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Full Length Research Paper

Intestinal phytase activity in chickens (*Gallus Domesticus*)

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The objective of the study was to adapt an assay and determine the effect of innate and dietary factors on intestinal phytase activity (IPA). The release of phosphorus, when an extract of intestinal mucosa was incubated with phytate as substrate, was used to measure phytase activity. Maximum IPA was detected between pH 5.6 and 6.2, and it was very low and non-detectable at pH 7.0-7.4. Activity was dependent on the presence of Zn^{2+} and Mg^{2+}, and IPA was inhibited by a commercial phosphatase inhibitor cocktail but not by L-phenylalanine, an inhibitor of alkaline phosphatase. The activity in the proximal portion of the duodenal loop was higher than in the distal portion. Newly hatched chicks exhibited IPA and neither age (1 to 8 weeks) nor gender had an effect on IPA in broiler chickens. However, a gender effect was evident in a layer strain; females had 94.7% higher IPA than males. Lowering the total phosphorus level of the broiler starter diet increased IPA. During the growing period, IPA was almost twice as high when the diet contained no supplemental inorganic Phosphorus. High dietary iron depressed IPA in fast- and slow-growing meat strains. The results indicate that phytase activity is present along the gastro-intestinal tract of chickens and that intestinal pH and nutrient composition are important modulators of intestinal phytase activity and phytate phosphorus utilization.

Key words: Intestinal phytase activity (IPA), inhibition, broilers, layers, phytate phosphorus.

INTRODUCTION

Phytate, a salt of phytic acid, is the main storage form of phosphorus in plants. Phytase, myo-inositol hexaphosphate hydrolase, [EC 3.1.3.8] hydrolyzes phytate to myo-inositol and inorganic phosphate via intermediate myo-inositol phosphates. The existence and role of intestinal phytase activity (IPA) in poultry has been controversial. Its presence was first observed in albino rats (Patwardhan, 1937) and later in rats, guinea pigs, and chickens (Krieger, 1938). Lowe and Steenbock (1936) failed to detect IPA in extracts from rats and chickens; and based on the results of balance experiments. Nelson (1976) concluded that IPA does not exist nor does it have a functional role in non-ruminants. In contrast, other results indicated the presence of phytase activity and a role for it in phosphorus utilization in chickens (Krieger, 1938; Lowe et al., 1939; Steenbock et al., 1958; Bitar and Reinhold, 1972; Maenz and Classen, 1998; Applegate et al., 2003; Marounek et al., 2008; Marounek et al., 2010). Ossification in granivores in the wild and plant phosphorus utilization in chickens fed corn-soy diets (Harms et al., 1962; Waldroup et al., 1964, 1965) lends credence to the latter results. The enzyme is localized in the brush border of the small intestine and facilitates P absorption by breaking down phytate (Davies and Flett, 1978).

Phytate hydrolysis in the chicken has been attributed to a non-specific alkaline phosphatase activity (Maddaiah et al., 1969) and to specific IPA (Maenz and Classen, 1998). The origin of intestinal phytase is also disputed; some ascribe it to innate secretion (Bitar and Reinhold, 1972; Biehl and Baker, 1997) and others consider it a product of gastrointestinal microflora (Wise and Gilburt, 1982; Kerr et al., 2000). The latter was not confirmed in germ-free rats (Miyazawa et al., 1996) and chicks dosed

with very high levels of antibiotics or cecectomized (Biehl and Baker, 1997). Since intestinal enzymes are modulated by dietary factors, it is possible that IPA may be affected by diet. Significant differences in chick IPA due to dietary treatments were detected in some studies (Krieger, 1938; Davies et al., 1970; Davies and Motzok, 1972; McCuaig et al., 1972; Applegate et al., 2003) but not in others (Maddaiah et al., 1969; Biehl and Baker, 1997). In laying hens, phytate phosphorus retention was influenced by age (Marounek et al., 2008). A possible explanation for the increased utilization of phytate phosphorus with age is the suggestion that more endogenous phytase is present in the gastrointestinal tract of older birds (Marounek et al., 2008; Marounek et al., 2010).

