiments were constructed by gradually increasing the P content of the basal diet with the use of phosphoric acid

(H_3PO_4). The P contents of the diets in the first standard curve were gradually increased up to 8.5 g/kg diet, and those for the second standard curve went up to 5 g/kg diet after it was discovered, in the first experiment, that there is no further increase in tibia ash above this concentration. The amount of filler was adjusted to take account of the H_3PO_4 that was added to each diet, as shown in Table 2.2.

2.1.2.5 Test ingredients

Seven feedstuffs commonly used in poultry feeds, as well as five monocalcium (MCP) sources, were selected for P digestibility determination. For each of the 12 ingredients, P digestibility was tested at two levels of inclusion of ingredient of the ingredient in the diet, resulting in 24 treatment combinations. The test ingredients and their levels of inclusion are shown in Table 2.2. In the Experiment 1, Maize, Fishmeal and MCP 5 were tested, and the rest of the ingredients were tested in Experiment 2. The MCP's varied in P and Ca contents as shown in Table 2.3. The test ingredients were added to the basal diet at two levels by substitution of filler. The amount of test ingredient used was based on the P content of the ingredient, the total P content of the final mix being 2.5 and 5 g/kg in each series. The Ca content of all test diets was maintained at 10 g/kg by adjusting the limestone addition to accommodate the Ca from the test ingredient.

2.1.2.6 Experimental design

The experiment was a completely random design (no blocking). Each treatment was replicated four times with each cage of ten birds representing a replicate. Two replicates consisted of males and the other two were females in all treatments. Feed was provided *ad libitum.*

2.1.2.7 Performance analysis

The body weight of all surviving birds in each pen was measured at the start and at the end of the experiment to determine the mean body weight of each bird and the weight gain. The average feed consumption per bird over the experimental period was also measured. This was obtained by weighing the feed supplied to each pen at the beginning of the experiment and that remaining at the end of the experiment. The difference between these

two weights divided by the number of live bird days over the experimental period was the average feed consumption per bird. The feed conversion efficiency (FCE) was calculated from weight gain and feed consumption (kg gain/kg feed consumed).

2.1.2.8 Tibia ash

The right tibia of each of five randomly selected chicks per pen was removed with a knife. Those from each pen were tied together by means of string and frozen to preserve them. On thawing they were boiled in distilled water for five minutes to soften the meat. The meat and cartilages were removed from the bone by hand. The bones were then dried at 70°C overnight and weighed. Each tibia was broken in the middle to expose the medullary bone prior to fat extraction. Fat was extracted from the bones, grouped on a pen basis, in boiling petroleum ether (72°C boiling point) for four hours. They were then air dried at room temperature for two hours to dissipate the petroleum ether, and thereafter dried at 70°C for one hour, after which they were weighed. The fat extracted bones were ashed in a muffle furnace at 600°C overnight. The tibia ash was weighed. The ash percentage was calculated as a percentage of fat extracted bone.

Table 2.1 Composition on as fed basis (g/kg) and calculated analysis of the basal diet used in the experiments

Ingredients	Quantities (g/kg)
Sugar	304.0
Soybean	462.0
Sunflower oil	84.0
DL-Methionine	2.5
Filler (Sunflower husks)	84.0
Celite	30.0
Limestone	24.6
L-Lysine HCl	0.5
Salt	0.8
Sodium bicarbonate	6.2
Vitamin and mineral premix	2.5
Calculated analysis	
Nutrient	Quantity
Metabolizable Energy	12.5 MJ/kg
Protein	215 g/kg
Calcium	10 g/kg
Total phosphorus	3 g/kg

2.1.2.9 Acid insoluble ash

Between one and three grams of the ileal contents and between two and three grams of each diet was weighed into ashed and weighed crucibles. The samples were dried in an oven at 70°C overnight. They were ashed by placing them in a cold muffle furnace, with the temperature set to rise slowly to 480°C, overnight. The crucibles containing ashed samples were put into beakers and 4M HCl was slowly poured into the beakers until the samples were wetted from underneath, and the crucibles were quarter filled with acid. This acid was boiled gently for 15 minutes, whilst ensuring that the crucibles did not boil dry and then the acid was removed under suction. The ash was rinsed with 4M HCl and then with distilled deionised water by suction. The crucibles were then put in a drying oven set at 70°C for two hours. They were then transferred to the muffle furnace and ashed

overnight at 480°C. The crucibles containing ash were cooled in a desiccator for 45 minutes and weighed. The quantity of the acid insoluble ash (AIA), expressed as a percentage of dry matter (DM) was then determined. The formulas for DM and AIA are as follows:

DM = (crucible + dry sample weight) - (crucible weight)

$$AIA\% = \frac{[(\text{crucible} + \text{Ash weight}) - \text{crucible weight}]}{DM} * 100$$

2.1.2.10 Calculation of digestible phosphorus

2.1.2.10.1 Ileal digestible phosphorus

The ileal contents from five chicks per pen were pooled and placed in a plastic bottle. The ileum was defined as that part of the intestine between the Meikel's diverticulum and the ileocecal junction. The ileal contents were freeze dried and later milled. The P content was determined in each ileal sample and in all diets using the method explained in AOAC (1990). The proportion P to AIA in the diet and in the ileal contents was determined. The difference between the proportion in the diet and ileal contents was the amount of P digested. This difference was expressed as a percentage of the P : AIA in the diet, i.e. digestible P (Ravindran, Cabahug, Ravindran and Bryden, 1999).

$$Digest.P = [((P / AIA)d - (P / AIA)i) / (P / AIA)d] * 100$$

Where: $(P/AIA)_d$ = ratio P to AIA in diet and $(P/AIA)_i$ = ratio P to AIA in ileal digesta.

2.1.2.10.2 Tibia ash

Once the tibia ash of the birds fed diets making up the standard curve, and those fed the test diets had been measured, an equation for a standard curve was derived with tibia ash percentage being regressed on the digestible P content of the diet. The tibia ash percentage from each pen was substituted in the standard curve equation to determine the digestible P content of the feed. This was then expressed as a percentage of the total P in the test ingredient, to determine the digestible P percentage.

Digestible P = (Ash percentage – constant)/gradient

$$\text{Digestible P\%} = \frac{\text{Digestible P} * 100}{\text{Total P in the test ingredient}}$$

Statistical analysis

Analysis of Variance was used to measure the significance of the differences between the treatments for all performance variables.

Table 2.2 Phosphoric acid, digestible phosphorus and filler contents on as fed basis (g/kg) added in diets for the standard curve of Experiments 1 and 2

Diet	Filler	H_3PO_4	Digestible P
Experiment 1			
Basal	84.0	0.00	0.0
1	80.9	3.10	1.0
2	77.4	6.60	2.0
3	74.6	9.40	3.0
4	71.6	12.4	4.0
5	68.4	15.6	5.0
Experiment 2			
Basal	84.0	0.00	0.0
1	83.1	0.90	0.3
2	82.1	1.90	0.6
3	80.9	3.10	1.0
4	79.9	4.10	1.3
5	79.3	4.70	1.5

Table 2.3 The calcium and phosphorus contents on as fed basis (g/kg) of the monocalcium phosphates tested in the experiments

MCP	Ca	P
MCP 1	215.7	185.7
MCP 2	181.0	180.9
MCP 3	168.2	176.5
MCP 4	130.0	195.6
MCP 5	135.0	143.5

MCP's 1-5 are monocalciumphosphates with different calcium and phosphorus contents

Table 2.4 The quantities (g/kg) of test ingredient, filler, limestone and basal diet included in test diets used in the experiments

Test ingredient	Test ingredient quantity	Filler	Limestone	Basal diet
Soya, 1	40.0	44.0	24.2	891.8
Soya, 2	80.0	4.0	23.8	892.2
Fishmeal, 1	39.0	45.0	12.3	903.7
Fishmeal, 2	84.0	0.0	4.9	911.1
Maize, 1	40.0	44.0	24.6	891.4
Maize, 2	80.0	4.0	24.6	891.4
Wheat, 1	40.0	44.0	24.5	891.5
Wheat, 2	80.0	4. 0	24.5	891.5
Gluten 60, 1	40.0	44.0	24.6	891.4
Gluten 60, 2	80.0	4.0	24.6	891.4
MBM[1], 1	10.0	74.0	20.9	895.1
MBM, 2	30.0	54.0	17.3	898.7
PBPM[2], 1	34.0	50.0	21.5	894.5
PBPM, 2	68.0	16.0	18.4	897.6
MCP[1] 1, 1	3.0	81.0	23.3	892.7
MCP 1, 2	6.0	78.0	22.0	894.0
MCP2, 1	3.0	81.0	23.3	892.7
MCP2, 2	6.0	78.0	22.0	894.0
MCP3, 1	3.0	81.0	23.3	892.7
MCP3, 2	6.0	78.0	22.0	894.0
MCP4, 1	3.0	81.0	23.3	892.7
MCP4, 2	6.0	78.0	22.0	894.0
MCP5, 1	10.0	74.0	20.3	895.7
MCP5, 2	20.0	64.0	16.1	899.9

MBM[1], meat and bone meal; PBPM[2], poultry by-product meal; MCP[1], monocalcium phosphate; ingredient, 1 or 2 = first or second level of phosphorus from the test ingredient. MCP's 1-5 are monocalciumphosphates with different calcium and phosphorus contents

2.1.3 Results

2.1.3.1 Standard curves

In the first standard curve the tibia ash percentage increased with increasing P, as H_3PO_4, up to 5 g/kg, after which there was no further increase in tibia ash percentage despite the

continued increase in P content of diets. This is what prompted the decision to decrease the maximum level of P added in standard curve diets to 5 gP/kg in Experiment 2. In the second standard curve the tibia ash percentage increased linearly with the increase in P content as shown in Table 2.5. The variances accounted for by the standard curves are 27.2 and 95.5% for standard curves 1 and 2, respectively, which improved to 98.2% when the two standard curves were combined, using the lowest three points of the first (i.e. up to 5 g P/ kg diet) and all points from the second standard curve. The combined standard curve is the one that was ultimately used to calculate the digestible P content of the ingredients. There was a concomitant decrease in coefficient of variation percentage (CV, %) of tibia ash percentage with the increase in P content of the diets.