The purpose of the study reported was to investigate the optimal conditions to determine IPA *in vitro* and to determine IPA in newly hatched chicks, changes with age, homogeneity of distribution and the effects of phosphatase inhibitors, gender, and diet.

MATERIALS AND METHODS

Study 1: adaptation of assay for intestinal phytase activity (IPA) in chickens

Low or negligible IPA was detected when the method of Iqbal et al. (1994) as described by Biehl and Baker (1997) was used. Hence, the initial objective was to adapt the assay to measure IPA in chickens. Experiment 1 assessed the optimal pH and ionic requirements for IPA extracted from chicken mucosa. Samples were assayed in triplicate using PIPES buffer adjusted to a pH range from 5.6 to 7.6 (Good et al., 1966). Mineral dependency of IPA was assessed by assay in triplicate using three concentrations each of $MgCl_2$ (0, 20, and 40 mmol/L) and of $ZnCl_2$ (0, 2, and 4 mmol/L). Experiment 2 determined the homogeneity of distribution of IPA in the intestine. Experiment 3 studied the effect of inhibitors and storage on IPA. The inhibitors tested were L-phenylalanine, an inhibitor of alkaline phosphatase, and a commercial inhibitor cocktail (Phosphatase Inhibitor Cocktail 2, Sigma P-5726). The effect of storage on IPA was assessed on the day of collection and after storage of mucosa at -20°C for 24 and 48 h.

Study 2: effect of age, type, and gender on IPA in chickens

Experiment 4 investigated the effects of age and gender on IPA in growing broiler chicks. Broiler chicks (n=48) were distributed at random in 6 cages, with three cages of each gender, in battery brooders in an environment-controlled room with air-flow of 2150 ft^3/min and 18 air changes/hour and 23 h photoperiod. One chick per pen was sampled weekly for IPA measurements from 1 to 8 weeks of age.

Experiment 5 examined IPA in egg type pullets and cockerels maintained in individual cages in an open-sided poultry house. Pooled tissue samples were used in this and all subsequent experiments. Four composite samples of each gender with three birds per composite sample were obtained. Experiment 6 determined if newly hatched chicks have IPA prior to exposure to feed and water and extensive colonization of the gut. Four composite duodenal mucosa samples were prepared using 8 newly hatched chicks per sample.

Study 3: effect of dietary variables on intestinal phytase activity in chickens

Samples of corn, soybean meal, dicalcium phosphate, and limestone were analyzed for total phosphorus (TP) and calcium content and assayed materials used in formulation of diets (NRC, 1994). Experiment 7 examined the effect of dietary P concentration and acidified drinking water on IPA in broiler chicks. Forty chicks were housed as in experiment 4, fed the control diet for a week and then the dietary treatments imposed from one to four weeks of age. Thirty-six chicks were randomly assigned to four blocks with a pen of three birds as the experimental unit. The treatments consisted of the control diet (6.5 g TP/kg); P-deficient diet (5.0 g TP/kg) prepared by reducing the content of dicalcium phosphate from 14 g to 5 g/kg; and the P-deficient diet with 0.00625 N HCL instead of the regular water.