2.1.3.2 Performance variables

All measures of performance, i.e. feed consumption, weight gain and feed conversion efficiency, increased with increasing content of each of the test ingredients except for the monocalcium phosphate (MCP) (Table 2.6).

2.1.3.3 Digestible phosphorus

The digestible P contents of the test ingredients at the two levels of P measured both by tibia ash and by ileal digestibility are shown in Table 2.7. The digestible P content of ingredients when determined using tibia ash decreased when the P content was doubled, except for maize, wheat, poultry by-product and gluten, which exhibited the opposite response. With the ileal digestibility technique, the digestible P content of all the diets increased as the P content of the

diets was doubled. The five monocalcium phosphate samples also exhibited a trend different from other ingredients when the ileal digestibility technique was used, the ileal digestible P decreasing as the P was doubled, except for MCP2 and MCP4 where the ileal digestible P increased as the P content increased.

Table 2.5 The tibia ash contents (g/kg) observed as the total P contents on as fed basis (g/kg) of the diets were increased by inclusion of graded levels of phosphoric acid in standard curve diets

Total P (g/kg)	Tibia ash (g/kg)	Standard Deviation	Coefficient of variation (%)
Experiment 1			
3.5	361	18.8	5.21
4.3	443	13.5	3.05
5.0	499	9.2	1.91
5.4	481	10.2	2.06
5.9	494	13.1	2.63
7.6	468	4.8	0.97
Experiment 2			
3.5	364	20.7	5.69
3.8	402	12.2	3.03
4.2	421	10.7	2.54
4.3	433	12.8	2.96
4.4	443	11.8	2.66

Table 2.6 Feed intake, weight gain and feed conversion efficiency observed for different test diets

Ingredient	Feed intake (g/bird.d)		Weight gain (g/bird.d)		FCE (g gain/ kg feed)	
Inclusion	Low[1]	High[2]	Low	High	Low	High
Soya	49	52	22	28	441	540
Fishmeal	57	65	38	44	590	678
MBM	56	58	29	33	514	580
Maize	53	55	24	27	461	488
Wheat	48	56	23	30	479	526
Plt by-pdt	50	57	25	32	507	570
Gluten 60	52	55	26	30	498	549
MCP1	53	59	27	31	506	530
MCP2	53	54	26	27	481	508
MCP3	54	56	25	30	481	524
MCP4	51	58	25	30	502	519
MCP5	67	65	39	39	585	601
EMS	**10.6**	**15.7**	**15.4**	**11.7**	**2260**	**2091**

[1] = Lower level of inclusion of the test ingredient, [2] = Higher level of inclusion of the test ingredient.

EMS = Error mean square MBM[1], meat and bone meal; PBPM[2], poultry by-product meal; MCP[1], MCP's 1-5 are monocalciumphosphates with different calcium and phosphorus contents

Table 2.7 The phosphorus digestibilities of the test ingredients at two levels for each, as determined by tibia ash and ileal digestibility techniques

Ingredient	Total P (g/kg)		Digestible P (by tibia ash) (%)		Ileal digestible P (%)	
Inclusion	Low[1]	High[2]	Low	High	Low	High
Soya	3.5	3.7	106.35 ± 8.70	110.02± 2.67	36.5 ± 11.6	66.5 ± 2.56
Fishmeal	4.0	4.7	110.40 ± 6.08	103.63± 1.99	53.1 ± 7.54	66.3 ± 3.56
MBM	4.8	6.1	88.17 ± 1.94	76.66± 1.92	53.9 ± 3.42	53.6 ± 0.89
Maize	3.5	3.6	100.49 ± 0.82	103.31± 6.36	60.9 ± 6.63	61.8 ± 7.02
Wheat	3.5	3.6	103.50 ± 4.01	113.50± 6.31	54.0 ± 9.09	68.4 ± 1.60
PBPM	3.6	3.9	99.86 ± 12.85	106.54± 5.41	63.4 ± 8.41	67.3 ± 6.26
Gluten 60	3.5	3.6	98.78 ± 3.26	105.40± 2.88	51.6 ± 7.73	65.5 ± 5.04
MCP1	4.0	4.6	102.74 ± 4.19	92.85± 6.72	60.0 ± 2.07	53.5 ± 7.15
MCP2	4.1	4.5	92.96 ± 2.83	87.98± 3.24	56.3 ± 3.37	56.6 ± 7.30
MCP3	3.9	4.6	100.58 ± 1.86	93.96± 2.80	57.8 ± 5.30	54.2 ± 3.91
MCP4	3.8	4.5	103.87 ± 1.74	92.62± 1.22	54.0 ± 7.13	58.7 ± 5.95
MCP5	4.7	9.5	105.33 ± 1.09	52.73± 0.74	42.6 ± 6.58	66.4 ± 3.19

[1] = Lower level of inclusion of the test ingredient, [2] = Higher level of inclusion of the test ingredient.

MBM[1], meat and bone meal; PBPM[2], poultry by-product meal; MCP[1], monocalcium phosphate; MCP's 1-5 are monocalciumphosphates with different calcium and phosphorus contents

2.1.4 Discussion

Daily weight gain and feed intake increased when the P content of the diets was increased, signifying a limitation in P content in diets because this was observed in diets where inorganic P sources were tested. There was an inconsistency in the results for digestible P, determined by the tibia ash technique, and by ileal digestible P technique. This leaves the debate about the accuracy of the tibia ash as a measure of P availability wide open, since the ileal digestible P also responded inconsistently to the increase in the P content of the diets. Generally, the standard curve and ileal digestibility techniques gave results that were unacceptably variable, i.e. the coefficients of variation were above 10%.

2.1.4.1 Tibia ash technique

The tibia ash content in Experiment 2 increased as the P content from H_3PO_4 in the diets increased, up to 5 g/kg diet, after which it decreased. Apparently the P content of the diets reached excess levels in terms of the requirements for maximum tibia ash and this might have resulted in the formation of indigestible complexes with Ca in the gut, thereby interfering with the absorption of both minerals (Rao and Roland, 1990; Smith, 1990 and Yi *et al.*, 1996). This resulted in the low variance accounted for by the standard curve obtained in Experiment 1, because the response curve was a broken stick but it was analysed as a linear curve. For Experiment 2 it was decided that the total P content of the standard curve diets should not exceed 5 g/kg diet, a decision which resulted in dramatically improved variance accounted for by the standard curve. The variances accounted for in second standard curve are high enough to justify the effectiveness of the phosphoric acid in increasing tibia ash percentage. The regression model for the first standard curve was off-set by failure of the tibia ash percentage to increase when the P content was increased above 5 gP/kg; this being made worse by the consequent decrease of tibia ash percentage when the P content was increased to 7.2 gP/kg. The decrease in coefficient of variation as the dietary inorganic P content of the standard curve diets increased can be attributed to the limited ability of birds to utilise phytate P. This observation is in line with the observations of Punna and Roland (1999) where it was observed that birds of the same strain vary in their ability to utilise phytate P. Birds of the same strain were fed on a P-deficient diet (1 gP/kg). Some of the birds on this diet grew "normally" while others exhibited P-deficiency symptoms.

The digestible P contents of ingredients as determined using the tibia ash technique were very high, except for the higher levels of five MCP's. The high standard deviations for these values show a very high inconsistency in the response of chicks in various replications of the same treatments. The reason for this variation is not clear, as there were no gradients, e.g. temperature, observed in the brooder room when the pilot trial was conducted. A possible explanation could be that in some cases the total P content of the final diet exceeded 5 g P/kg. It was observed that there was no further improvement in the tibia ash content above 5 g P/kg total P. In some diets for the standard curve the P content went above this mark. This could have affected the regression equation obtained.

2.1.4.2 Ileal digestible phosphorus technique

The ileal digestible P values observed were unrealistic in the sense that for some ingredients of plant origin their values were higher than those of animal and inorganic origin. One would expect a reverse situation to this because of the phytate contained in ingredients of plant origin. This situation can be blamed on the use of soybean oil cake as a source of protein in diets. In the final diets the ingredients, especially those of plant origin, which have very low P content, provided very little P (Table 2.4). The digestible P therefore analysed was mainly from the soyabean oilcake. This can be considered as one of the shortcomings of the experiment.

The use of soybean oilcake as a source of protein in diets of the experiments where digestible P is investigated was heavily criticized by J. D. van der Klis (2001) in our personal communication. He stated that since soybean contains phytate, this would interfere with the digestible P values of the test ingredients. This fact is supported by the results of the experiment by Harrold *et al.* (1983), in which it was observed that the presence of phytic acid in the basal diet reduced the availability of P added from a highly available source, in their case K_2HPO4. This would explain the unrealistic results for digestible P obtained in this experiment. The phytate could have had a negative impact on the diets making up the standard curve where phosphoric acid, which is a highly available source of P, was added to the diets containing soybean oil cake, which was used as a source of protein.

2.1.5 Conclusions

This experiment proved to be a learning experience in the measurement of digestible P in feed ingredients. Some valuable lessons were learned, which enabled the technique to be improved in subsequent experiments. The first lesson was that P contents in excess of 5 g/kg are too high to draw a linear standard curve and to precisely predict the digestible P content of ingredients. Secondly, the use of the tibia ash method for determining the digestible P content of ingredients needs further investigation, since the results obtained from this method contradicted in many instances with those obtained from the ileal digestible P method.

The most pronounced shortcoming was apparently the use of an unsuitable source of protein in the basal feed i.e. soyabean oilcake. This ingredient has been used before in P digestibility assays by Leske and Coon (1999). A different source of protein that contains minimal P content would need to be used in further studies since the soyabean oilcake seems to tamper with the estimation of P digestibility. This would lead to the formulation of a basal diet with low P content. This means therefore that the test ingredient would be included in the final diet in such a way that it would be the main source of P. In addition, the P from this alternative protein source should be highly available to the bird, i.e. it must be phytate free. Whey powder contains very little P (0.7%) compared to soyabean oilcake (0.15%), and was the protein chosen by van der Klis and Versteegh (1996) in his assays of the digestible P content of feed ingredients. Subsequent experiments in this investigation therefore made use of whey powder in the hope that the results would be less variable and more meaningful.

Chapter 3

Effectiveness of phytase in improving nitrogen and phosphorus digestibilities

3.1 Introduction

Phytate (myo-inositol hexaphosphate) is the main storage form of phosphorus in ingredients of plant origin (Maenzi and Classen, 1998; Leske and Coon, 1999; and Yan, Kersey and Waldroup, 2001) and studies of its content in feed ingredients commonly fed to monogastric animals have shown that approximately 60 to 80% of the total P content of grains and seed is in form of the phytate P (Lynch, 1996 and Sebastian *et al.*, 1998). It has six inorganic phosphate groups that are poorly utilised by monogastric animals, which contribute to the excessive excreta P resulting in pollution problems in countries of intensive poultry production (Eeckhout and Paepe, 1994; van der Klis and Versteegh, 1996; Maenzi and Classen, 1998; Sebastian *et al.*, 1998 and Yan *et al.*, 2001). Digestion studies with broilers and pigs have indicated that digestible P content varies between ingredients; this variability is influenced by the varying endogenous phytase activities and phytate contents. An example of this is phytase activities of wheat and barley, which have phytase activities of 1190 and 580 phytase units/kg, respectively, while corn and soyabean have low phytase activities of 15 and 40 phytase units/kg, respectively (Lynch, 1996 and Viljoen, 2001).

Besides having a negative effect on P digestion, the phytate also reduces amino acid digestibility by affecting both the substrate (amino acid) and protease enzymes. It exerts its effect by forming complexes with proteins that are more resistant to proteolytic digestion than the protein alone and by inhibiting a wide range of proteolytic enzymes by virtue of its ability to chelate Ca ions, which are essential for activation of these enzymes (Sebastian *et al.*, 1998). The negative effect of phytate on amino acid and protein digestibility, however, still remains equivocal. Thompson and Serraino (1986) observed no negative effect of phytate on amino acid digestibility in their experiments with rats fed on diets with gradually decreasing rapeseed phytate content. In contrast, Knuckles *et al.* (1985) observed that phytate significantly decreased pepsin digestion of both casein and bovine serum

albumin in a linear fashion, and hydrolysing the phytate reduced this inhibitory effect. The extent to which the inhibition of enzyme activity by phytate contributes to its overall antinutritional effect remains unclear (Sebastian *et al.,* 1998). Therefore, further research is warranted in order to understand the full effects of phytate on the protein quality of feedstuffs. Putting this issue beyond doubt will help in attempts to reduce the amount of ammonia emitted from poultry enterprises, which has negative effects on both the environment and the birds. Ammonia is a source of unpleasant odours for the general public, as suburban and rural residents interface with agriculture (Ferguson *et al.*, 1998 and Nahm, 2000). Recent reports have also suggested a detrimental effect of ammonia exposure on the welfare of poultry by causing irritation to the mucous membranes in the eyes and the respiratory system, thus increasing the susceptibility to respiratory disease and reducing feed intake, feed conversion efficiency and growth rate (Kristensen and Wather, 2000).

Phytase (myo-inositol hexaphosphate phosphohydrolase) hydrolyses phytate to inositol and six inorganic phosphates, and its commercial production for use as an exogenous enzyme supplement for diets is most easily achieved using microbial cultures (Sebastian *et al.*, 1998). It can be used to reduce or completely eliminate the antinutritional and consequently detrimental environmental effects associated with phytate. Sohail and Roland (1999) observed that supplementing 300 phytase units/kg of a P deficient diet (0.225% non-phytate P (npP))was effective in preventing P deficiency symptoms such as reduced weight gain, FCE and feed intake, which show the potential of reducing P excretion by reducing dietary P content and addition of phytase without compromising production. This is further affirmed by the observations of Yan *et al.* (2001) where npP requirements to optimise tibia ash body weight gain and FCE were reduced from 0.33, 0.186, and 0.163% to 0.24, 0.151 and 0.109%, respectively, when 800 phytase units/kg diet was included in the diets. It was also observed that fecal P levels were markedly reduced at the lower P levels, especially when phytase was included in these diets. Broz *et al.* (1994) observed a significant increase in both growth rate and feed intake when graded levels of phytase (125, 250 and 500 PU/kg diet) of phytase were included in diets, which were attributed to improved utilization of dietary P. There are still publications disputing the effectiveness of phytase in improving P utilisation of birds. Ledoux, Zyla and Veum (1995) and Keshavars (2000) did not observe any improvement in P utilization with inclusion of phytase in diets in their experiment with turkey hens and growing pullets, respectively. If the observed

improvement of P utilisation with phytase inclusion in some experiments is anything to go by, then inclusion of phytase into practical broiler diets will allow for the reduction or complete omission of additional dietary inorganic P (Broz *et al.*, 1994). This will have positive effects both economically and environmentally, by reducing inorganic P supplementation to diets and reduced excreta P content, respectively (Bosch *et al.*, 1997)

A large part of the N excrete is a consequence of an imbalance in the amino acid make up of the dietary protein or inefficiencies in their digestion and absorption (Nahm, 2000). Theoretically phytase supplementation should also be able to release phytate-bound protein for utilization, but published data to support this hypothesis are scarce and inconsistent in terms of the amino acids for which the digestibilities are improved (Sebastian *et al.*, 1998). A number of researchers have reported an improvement in ileal digestibilities of all essential amino acids (Zhang, Roland, MacDaniel and Rao, 1999 and Ravindran, Cabahug, Ravindran, Selle and Bryden, 2000) but some have observed improvement in digestibilities of some amino acids (Namkung and Leeson, 1999). Ravindran *et al.* (1999) observed a significant improvement in the digestibilities of protein and amino acids of all feedstuffs investigated, but the magnitude of response varied depending on the feedstuff and amino acid considered. Of note in these experiments were relatively high increases in digestibilities of valine and threonine in all feedstuffs. These reports imply that phytase has a potential of addressing environmental concerns associated with P and N losses in poultry manure.

The objectives of this experiment were to determine the digestible P contents of various feedstuffs used in poultry diets by the local industry. Whey powder was included in place of soyabean oilcake meal in the basal feed, to determine the effectiveness of phytase in improving P and N digestibilities, and to measure the effect that phytate might have on the activities of various digestive enzymes.

3.2 Materials and methods

3.2.1 Pre-trial period

Sexed day-old Cobb broiler strain chicks were allocated, ten to a cage, to 96 wire cages (48 pens with males and 48 pens with females), the sexes being separate. The birds were fed a

commercial starter diet for 10 days. Water was made available to the birds through nipple drinkers. Water and feed were provided *ad libitum*. The birds were on a 23L : 1D lighting programme and the temperature was started at 32°C and was reduced by 0.5°C every second day.

3.2.2 Trial period

The experiment commenced when the birds were 10 days old and the birds remained on the experimental diets for 10 days. During this period water and feed were provided *ad libitum*, the temperature was maintained at 26°C and the birds were on a 23L : 1D lighting programme.

3.2.3 Diets

A semi-synthetic diet with the lowest possible P content (1.6g/kg) was formulated as a basal diet; it was formulated in such a way that it met all nutritional requirements of the chicks, except P (Table 3.1). Three test ingredients, i.e. maize, soya and lupin, were chosen for this experiment. There was no basic criterion for the choice of these ingredients, but they were as diverse as possible in terms of the nutrients they supply, e.g. energy or protein sources. The substitution technique was used for test diets, where the test ingredients were substituted for the equivalent nutrients in the feed up to a limit set by the P content in final mix, which was to be 4.6 g/kg diet (Table 3.1). The objective of this was to make use of as much of the P from the test ingredient as possible. Supplementation with inorganic P was minimized or eliminated in some diets. The Ca content was maintained at 10 g/kg in all diets. The diets containing 4.6 gP/kg diet were blended 1:1 with the basal diet to obtain diets with a lower P content, i.e. 3.2 g/kg diet. After thorough mixing, the diets containing the test ingredients were divided in half and phytase was included in one half of each diet at the manufacturer's recommended rate of 750 phytase units/ kg diet, obtained by adding 300 g Ronozyme®/tonne (Roche, Johannesburg) diet. The phytase was included on top of the formulation, i.e. the phytase was not allowed for during formulation of all diets. For the determination of ileal digestibility, an acid insoluble ash (celite) was included as an indigestible marker at a level of 30 g/kg in each test diet.

3.2.4 Experimental design

The experiment was a completely random design (no blocking). Each treatment was replicated four times, with each cage of ten birds representing a replicate. Two replicates consisted of males and the other two were females in all treatments.

3.2.5 Performance variables

The body weight of all surviving birds in each pen was measured at the start and at the end of the experiment to determine the mean body weight of each bird and the weight gain. The average feed consumption per bird over the experimental period was also measured. This was obtained by weighing the feed supplied to each pen at the beginning of the experiment and that remaining at the end of the experiment. The difference between these two weights divided by the number of live bird days over the experimental period was the average feed consumption per bird. The feed conversion efficiency (FCE) was calculated from weight gain and feed consumption (kg gain/kg feed consumed).

Methods for measuring **tibia ash**, **acid insoluble ash**, and the **calculation of digestible P** were the same as described in **Chapter 2**. Calculation of digestible P from the standard curve for tibia ash had to be omitted in this Experiment due to high mortality rate in pens where bird were on standard curve diets, and feeding of these diets had to be called off. Due to these circumstances the tibia ash content of the birds in test diets was only used to compare the relative availability of P from these diets since P is one of the main components of the bone.

3.2.6 Amino acid analysis

The amino acid contents of the diets and in the ileal samples were determined by means of the chromatography technique used in our laboratory.

3.2.7 Amino acid Digestibility

Due to the limited amount of ileal samples collected on each treatment, amino acid digestibilites were analysed only in lupin diets with and without phytase, which produced more ileal output than the other two ingredients tested. The digestibility was calculated using the celite as an acid insoluble ash marker, as:

$$Digest.aa = [((aa / AIA)d - (aa / AIA)i) / (aa / AIA)d] * 100$$

Where: $(aa/AIA)_d$ = ratio amino acid to Acid Insoluble Ash in diet; and $(P/AIA)_i$ = ratio amino acid to Acid Insoluble Ash in ileal digesta.