Experiment 8 determined the effect of P deficiency and feed grade phytase during the grower period on IPA activity in broiler chicks. Chicks were brooded and fed a starter diet from day-old to 3 weeks. At 3 weeks, thirty-six chicks were housed in twelve grower cages (three birds per cage) and randomly assigned to one of three dietary treatments from 3-6 weeks of age. The treatments consisted of the control grower diet (6.0 g TP/kg), a P-deficient grower with no supplemental inorganic P (4.0 g TP/kg), and the P-deficient grower diet with 1250 phytase units/kg (Natuphos®, BASF). Experiment 9 investigated the effect of dietary iron on IPA in fast-growing broiler chicks and the slow-growing random bred Ottawa Meat Control strain. The two strains of chickens were reared to 3 weeks. Poultry diets contain high levels of iron especially if they contain poultry by-products and hence the control diet was supplemented with ferrous sulfate to generate experimental diets with 860 and 3600 mg Fe/kg. The three diets were fed to three blocks of broiler chicks and the control and 860 mg Fe/kg diet fed to three blocks of the slow-growing random bred Ottawa Meat Control strain from 3 to 6 weeks of age. A pen of three birds constituted the experimental unit. Feed and water were provided ad libitum for the duration of the experiments.

Preparation of tissue homogenates and assay for intestinal phytase activity

The duodenum was flushed with saline, the mucosa scrapped, 0.2 g mucosa was homogenized in 20 ml PIPES buffer (10 mmol, pH 6.1), centrifuged at 7500 rpm for 4 min at 4°C, and the supernatant was diluted (1:15) with buffer and the procedure of Iqbal et al. (1994) was followed. The diluted supernatant (2 ml), a mixture (0.2 ml) of magnesium chloride (40 mM) and zinc chloride (4 mM) was added into each tube followed by 0.1 ml substrate {corn phytic acid, (Sigma P-3168) 30 mM with 0.125 g penicillin (Sigma-P 7794) and 0.017 g streptomycin sulfate (Sigma S-9137)}. The tubes were incubated at 37°C for 1 h in a shaking water bath and P in the incubation mixture was determined with malachite green reagent (MGR) (0.34 g malachite green in 34 mmol/L ammonium molybdate in 0.5 L 5N HCl and adjusted to 1 L and stored in an amber bottle). To 0.5 ml of the incubation mixture or standard solution 1.5 ml urea (Sigma U-0631, 36 g/100 ml) and 4 ml MGR were added. The contents were mixed, color development allowed for 10 min and absorbance measured at 640 nm. Phosphorus concentration in each tube was computed, the sample corrected for blank and dilution and phytase activity expressed as nanomoles P released mg^{-1} min^{-1}.

Table 1. Phosphatase inhibitors and chicken intestinal phytase activity.

Treatment	Phytase activity[1] (mean ± SD)
Control	40.3 ± 0.98
+ L-phenylalanine[2], 20 µl	42.5 ± 0.85
+ Inhibitor cocktail[3], 10 µl	34.7 ± 0.96
+ Inhibitor cocktail, 20 µl	20.7 ± 1.27
+ Inhibitor cocktail, 30 µl	8.3 ± 0.96

[1]Intestinal phytase activity based on three pooled replicates and expressed in nmol P released mg $^{-1}$ min^{-1}. [2]L-phenylalanine an inhibitor of alkaline phosphatase. [3]Phosphatase Inhibitor Cocktail 2 (Sigma-5726). An inhibitor of acid and alkaline phosphatase as well as tyrosine protein phosphatases. The response of IPA to the inhibitor was described by a significant linear function y = 42.50 – 1.10x (R^2 = 0.97), where x = volume of inhibitor cocktail added.

Experimental design and statistical analyses

Analysis of variance for a complete randomized design was used to determine the effects of age and gender on intestinal phytase activity. Analysis of variance for a randomized complete block design was used to determine the effects of dietary treatments on intestinal phytase activity. Statements of statistical significance were based on $\alpha<0.05$. The values in tables are given as the mean ± SD.