3.2.8 Tissue collection and preparation

At the end of the experiment, the birds were slaughtered by asphyxiation with carbon dioxide and then dissected to expose the visceral organs. The jejunum (part of the intestinal tract extending from the ligament of Treitz to Meikel's diverticulum) and the pancreas were removed for analysis. The tissues were flushed with ice-cold normal saline water and the jejunum was slit longitudinally. The mucosa was rinsed of digesta with PBS and wrapped in aluminium foil, then snap-frozen in liquid nitrogen for use in the preparation of mucosal homogenate on which the activities of digestive enzymes are studied. Birds were not gassed in large numbers, because intestinal and pancreatic enzyme activities tend to decline rapidly after death; a maximum of two replicates with five birds were killed and sample collection was completed within a few minutes.

Fresh frozen intestinal and pancreatic samples were cut into small pieces and defrosted in buffer (100 mM mannitol, 2 mM *N*- [2-hydroxyethyl] piperazine-*N*- [2-ethanesulfonic acid] (HEPES)/Tris, pH 7.1). About 25 ml of buffer were used for 1-2 g tissue. The mixture was vibromixed for 2 x 30 seconds and filtered through a Buchuner funnel, 1mm pore size. The filtrate was then blended with a homogeniser for 30 seconds at high speed. One ml of homogenate was taken for assessment of protein and brush-border membrane enzyme activity.

3.2.9 Tissue protein content

The recovery rate of the membranes was assessed by estimating the protein concentrations in homogenates according to the modified techniques of Bradford (1976). The assay utilizes the red form of Coomassie Brilliant Blue G-250 (CBB), which turns blue on binding to protein. The reaction was started by adding 2ml of CBB to 40µl of dilute vesicles or homogenate, vibromixing and reading at 595 nm after 5 minutes but within 1 hour. Data generated was analysed with the aid of computer software, Lowry (Elsevier BIOSOFT, Cambridge, UK).

3.2.10 Alkaline phosphatase

Alkaline phosphatase (AP) was assayed in line with modified methods described by Forstner, Sabesin, and Isselbacher (1968) and Holdsworth (1970). The assay system consists of 50 mM $MgCl_2$, 50 mM Tris (pH 10.1) and the substrate, 10 mM paranitrophenol phosphate (PNP Sigma 104). The reaction was initiated by incubating 25 µl of homogenate with 0.8 ml of 50 mM Tris buffer, pH10.1, 0.1 ml of 25 mM $MgCl_2$ and 0.1 ml of PNP at 39°C for 20 minutes. The reaction was terminated by 0.1 ml 40 % trichloroacetic acid. Further colour development was accomplished by adding 2.0 ml of 0.4 M NaOH to 0.1 ml of the primary reaction mixture, which was then vibromixed and read at 410 nm.

Table 3.1 Composition (g/kg) and laboratory analysis of the basal diet and three test diets used in the experiment

Ingredients	Basal diet	Maize diet	Soya diet	Lupin diet
Sugar/Starch	501.9	-	216.3	-
Whey powder	221.9	170.2	-	29.4
Vit+min premix	2.5	2.5	2.5	2.5
Limestone	2.1	18.2	21.8	20.4
Monocalcium phosphate	140.0	6.4	3.9	-
Salt	30.0	2.1	2.2	6.4
Plasterer's sand (filler)	1.7	20.0	59.2	20.0
Celite	3.9	30.0	30.0	30.0
Tryptophan	-	-	0.1	-
Arginine	2.4	1.0	-	-
DL-methionine	73.3	-	-	0.9
Sodium bicarbonate	-	2.3	2.1	1.82
Sunflower oil	-	32.3	100.0	67.7
Maize meal	-	32.3	-	-
Soya oil-cake	-	-	561.4	-
Lupin	-	-	-	818.6
Analysis:				
Protein	202.6	204.0	281.1	277.1
Calcium	10.6	10.7	10.5	10.1
Phosphorus	1.6	4.6	5.3	4.6

3.2.11 Sucrase and maltase

The disaccharidases, α-glucosidase (maltase) and β-fructofuranodase (sucrase) were assayed using the method described by Dalhqvist (1964). The incubation mixture was freshly prepared 100 mM maltose and sucrose, respectively, in succinate buffer (4 mM sodium succinate, 90 mM sodium choride, pH 6.0). Homogenates, 25 µl, were incubated in

500 µl of substrate-buffer for 30 minutes at 39°C. Incubation was terminated by pippeting in five fold of 0.2 % Triton X-100 (w/v) in 0.5 M Tris buffer, pH 7.02 at 39°C. Incubation released glucose, which was then estimated by the GOD-Perid test kit (glucose oxidase, Boehringer-Mannheim Pty. Ltd., Australia). The amount of glucose released was measured colorimetrically at 610 nm after 30 minutes of colour development at room temperature.

3.2.12 Amylase

A substrate was prepared by adding 2 mg/ml soluble starch (Difco) in 50 mM phosphate buffer (pH 7.2) and boiled to dissolve. The original method used a reagent, dinitrosalicylic acid (DNS), made from several chemicals and large volumes of this were required for each assay. The modification retained the first step of Miller *et al* (1960), in which the substrate, starch was digested to disaccharides and relatively small amounts of glucose. The glucose was the main sugar measured by DNS. This can be more rapidly done using the Glucose kit supplied by Roche Diagnostics, the same kit used in the second stage of determining maltase and sucrase activities. Incubating starch with microbial amylase created the standard. In 1.5 ml reaction mixture, add 0.5 ml dilute sample and then make up to 1 ml with 50 mM phosphate buffer, pH 7.2. Add 500 µl (1mg starch) pre-warmed substrate and incubate for one hour at appropriate temperature (39°C). The reaction was terminated with sucrase/maltase solution C, then the glucose formed was measured as per disaccharides (Miller, Blum, Glennon and Briton, 1960).

3.2.13 Pancreatic lipase

The substrate was prepared by suspending 0.5% tributyrin (TB, Kodak) in 100mM Tris-25 mM $CaCl_2$ buffer (pH 8.0). The stock standard was prepared by dissolving a standard microbial lipase in 50 mM Tris/7.5 mM $CaCl_2$, pH 8.0 to yield 0.5 U/µl). The desired concentration (0.05 U/µl) was obtained by diluting 1 in 10, using the buffer. Of this working solution, 100 and 200 µl may be used; these quantities are equivalent to 5 and 10 U, respectively. 100 µl sample was incubated in 2 ml of pre-warmed (39°C) substrate for 30 minutes, and the reaction was terminated with 100 µl of 40 % TCA then the absorbance was read at 450 nm (Smeltzer, Hart and Iandolo, 1992).

3.2.14 Phytase activity

A substrate was prepared by dissolving 0.1mM sodium phytate in 50 mM Tris/50 mM HEPES/50 mM MES (THM buffer). The incubation mixture was set up to consist of 40µl dilute sample; 1ml of buffer; 100 µl 50 mM $MgCl_2$ and 100 µl of substrate (N.B. add substrate last). The blank contained all buffers and substrate but the sample was replaced with distilled water or ROH. The mixture was allowed to stand for 20 minutes then the reaction was stopped by rapidly pipetting in 100 µl ice-cold 50 mM TCA after which it was centrifuged and the inorganic P produced was determined. This was done by taking 1 ml of the supernatant and add 1ml ROH, 100 µl Fiske and SubbaRow solution and 0.25 ml acid molybdate. The mixture was incubated at room temperature for 20 minutes then the absorbance was read at 660 nm (Maenz and Classen, 1998).

3.2.15 Chymotrypsin

A substrate in form of 0.1 mM solution of succinyl- $(ALA)_2$- Pro-Phe-*p*-nitroanilide (SAPNA) was prepared, and 50 mM Tris-HCl buffer and 20 mM $CaCl_2$ (pH 7.5) was added to allow the substrate to dissolve. For the standard, graded concentrations of *p*-nitroaniline were used. 30 µl of sample were added to 1.5 ml fresh substrate solution and incubated at 39°C for 10 minutes; the reaction was terminated with 250 µl of 30% acetic acid and the absorbance was read at 410 nm against a water blank. Calculation:

Activity was expressed in SAPNA units/mg as:
($A_{410\ nm/min}$ x 1000 x vol of reaction mixture)/8800 x mg protein in the assay), where 8800 was the extinction coefficient of *p*-nitroaniline (Seviere-Zaragoza, del Toro and Garcia-Carreno, 1997).

Statistcal Analysis

The analysis on variance (Genstat) was used to analyse the effect of phytase inclusion on ileal digestibility of each of the essential amino acids.

3.3 Results

3.3.1 Performance variables

There was no significant effect of phytase on weight gain in any of the treatments, though maize and soya at lower P levels showed a positive response to enzyme inclusion (Table 3.2). The lupin diets showed a non-significant negative response to enzyme inclusion at both levels of P. Soya diets showed a significantly higher gain compared to lupin and maize (Table 3.2). There was a significant difference in feed consumption between ingredients, with birds consuming more of soya diets than maize and lupin diets (Table 3.2). Other factors, i.e. enzyme and P level, did not have a significant effect on feed consumption. FCE was significantly higher in birds on lower P diets than those on higher P (Table 3.3), and the FCE was significantly higher for soya diets than maize or lupin diets.

3.3.2 Digestible phosphorus

The digestibility of the P contained in the three test ingredients at two inclusion levels and the effect of phytase enzyme on these digestibilities is given in Table 3.4. There was a significant difference between ingredients in terms of P digestibility, with soya diets having higher P digestibility than maize and lupin. Increasing P level from 3.2 to 4.6 g/kg significantly decreased P digestibility in all ingredients. Phytase inclusion improved P digestibilities of maize and soya diets at both P levels, though non-significantly for maize. A significant improvement with phytase inclusion was observed only at the higher level of soya, where an improvement of 15% in P digestibility was obtained. In lupin diets there was a non-significant reduction in P digestibility at both levels of P. The P digestibilities obtained in diets with 3.2 g P/kg diet is considered the actual digestibility since these are below the plateau of the known P requirement (4.5 g/kg).

Table 3.2 Weight gain (g/bird day), feed intake (g/bird day) and feed conversion efficiency (g weight gain/kg feed intake) of the birds in test diets with (+) and without (-) phytase

Test ingredient	Tot. P (g/kg)	Weight Gain		Feed Intake		FCE	
Phytase		-	+	-	+	-	+
Maize	3.20	16.5	19.0	34.5	37.0	480	511
	4.60	18.0	17.8	37.5	35.3	477	498
Soya	3.40	25.8	27.0	45.5	47.0	564	573
	5.30	27.3	27.0	50.5	50.8	538	524
Lupin	3.20	25.5	23.3	42.5	41.5	597	561
	4.60	21.5	20.5	41.8	42.0	511	483
Main effects of Phytase		21.5	22.4	42.0	42.3	528	525
SED		**1.32**		**1.92**		**17.8**	
Source of variation		**Prob.**		**Prob.**		**Prob.**	
Enzyme		0.72		0.96		0.58	
P level		0.83		0.09		<0.01	
Ingredient		<0.01		<0.01		<0.01	

Table 3.3 The average feed conversion efficiency observed for the two phosphorus levels in all ingredients

Dietary P content (g/kg diet)	FCE
3.2	547 ± 36
4.6	505 ± 42

3.3.3 Tibia ash

The effects of test ingredient, phytase enzyme addition and content of total P on the tibia ash content are shown in Table 3.4. There was a significant ($P<0.01$) difference in tibia ash content between test ingredients, with maize diets resulting in highest tibia ash compared to soya and lupin diets. There was a significant ($P<0.01$) improvement in tibia ash when the P content of the diets was increased. The enzyme had a non-significant ($P>0.05$) effect on tibia ash content; however there was a general improvement in tibia ash in diets with phytase except for the higher levels of P in maize and soya diets, where phytase inclusion reduced tibia ash content.

Table 3.4 Ileal digestible phosphorus content (g/kg) of test diets with (+) and without (-) phytase enzyme and tibia ash content of the birds on them

Test ingredient	Dietary P (g/kg)	Digestible P		Tibia ash (g/kg)	
Phytase		-	+	-	+
Maize	3.20	65.2	72.2	473	480
	4.60	55.3	59.9	506	498
Soya	3.40	73.8	77.7	440	448
	5.30	53.3	68.4	472	448
Lupin	0.32	75.2	75.0	428	435
	0.46	57.3	54.5	459	485
Main effects of Phytase		62.5	67.7	463	466
SED		**4.31**		**0.97**	
Source of variation		Prob.		Prob.	
Ingredient		**0.03**		**<0.01**	
P level		**<0.01**		**<0.01**	
Enzyme		**0.01**		**0.20**	

3.3.4 Amino acid digestibilities

The ileal digestibilities of amino acids in lupin diets were not statistically altered by inclusion of phytase (Table 3.5). The digestibilities of all amino acids, other than tyrosine, showed non-significant improvements with the addition of phytase.

Table 3.5 Mean amino acid digestibilities on as fed basis (%) ± standard deviation of lupin diets with and without phytase

Amino acids	No phytase	With phytase
Aspartic acid	86.19 ± 0.42	87.26 ± 1.45
Threonine	80.54 ± 1.17	81.06 ± 1.79
Serine	83.99 ± 2.17	84.29 ± 1.98
Glutamic acid	91.22 ± 0.27	91.53 ± 1.42
Proline	85.17 ± 0.77	86.21 ± 0.90
Glycine	81.72 ± 0.39	83.01 ± 1.75
Alanine	81.55 ± 0.75	83.00 ± 1.97
Valine	81.57 ± 0.47	82.73 ± 1.82
Methionine	88.15 ± 2.33	89.76 ± 2.00
Isoleusine	84.93 ± 1.41	85.24 ± 2.06
Leusine	88.34 ± 0.10	88.80 ± 1.43
Tyrosine	89.24 ± 0.80	89.06 ± 1.35
Phenylalanine	86.67 ± 0.78	87.32 ± 1.68
Histidine	87.72 ± 1.17	87.87 ± 0.92
Lysine	87.56 ± 0.43	88.41 ± 1.61
Arginine	92.90 ± 1.18	93.53 ± 0.64

3.3.5 Enzyme activities

There was a significant ($P<0.01$) difference in activities of lipase and amylase between ingredients, with lupin diets having highest activities compared to maize and soya diets (Tables 3.6). There was no significant effect on activities of other digestive enzymes in this experiment by all the factors considered (Table 3.6). The chymotrypsin activity was non-

significantly improved with phytase inclusion at both P levels of maize and lupin diets, whereas that with soya diets was decreased.

Table 3.6 Enzyme activities of digestive enzymes in chicks at two levels of inclusion of test ingredients, with and without phytase

Treatment	Chymo.[1]	Lipase[2]	Amylase[3]	Maltase[4]	Sucrase[4]	Phytase[5]	AP[6]
Maize, 1	1.03	0.03	1.25	3.16	0.05	1.67	1.12
Maize, 2	0.99	0.02	0.92	2.37	0.04	2.38	1.28
Maize,1 +Ph[7]	1.07	0.02	1.10	2.41	0.04	2.03	1.67
Maize,2 +Ph.	1.04	0.02	1.28	2.24	0.04	1.80	1.40
Mean	**1.03**	**0.02**	**1.14**	**2.55**	**0.04**	**1.97**	**1.37**
Soya,1	0.96	0.03	1.47	2.23	0.05	1.92	1.49
Soya,2	1.10	0.03	1.27	2.05	0.04	1.93	1.26
Soya,1+ Ph.	0.90	0.03	1.42	1.99	0.03	2.29	1.24
Soya,2+ Ph.	0.90	0.02	1.67	1.82	0.04	2.49	1.32
Mean	**0.97**	**0.03**	**1.46**	**2.02**	**0.04**	**2.16**	**1.33**
Lupin,1	0.13	0.03	1.25	2.00	0.03	1.99	1.21
Lupin,2	0.84	0.03	1.54	2.21	0.03	1.78	1.05
Lupin,1+Ph.	0.78	0.02	2.28	2.64	0.04	2.01	1.39
Lupin,2+Ph.	0.96	0.04	1.09	2.22	0.03	2.39	1.59
Mean	**0.68**	**0.03**	**1.54**	**2.27**	**0.03**	**2.04**	**1.31**
	SED	**0.17**	**0.01**	**0.25**	**0.47**	**0.01**	**0.60**
							0.24
SOV	Prob.	Prob.	Prob.	Prob.	Prob.	Prob.	Prob.
Ingredient	**0.36**	**0.01**	**0.01**	**0.30**	**0.21**	**0.48**	**0.98**
P level	**0.64**	**0.30**	**0.25**	**0.28**	**0.52**	**0.35**	**0.81**
Enzyme	**0.35**	**0.25**	**0.12**	**0.63**	**0.79**	**0.29**	**0.07**

[1] = µmole nianiline/mg protein/minute; [2] = lipase units; [3] = ρmole glucose/mg protein/minute; [4] = µmole glucose/mg protein/minute; [5] = µmole P_i/mg protein/minute; [6] = µmole PNP/mg protein/minute; Ph. = phytase. SOV = source of variation, AP = Alkaline Phosphatase. Chymo.=Chymotrypsin

3.4 Discussion

The inclusion of the whey powder improved the theoretical relevance of the digestible P values obtained compared to when the soyabean was used as a protein source, i.e. the ingredients with high phytate content had lower P digestibility than those with lower phytate content, and these were comparable to the values obtained by other researchers.

3.4.1 Ileal digestible phosphorus

The significant difference in P digestibilities between ingredients observed in this experiment can be attributed to two factors, one being the phytate content and the second being the phytase activity; these two factors vary between ingredients (Eekout and Paepe, 1994). The digestible P contents of soya and lupin observed in this experiment are comparable to those observed by van der Klis and Block (1997), where values observed were 61 and 68%, respectively. However, the digestibility value of P for maize differed considerably to that of van der Klis and Block (1997), i.e. 30% compared to 65.2% observed in this experiment. The common aspects between these two experiments are the relative values of P digestibility of the three ingredients, i.e. in both experiments the digestible P value of lupin was the highest, followed by soya then maize. The difference in the actual values can be attributed to the difference in conditions under which the two experiments were conducted, i.e. the other experimental diets contained 5g Ca/ kg diet and the difference in genetic make up of their test ingredients and those tested in this experiment.

The decrease in P digestibility as the P content of the diets increased was also reported by Waldroup (1999), where it was observed that as the dietary P levels increased from deficient to excessive, fecal P levels increased gradually up to a point at which tibia ash is at a maximum, whereafter it increases steeply. Similar results were observed by Sloan *et al.* (1995) with laying hens, where the reduction of dietary P content from 0.65 to 0.45% reduced the fecal P content from 2.78 to 1.89%, and the egg shell quality and egg production were not affected by the dietary P content. The digestible P content decreased as the dietary P content increased, suggesting that at higher levels, the P provided in diets could have been more than the requirement to maximise tibia ash content, therefore resulting in excess P being excreted and therefore reducing the percentage of P that is

retained (Waldroup, 1999). The results of this experiment introduce a hope of reducing the P content of diets without affecting performance in a negative way. This is based on the fact that there was no significant difference in weight gain between birds on 0.46% P diets and those on 0.32% P diets and the birds on the latter diets had a significantly higher FCE than those on the former diets. Regardless of the strategy one uses in an attempt to reduce P content of the excreta, the biologically available P content of the diet must not exceed the amount needed by the chick to maximise performance, for if it does there will be increased excretion of P (Waldroup, 1999).

The improvement of P utilisation by inclusion of phytase observed in maize and soya diets in this experiment is in line with observations of similar experiments (Simon *et al.*, 1990; Perney, Cantor, Straw and Herkelman 1993; Broz *et al.*, 1994; Ledoux *et al.*, 1995; and Yan *et al.*, 2001). Broz *et al.* (1994) observed a dramatic increase in plasma inorganic P content in phytase treated birds and reduced P concentration in chick excreta (by between 8.7 and 10.1%). The excreta P content was also reduced in this experiment by virtue of improvement of P digestibility in phytase treated diets. The highest improvement in P digestibility by the inclusion of phytase in the experiment was observed at a higher level of P in soya diets, which is in contrast to what was observed by other researchers who observed greater improvements at lower P levels (Yi *et al.*, 1996 and Yan *et al.*, 2001). The absence of improvement in P digestibility in lupin diets can be attributed to the very small amount of phytate P (20% of the total P) in lupin (Eeckhout and Paepe, 1994). Maize and soya have phytate P contents of 68 and 53% of the total P content, respectively; meaning the substrate for phytase enzyme was in abundance in the diets containing these two ingredients. The knowledge of variability of ingredients in response to phytase inclusion is crucial in attempts to reduce excreta P contents because disastrous generalisations will be avoided. If the improvement in P digestibility that is expected from ingredients is known, then this can be used in the formulation of diets in such a way that the P supplied in the diet is the amount that is required by the bird after phytase supplementation. The inclusion of phytase in diets may, therefore, result in a reduction or possibly total omission of inorganic P supplementation (Ledoux *et al.*, 1995; Yi *et al.*, 1996 and Keshavarz, 2000).