RESULTS

Intestinal phytase activity was dependent on pH and [Zn^{2+}] and [Mg^{2+}] and varied with storage and region in the intestine. Maximum IPA was detected at pH 5.6-6.4 (59.7 nmol/mg protein/hr), activity was significantly reduced at pH 6.6 (45.8 nmol mg^{-1}min^{-1}), it was negligible at pH 7.0 (6.0 nmol mg^{-1}min^{-1}), and not detectable at pH 7.6. Phytase activity was not detected in the absence of both Mg^{2+} and Zn^{2+}. When Zn^{2+} alone was present in the incubation mixture, IPA was low (2.8 nmol mg^{-1}min^{-1}) while activity was higher when Mg^{2+} alone was present (11.9 and 14.5 nmol mg^{-1}min^{-1}). A plateau in IPA was noted with combinations of 20-40 mM $MgCl_2$ and 2-4 mM $ZnCl_2$ and was 39 nmol mg^{-1}min^{-1}. A significant linear decrease (10% per 24 h storage) in IPA occurred when mucosa was stored. Significant regional differences were detected in IPA; with highest activity in the duodenum (31.9 nmol mg^{-1}min^{-1}) with lower IPA in the jejunum (19.5 nmol/mg protein/hr ± SEM 0.66 and n=3) and ileum (11.4 nmol mg^{-1}min^{-1}± SEM 0.26 and n=3).

Activity was about three-fold higher in the proximal duodenum when compared to the distal portion (45.7 versus 18.1 ± SEM 0.83 and n=3). The rate of reaction was linear with respect to time for 1.5 h. Based on these results, the IPA assay was conducted on the day of tissue collection with a sample from the entire duodenum using a pH 6.1 PIPES buffer with 40 mM $MgCl_2$, 4 mM $ZnCl_2$, and phytic acid as substrate with the reaction mixture incubated for 1 h. The phosphatase inhibitor cocktail significantly inhibited IPA in chickens in a linear dose-dependent manner. In contrast, the alkaline phosphatase inhibitor, L-phenylalanine, did not influence IPA (Table 1).

Mucosal phytase activity was not significantly affected by age or gender of broiler chickens from day-old to 8 weeks of age (Table 2). A significant gender effect was detected in mature egg-type pullets and cockerels; the females exhibited 94.7% higher IPA than males (*P<0.05*). In newly hatched chicks, killed before receiving any feed or water, average phytase activity was 29.6 nmol mg protein^{-1} min^{-1}.

Birds fed the low level of TP (5.0 g/kg) during the first 3 to 4 weeks had 11% higher (*P<0.05*) phytase activity compared to those fed the control starter diet (Figure 1). The group that received the acidified water instead of regular water had 43% higher phytase activity (*P<0.05*) compared to the control. During the grower period (Figure 2), birds fed the P-deficient diet (4.0 g/kg TP) produced 50% more phytase enzyme compared to those fed 6.0 g/kg TP diet (*P<0.01*). Birds fed the P-deficient diet with phytase produced 16% less intestinal phytase than those fed the diet without exogenous phytase (*P>0.05*).

Iron supplementation at both levels significantly (*P<0.05*) reduced intestinal phytase activity in fast-growing commercial broiler chickens (Figure 3). Birds fed the control diet had 40% higher activity compared to those fed the iron-supplemented diets. The highest level of iron did not further depress IPA. The same response was obtained when slow-growing meat strain birds were fed the diet with 860 mg/kg iron (Figure 3). Phytase activity was 51% higher in the controls compared to the group that received 860 mg/kg iron diet (*P<0.05*). Phytase activity in birds fed the control diet was significantly higher in fast-growing birds than in the slow-growing meat strain.

DISCUSSION

Iqbal et al. (1994) determined mucosal phytase activity in rats and humans at pH 7.1 while Biehl and Baker (1997) used the same procedure to determine the activity in chicks. We used the same procedures to measure IPA in

Table 2. Effect of age and gender on intestinal phytase activity (IPA) in chickens.