3.4.2 Tibia ash

The tibia ash content observed in this experiment did not show a consistent response to phytase inclusion to the diets. However, the response to the increase in dietary P content was consistent. The response in tibia ash content to phytase inclusion in this experiment was inconsistent with that of the ileal digestible P, as in some cases the tibia ash content increased with the inclusion of phytase while the ileal digestible P decreased, e.g. in lupin diets. The opposite of this was observed at higher P levels of both maize and soya diets, i.e. the tibia ash decreased while ileal digestible P increased. The response observed in the former situation can be attributed to the ability of the phytase to increase the digestibility of other nutrients besides phytate P, such as amino acids and trace elements; so since there was no improvement in P digestibility, the increase in tibia ash could have been from these nutrients and not from P (Keshavarz, 2000). The reason for the second trend of response of tibia ash content as related to ileal digestible P is not clear, as it has never been reported before. The only way to try to explain it is that since the diets on which this decrease in tibia ash content was observed were those with higher P content in maize and soya diets, i.e. 4.6 and 5.3 g/kg, respectively, the increase in P retention by phytase must have resulted from of post-absorption reaction(s) which might have interfered with mineralisation of the bone. This affirms the criticism of use of bone ash content as a measure of availability of P, based on the fact that the ash content of the bone is also influenced by other factors like the presence of sufficient Ca to build the bone material with P and acid-base equilibrium (van der Klis, 1993a). The reports in responses of tibia ash to phytase have always been contradictory, with some researchers observing significant improvements in tibia ash with phytase inclusion to diets (Yi *et al.*, 1996 and Yan *et al.*, 2001) and some not observing any improvement (Perney *et al.*, 1993 and Keshavarz, 2000). In the latter experiments there was a non-significant effect of phytase on tibia ash content whereas the improvement of P retention was significant, as was the case in this experiment. This further questions the credibility of tibia ash as a parameter to estimate P digestibility.

3.4.3 Performance variables

The ineffectiveness of phytase to improve performance in this experiment has been reported before (Perney *et al.*, 1993 and Zhang *et al.*, 1999). In contrast, Broz *et al.* (1994) and Sohail and Roland (1999) observed a significant improvement in body weight gain

when graded levels of phytase of up to 500 and 600 phytase units/kg diet, respectively, were included in diets. This is strange, considering that in this experiment the phytase was included at 750-phytase units/kg diet and yet no improvement was observed in performance. This may be blamed on differences in composition of diets used in these experiments and the age of chicks at the start of the experiments; the other experiments were started at day-old.

The improvement in amino acid digestibility brought about by the addition of phytase in this experiment, is well documented (Ravindran *et al.*, 1999; Namkung and Leeson, 1999; Zhang *et al.*, 1999; Ravindran *et al.*, 2000 and Ravindran, Selle, Ravindran, Morel, Kries and Bryden, 2001). The response to phytase inclusion in terms of amino acid digestibility was non-significant which is similar to the observations of Zhang *et al.* (1999). The reason for this kind of response could be the lower phytase inclusion in the diets of this experiment and that of Zhang *et al.* (1999), where 600 PU/kg diet was supplemented. In the experiments where significant responses were observed graded levels of phytase were included in diets up to between 1000 and 1200 PU/kg diet.

3.4.4 Phytase

There was no effect of the factors considered here on the intestinal phytase activity, this being in contrast to the results reported by Lopez, Vallery, Levrat-Verny, Coudray, Demigne and Remesey (2000). In their experiment they monitored the phytase activity of rats fed on dietary phytic acid and on wheat bran diets. They observed that the intake of phytic acid enhanced the phytase activity in the upper parts of the small intestines (duodenum and jejunum), whereas the wheat bran diet activated ileal phytase. This implies that if there is phytase included in diets, the intestinal phytase activity should decrease since the phytate in diets is hydrolysed but this was not the case in this experiment. This can be argued by stating that the physiological responses of rats could have differed to those of other monogastrics in this respect. The observations of this experiment can be related to those of Pointillart, Fourdin and Fontaine (1984) where the higher P availability of triticale diets was attributed to its higher phytase activity compared to that of Maize and the intestinal phytase activity was similar in pigs on either diet. This means that the phytase activity is independent of the phytase in the digesta.

3.4.6 Intestinal Alkaline phosphatase (AP)

The absence of a significant response in AP with phytase inclusion is in contrast to the observations other researches, who reported a decrease in AP activity with the inclusion of phytase in diets (Huff, Moore, Waldroup, Waldroup, Balog, Huff, Rath, Daniel and Raboy, 1998 and Wu, Jiang, Wu, Dai and Fang, 1999). Huff *et al.* (1998) attributed the observation to the down regulation of AP resulting from the increased P availability by phytase. However there are some researchers who reported the AP activity not being affected by the dietary phytase as it was the case in this experiment (Pointilart *et al.*, 1987 and Qu, Zhan and Song, 1999).

3.4.6 Pancreatic Amylase

The significant ($P<0.01$) differences observed in pancreatic activities of birds on different test ingredients were caused by other factors besides than the phytate content of the test ingredients since the phytase inclusion did not have a significant ($P<0.01$) effect on amylase activity and the test ingredient with highest amylase activity is the one with the least phytate content, i.e. Lupin. The phytate chelates the Ca^{2+} ions, which are essential for the activity and stability of amylase therefore inhibiting the activity of the enzyme (Deshpande and Cheryan, 1984 and Sebastian *et al.*, 1998). Considering this information, it was expected that the phytase inclusion in diets will improve the activity of amylase, but in this experiment there was no clear trend observed. In contrast to this expectation Martin, Nolan, Nitsan and Farrel (1998) observed a reduction in amylase activity of the intestinal contents of ducklings when between 1000 and 1500 phytase units/kg diet were included in diets. This leaves the issue of phytase and amylase activity equivocal, or maybe a consistent trend can be observed if phytase included in diets can be increased from 750 phytase units, as it was in this experiment, to between 1000 and 1500 PU/kg diet.

3.4.7 Chymotrypsin activity

The response of chymotrypsin activity with inclusion of phytase explains the trend towards improvement observed in digesibilities of amino acid in lupin diets. This implies that the phytase reduces the inhibitory effect, which the phytate has on the activity of chymotrypsin (Sathe and Sze-tao, 1997).

3.4.6 Intestinal Sucrase activity

The observed absence of the effect of phytase inclusion in diets in intestinal sucrase activity is in line with the observations of Onomi, Katayama and Sato (1999) who observed that the dietary phytate had no influence on intestinal activity of sucrase.

3.5 Conclusions

The use of whey powder in diets as a source of protein greatly improved the relevance of the digestible P result compared to those obtained in Chapter 1 (Table 1.7). The digestible P content of ingredients of plant origin varies from ingredient to ingredient, and it decreases as the P content of the diet is increased. The P content of broiler diets can be reduced, within certain limits, without negatively affecting the performance of the birds; consequently the excreta P will be reduced. Phytase improves P digestibility of plant ingredients but the margin of improvement varies from ingredient to ingredient. This variability is associated with the phytate content of the ingredients: those with higher phytate content having higher improvement margins than those with lower phytate content. Tibia ash content appears to be an unreliable parameter for estimating P digestibility, and further use of this parameter in experiments such as the present needs to be reviewed.

The improvement of amino acid digestibility when phytase is included in diets is linked to the improvement of chymotrypsin activity when this enzyme (phytase) is supplemented. The improvement of both amino acid digestibility and chymotrypsin activity was non-significant in this experiment, so in future the effect of inclusion of higher levels of phytase in diets should be investigated.

Chapter 4

Determination of digestible phosphorus content of ingredients in diets with reduced calcium content

4.1 Introduction

Phytate has an ability to chelate cations like Ca, zinc (Zn) and magnesium (Mg) and in the process, the digestibility of P, which is part by reduced, specifically reduced by Ca^{2+} (Sebastian *et al.*, 1998). In an experiment by van der Klis (1994) it was observed that when the Ca content of the diet was increased from 5 g/kg diet to 8.3 g/kg diet the breakdown of phytate in peas and soybean diets was decreased from 38% and 69% to 28% and 36%, respectively. This means that the breakdown of phytate at lower dietary Ca levels (5 g Ca/kg) is higher than at raised Ca levels, which are commonly used in practice (van der Klis, 1994). This implies that higher digestible P contents are to be expected from ingredients at lower diatary Ca levels than at higher ones.

The negative effect of Ca on P digestion can be attributed to the Ca: phytate P ratio (Sebastian *et al.*, 1998). The Ca: tot P ratio of 2:1 impairs the digestion of phytate because of the formation of an insoluble Ca phytate complex in the intestines. The Ca in this complex blocks the access points of intestinal phytase enzyme, therefore rendering the phytate less degradable and consequently the digestible P content of the diet is reduced. Birds consuming a diet with a Ca: tot P ratio of 1:1 exhibited a higher P digestibility than those fed a diet where the ratio was 2:1. Widening the Ca: total P ratio in diets from 1:1 to 2:1 decreased the digestibility of P from plant ingredients to a greater extent than from inorganic supplements such as dicalcium phosphate, which do not contain phytic acid (Sebastian *et al.*, 1998). Shafey *et al.* (1990) also observed a negative effect of Ca increase in P utilization, where an increase in Ca content from 12 to 32 g/kg diet reduced weight gain and tibia P content.

Older birds have a greater ability to hydrolyze phytate than do chicks because there is more phytase activity present in the gastrointestinal tract of older birds (Sebastian *et al.*, 1998).

Based on this fact, it is therefore logical to say that if the effect of exogenous phytase enzyme inclusion in diets is to be investigated, the birds should be put on the test diets at day-old before they develop their own effective phytase activity.

The objectives of this experiment were to test the effect of different dietary calcium contents on P digestibility, and to ascertain whether phytase enzyme has a greater activity on P digestibility when chicks are fed this enzyme from day-old, rather than from one week of age, which was done in the previous trial.

4.2 Materials and methods

4.2.1 Trial period

Sexed day-old Cobb broiler strain chicks were allocated ten to a cage to 96 wire cages, with sexes being separated, i.e. 48 pens with males and 48 with females. Water, being made available by nipple drinkers, and experimental diets were provided *ad libitum*. The birds were on a 23L : 1D lighting programme and the temperature was started at 32°C and reduced by 0.5°C every second day up to 26°C where it was maintained throughout the experiment. The experiment lasted for 18 days.

4.2.2 Diets

In these experiment eight ingredients of plant origin, one of animal origin (fish meal) and four monocalcuim phosphates (MCP) were tested for the digestible P content. The ingredients of plant origin consisted of four cereals, i.e. wheat, wheat bran, sorghum and maize; and four protein sources, i.e. lupin, sunflower oil cake, soya oil cake and canola (Table 4.1). The four MCP's had different Ca and P contents as shown in Table 2.3, the diets with these are shown in Table 4.2.

The test ingredients were substituted for equivalent nutrients in the basal diet up to a limit set by the P content in the diet, i.e. 3.5 g/kg diet. The objective of this was to make use of as much of the P from the test ingredient as possible. In all the diets the Ca content was maintained at 5 g/kg diet. All the diets containing plant test ingredients or fish meal were divided into two, and phytase was included in one half. The phytase was included on top of

the formulation, i.e. the phytase was not allowed for during formulation of the diets; and it was included at the manufactures recommended rate of 750 phytase units/kg diet, obtained by adding 300 g Rhonozyme®/tonne (Roche, Johannesburg) diet. For the determination of ileal digestible amino acids and P, an acid insoluble ash (celite) was included as an indigestible marker at a level of 30 g/kg of each diet.

4.2.3 Variables measured

The same procedures were used in this experiment as those described in Chapter 2.

Table 4.1 The composition (g/kg diet) and nutrient analysis of test diets used in this experiment

Ingredients	Canola	Fishmeal	Lupin	Maize	Sorghum	Soybean Oilcake	Sunflower Oilcake	Wheat	Wheat Bran
Sugar/Starch	514.7	422.7	166.9	-	-	369.0	431.4		417.7
Whey	175.1	123.1	68.3	152.4	119.4	50.0	105.1	77.5	160.7
DL methionine	-	-	12.2	-	-	0.1	-	0.6	0.2
L-lysine HCl	-	5.5		7.9	6.9	11.3	11.7	6.7	13.1
Vit+min Premix	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Limestone	2.0	1.0	6.7	7.7	8.7	7.7	7.6	9.9	7.5
Plasters sand	-	230.3	136.1	17.5	30.7	143.8	110.2	-	81.6
Salt	2.7	-	1.9	-	-	-	-	-	-
Tryptophan		2.1	2.0	-	2.4		2.3	2.4	2.2
Arginine	-	1.0	-	1.9	4.9	-	-	4.3	12.1
Sodium bicarbonate	0.1	3.0	2.4	4.6	4.6	4.7	4.7	4.0	4.6
Mcp	0.5		4.2	-	-	3.2	-	-	-
Sunflower oil	80.0	80.0	80.0	-	-	80.0	80.0	45.8	80.0
Canola	212.5			-	-	-	-	-	-
Fishmeal	-	129.2		-					
Lupin	-	-	527.9	-	-	-	-	-	-
Maize meal	-	-	-	805.6	-	-	-	-	-
Sorghum	-	-	-	-	819.6	-	-	-	-
Soybean oil-cake	-	-	-	-	-	363.0	-	-	-
Sunflower oil-cake	-	-	-	-	-	-	243.6	-	-
Wheat	-	-	-	-	-	-	-	846.5	
Wheat bran	-	-	-	-	-	-	-	-	217.8
Analysis									
Protein	217.4	200.2	227.9	210.4	205.3	204.3	192.6	216.7	206.4
Calcium	6.6	6.1	6.4	6.5	5.8	3.9	6.1	6.8	6.0
Phosphorus	3.5	3.4	3.2	2.7	2.8	2.8	2.9	3.5	3.3

Table 4.2 The composition (g/kg) of the test diets in which different monocalcium phosphates were used as test ingredients

Ingredients	MCP1	MCP2	MCP3	MCP4
Sugar/Starch	480.7	480.7	480.7	480.7
Whey	209.6	209.6	209.6	209.6
L-lysine HCl	10.8	10.8	10.8	10.8
Vit+min premix	2.5	2.5	2.5	2.5
Limestone	-	0.1	1.2	2.8
Plaster's sand	195.0	193.8	193.3	192.8
Tryptophan	1.9	1.9	1.9	1.9
Arginine	4.3	4.3	4.3	4.3
Sodium bicarbonate	4.7	4.7	4.7	4.7
Sunflower oil	80.0	80.0	80.0	80.0
MCP1	10.4	-	-	-
MCP2	-	10.7	-	-
MCP3	-	-	11.0	-
MCP4	-	-	-	9.9
Analysis				
Protein	210.5	211.6	205.3	199.6
Calcium	5.2	5.4	5.8	5.6
Phosphorus	3.9	4.1	4.3	4.2

4.3 Results

4.3.1 Performance Variables

The weight gains, feed intake and feed conversion efficiency of birds in diets with or without phytase are shown in Table 4.3. There was a significant ($P<0.001$) difference in weight gain observed for the different ingredients but the enzyme and enzyme by ingredient interaction effects were non-significant ($P>0.05$). The semi-synthetic diets seem

to be unpalatable to birds, they did not consume these diets at all in this experiment, as a result the treatments in which the digestible P contents of MCP's were to be determined had to be cancelled from the experiment. There was a significant difference in feed intake as affected by ingredient and enzyme, with these being significant at $P<0.001$ and $P<0.05$, respectively. The lupin diets had the highest intake and those with phytase having higher feed intake than those without. There was a significant difference in FCE between ingredients; the highest FCE values were obtained from the wheat bran diet. The phytase and the phytase by ingredient effects were non-significant ($P>0.05$).

4.3.2 Digestible phosphorus

The digestible P contents of diets with or without phytase are shown in Table 4.4. There was a significant ($P<0.001$) difference in digestible P content between ingredients, with fishmeal diets having the highest. Phytase had a non-significant ($P>0.05$) effect on P digestibility, but the phytase by ingredient interaction was only significant at $P\leq0.069$. The phytase had a negative effect on P digestibility of some ingredients, e.g. maize, sorghum and lupin; however it had a positive effect on wheat, wheat bran, soya oilcake, sunflower oilcake and canola but there was no effect observed on fish meal P digestibility.

Table 4.3 Weight gain (g/bird day), feed intake (g/bird day) and feed conversion efficiency (FCE, g gain/kg food) of birds given test diets with (+) or without (-) phytase

Ingredients	Weight gain		Feed intake		FCE	
Phytase	-	+	-	+	-	+
Maize	6.3	6.2	18.3	20.6	333	305
Sorghum	4.7	4.8	20.3	18.8	239	250
Wheat	11.5	11.5	27.8	27.0	410	426
Wheat bran	11.5	10.8	24.8	24.3	480	445
Soya oil - cake	8.3	8.3	23.3	26.0	350	315
Sunflower oil-cake	8.8	8.8	27.8	28.3	312	303
Canola	7.3	8.0	19.0	20.8	378	384
Fish meal	8.8	6.8	20.8	24.5	292	289
Lupin	8.3	10.5	26.5	33.0	317	319
Main effects of phytase	8.0	8.3	23.0	24.7	349	337
SED	**0.81**		**2.11**		**28.74**	
Source of variation	Prob.		Prob.		Prob.	
Ingredient	<0.001		<0.001		<0.001	
Phytase	0.301		0.022		0.210	
Ingredient*Phytase	0.314		0.155		0.849	

Table 4.4 The ileal digestible phosphorus contents (g/kg diet) of the test diets without (-) or with (+) phytase

Ingredients	Total P	Ileal digestible P	
Phytase		-	+
Maize	2.7	805	731
Sorghum	2.8	835	560
Wheat	3.5	631	652
Wheat bran	3.3	699	745
Soya oil-cake	2.8	714	801
Sunflower oilcake	2.9	731	776
Canola	3.5	771	829
Fish meal	3.4	869	867
Lupin	3.2	625	5.7
Main effects of phytase		742	724

SED	**5.9**
Source of variation	Prob.
Ingredient	<0.001
Phytase	0.423
Ingredient*Phytase	0.069

4.3.3 Enzyme activities

The activities of enzymes tested are shown in Table 4.5. There was a non-significant (P>0.05) effect of all the factors considered on chymotrypsin activity, but the main effects of phytase showed a non-significant improvement in the activity of chymotrypsin (Table 4.5). The phytase non-significantly (P>0.05) improved the activity of chymotrypsin in all test diets except maize and sorghum diets (Table 4.5). There were also non-significant

effects of the factors considered on lipase activity, but the main effects of phytase showed a decline in lipase activity with the inclusion of phytase (Table 4.5). There was a significant ($P<0.05$) difference in amylase activity observed between the test ingredients. However, there was a non-significant ($P>0.05$) effect of phytase and phytase by ingredient interaction on amylase activity (Table 4.5). The main effects of phytase showed a general improvement in amylase activity with phytase inclusion.

There was a non-significant ($P>0.05$) effect of all the factors on AP activity. However, the phytase inclusion showed a non-significant decline in AP activity with phytase inclusion (Table 4.5). There was no significant ($P>0.05$) effect of any of the factors considered on the phytase activity. However the main effects of phytase inclusion showed a non-significant improvement of intestinal phytase activity (Table 4.5).

Table 4.5 Enzyme activities observed in birds on test diets without (-) or with (+) phytase

Ingredients	Chymotrypsin[1]		Lipase		Amylase[3]		AP[6]		Intestinal Phyta	
	-	+	-	+	-	+	-	+	-	+
Maize	0.81	0.69	0.017	0.014	1.81	1.28	1.63	1.27	2.61	3.00
Sorghum	0.70	0.62	0.022	0.021	3.68	2.62	1.62	1.23	2.69	2.34
Wheat	0.62	0.86	0.047	0.016	4.03	4.55	1.37	1.46	2.94	3.51
Wheat bran	0.65	0.69	0.021	0.025	4.26	3.56	1.16	1.33	2.82	2.16
Soya oilcake	0.58	0.67	0.022	0.017	1.80	3.21	1.36	1.29	2.43	2.74
Sunflower oilcake	0.56	0.66	0.022	0.017	3.78	4.53	1.32	1.48	2.50	3.31
Canola	0.89	0.96	0.022	0.017	1.29	3.28	1.39	1.31	1.80	3.25
Fishmeal	0.74	0.82	0.019	0.012	2.41	3.70	1.45	1.40	2.13	3.41
lupin	0.66	0.69	0.024	0.021	2.29	1.76	1.23	1.13	2.19	2.28
Main effects of phytase	0.69	0.74	0.021	0.018	2.81	3.17	1.39	1.32	2.46	2.89
SED	**0.05**		**0.003**		**0.55**		**0.04**	**0.50**		
Source of variation		Prob.		Prob.		Prob.		Prob.		Prob.
Ingredient		0.37		0.416		0.017		0.436		0.833
Phytase		0.43		0.086		0.400		0.261		0.112
Ingredient*Phytase		0.94		0.092		0.648		0.307		0.675

[1] = µmole nianiline/mg protein/minute; [2] = lipase units; [3] = ρmole glucose/mg protein/minute; [5] = µmole P_i/mg protein/minute; [6] = µmole PNP/mg protein/minute.

4.4 Discussion

4.4.1 Digestible phosphorus

The significant differences in P digestibility between ingredients observed in this trial can be attributed to the variability in the phytate content of these ingredients. Unlike the ingredients of plant origin, the animal and inorganic P sources do not contain phytate, which is responsible for reduced P digestibility observed in plant ingredients (Eeckhout and Paepe, 1994). This being confirmed by the fact that the fishmeal had the highest digestible P content in this experiment. The reduction of Ca content improved the observed digestible P content of maize compared to that measured in Chapter 2, i.e. 65.2% against 80.5%. Similar improvement was observed in an experiment by Punna and Roland (1999) where an increase in dietary Ca content reduced the phytate P utilization by the birds. The P digestibilities of soya and lupin decreased in this experiment compared to those of Chapter 2, i.e. 73.8 and 75.2, respectively. This observation can be attributed to the variability of phytate content of the ingredients, with the maize having the highest phytate content (68%) followed by soya (52%) then lupin (20%) (Eeckhout and Paepe, 1994); it is to be expected that the reduction of Ca would have a greater effect on the ingredient with high phytate content, in this case maize, and lesser or no effect on those with lower phytate (van der Klis, 1994).

The addition of phytase had varying effects on P digestibility, with the digestible P content being reduced in some ingredients. The reason for the negative effect of phytase on P digestibility has never been reported but it can be speculated that it may be a result of an interaction between Ca and phytate P since this was not observed in Chapter 2 with maize diets. This negative effect of phytase on P digestibility can be attributed to the reduction of Ca content of diets. For example, in Chapter 2 where the Ca content of the diet was 10 g/kg the maize diets did not exhibit reduction in P digestibility with phytase inclusion. The reduction of P digestibility with phytase inclusion could be the result of the P released from the phytate through the action of phytase. The P released could have offset the inorganic P: Ca ratio. The inorganic P: Ca ratio is critical in the absorption of both minerals in the gut (Sebastian *et al.*, 1998); therefore, the phytase might have released more inorganic P from the phytate, which might have interacted with Ca and resulted in the

reduction P absorption. The phytase did not have any effect on P digestibility in fishmeal diets, nor was this expected, as fishmeal does not contain phytate.

4.4.2 Performance variables

The results of this experiment are similar to those of Chapter 2, except for the significant effect of phytase on feed intake. The improvement in feed intake with the addition of phytase observed in this experiment agrees with the observation of Broz *et al.* (1994) and Zhang *et al.* (1999). In contrast, Ravindran *et al.* (2001) observed no influence on feed intake with the addition of phytase. The improvement in food intake measured in this experiment could well be due to the improved Ca: P balance in the diets used in this experiment compared to that used in Experiment 2 (Chapter 3).

4.4.3 Enzyme activities

The phytase inclusion improved the chymotrypsin activity even in the fishmeal diets; this was unexpected since the fishmeal does not contain any phytate. The phytase inclusion to diets non-significantly reduced the AP activity, which is in agreement with the observations of Huff *et al.* (1998) and Wu *et al.* (1999). The main effects of phytase show an improvement in amylase activity by inclusion of phytase in diets; this is to be expected since the phytate tends to reduce the activity of amylase by chelating Ca^{2+}, therefore inclusion of phytase should reduce this negative effect (Deshpande and Cheryan, 1984). The improvement of amylase activity with phytase inclusion was also observed in fishmeal diets, which in a way disputes the theory that the improvement of amylase activity is brought about by the release of Ca^{2+} from the phytate by phytase. The independence of intestinal phytase activity from dietary phytase content also showed in this experiment.

The improvement of P digestibility observed when dietary Ca is reduced is mainly due to the reduction of the extent of formation of Ca-phytate complex that is difficult to hydrolyse by the birds' intestinal phytase (Van der Klis, 1994). This means that it is easier for the intestinal phytase to release P from the phytate at lower Ca levels (5 g/kg) than at higher levels (10 g/kg) since the access points for the action of this enzyme are not blocked by the Ca. Therefore, it was logical to expect that the digestive enzymes like chymotrypsin and

amylase will be negatively affected by the Ca-phytate complex will be more active at lower Ca levels. But this was not the case when comparing the activities in this experiment and those in Chapter 3. This can be considered with some doubt since these were two separate experiments

4.5 Conclusion

The reduction of Ca dietary content from 10 g/kg to 5 g/kg diet improved the P digestibility as was observed in this experiment with maize, but this is equivocal because the P digestibilities of lupin and soya decreased compared to those observed in Chapter 3. More experiments need to be conducted to verify the effect of dietary Ca content on P digestibility. The results of this experiment were compared to those of Chapter 3, something that is very subjective to do, since the two experiments were conducted separately. In addition to this, the improvement was only observed in maize but not for soya and lupin. This experiment was conducted under standardized conditions specified by Van der Klis (1996), where it was stated that the dietary Ca content should be 5 g/kg. The improvement of P in this experiment compared to when the dietary Ca content was 10 g/kg was a chance observation, i.e. it was not the subject of the experiment. A proper experiment where the effect of dietary Ca content is investigated needs to be conducted. The information on this aspect of nutrition is crucial in diet formulations, where the decision to supplement with inorganic P sources need to be taken. Perhaps future experiments could test the 5 and 10 g Ca/kg in the same experiment, and an experiment to test the effect of phytase addition at day 0 and day 7 would divulge crucial information about the suitable age of supplementation with phytase.

The reduction of dietary Ca content can result in decrease of digestible P content when phytase is included in diets with some ingredients e.g. maize, sorghum and lupin. The ingredients vary in their response to phytase supplementation; in this experiment there was improvement of P digestibility for some ingredients while for other some there was reduction. This negative effect of phytase on P digestion needs to be further investigated to identify the ingredients that continually exhibit this trend; such information is important where the decision on whether or not to supplement with phytase needs to be taken. The phytase has no influence on the P digestibility of ingredients of animal origin and these have higher P digestibility than ingredients of plant origin, however in this experiment the

fish meal showed similar response to the ingredients of plant origin in terms of enzyme activities, specifically amylase and chymotrypsin. Definitely these improvements did not involve phytate, therefore the pathway that resulted in this effect needs to be investigated.

The reduction of dietary Ca content in diets hindered the growth and development of the birds in this experiment, in future the experiments with determination of digestible P involving reduction of Ca should be done separately from phytase experiments; this is because the former experiments are started at 10 days of age and the latter experiments at day one.

General discussion

There were many valuable observations from the three experiments conducted for this thesis; the first experiment gave some very vague results but the relevance of the results to the literature review improved in subsequent experiments. An important lesson learnt from Chapter 2 is that the use of a plant protein source as part of the basal diet in experiments like these interferes with the results, and that the total P content in test diets should be kept below 5 g/kg because above this level there is no response.

The digestible P contents observed in Chapters 3 and 4 was in line with the observations of other researchers; and they proved once again to vary from plant to plant. This affirms the call for a more accurate P quantification technique to be used in feed formulations; while it also vehemently refutes the previous assumption about P availability from plant and animal ingredients the results of Chapter 4 prove that though the P from animal ingredients is not 100% digestible, it is more digestible compared to plant ingredients. While the phytase inclusion to fishmeal diets, representing animal sources did not have any effect in P digestibility, the response of plant ingredients varied. This should be paid a lot of attention to when this enzyme is to be supplemented in diets to prevent unnecessary supplementation or under supplementation.

The phytase had a non-significant improvement of amino acid digestibility and enzyme activity, which requires further experiment done on this field with higher levels of phytase inclusion to find the point at which significant results can be obtained. The improvement of amino acid digestibility varies between amino acids; further experiments should be done to identify those that are constantly influenced by pyhtase inclusion so that their inclusion in diets can be reduced accordingly if phytase is included in diets. This can play a crucial role in the reduction of ammonia gas emitted from poultry enterprises, and can be economical since amino acids are expensive nutrients. The improvement of amylase and chymotrypsin activities by inclusion of phytase in fish meal diets in Chapter 4 needs to be investigated further in order to explain how this came about.

The tibia ash emerged as an unreliable parameter for estimating the availability or digestibility of P. Hence, the use of it in P experiments needs to be reviewed. Standardized

conditions for the determination of P digestibility that more or less simulate the practical ones, should be put in place in order to minimize the variability and irrelevance of the experimental values obtained to on-farm conditions. These conditions should include Ca and P contents of the diets, composition of the basal diet and the age at which the birds are to be put on the diets. The age of the birds at which the digestibility is determined in experiments is crucial, since the bird's ability to degrade phytate increases with age.

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