Chicken type	Age (wks)	IPA[1] (mean ± SD)[1]
Broiler[2]	At hatch	29.9 ± 1.89
Broiler	1	46.3 ± 5.87
	2	38.4 ± 5.74
	3	59.3 ± 8.24
	4	55.4 ± 8.30
	5	60.3 ± 6.04
	6	48.1 ± 7.43
	7	44.1 ± 6.35
	8	46.3 ± 6.47
SEM		
Layer[3]		
Pullets	26	32.9[a]± 1.67
Cockerels	26	16.9[b]± 1.63

[1]Intestinal phytase activity expressed in nmol P released/mg protein/hour. [2]Each value for the broiler chickens was based on six individual birds. [3]Each value for the egg type birds was based on pooled replicates. [ab]Means in the same column with different superscripts differ significantly (*P<0.05*).

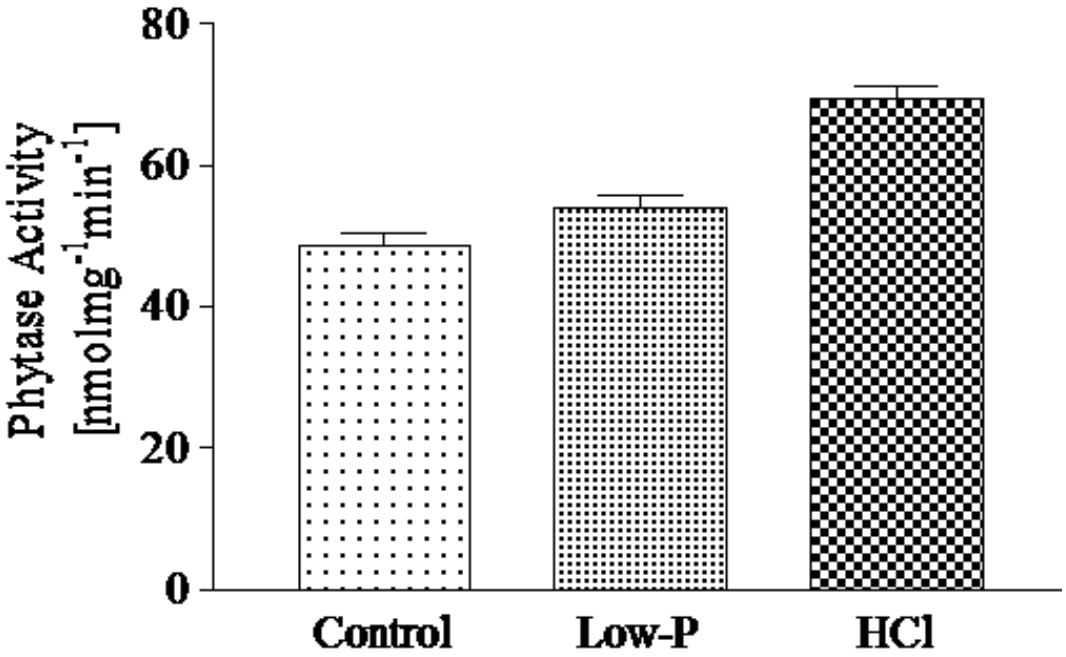

Figure 1. Effect of inorganic P and acid on intestinal phytase activity during starter period. The bars represent the mean ± SEM of 4 determination of phytase activity; mucosa from three birds was pooled in triplicates.

chicks and detected negligible activity at pH 7.1. The digestive tract of birds is acidic and the pH of the duodenum of chicken is about 6.1 (Hill, 1971). Mammals have a higher pH in the intestinal tract compared to birds because the bile in mammals is alkaline, (pH about 7.5 to 8.5). Thus, we tested mucosal phytase activity across a pH range from 5.6 to 7.6 using PIPES buffer (pK_a 6.8); and found maximal activity at pH 6.1. We observed higher phytase activity in the chick's small intestine than previously reported (Biehl and Baker, 1997). These results are in agreement with published evidence that optimal IPA occurred at a pH between 5.5 and 6.5 and at pH 6.0, IPA was distinct from that of nonspecific phosphatase since the brush border membrane contains no nonspecific phosphatase at this pH (Maenz and Classen, 1998). The optimum pH for chicken IPA

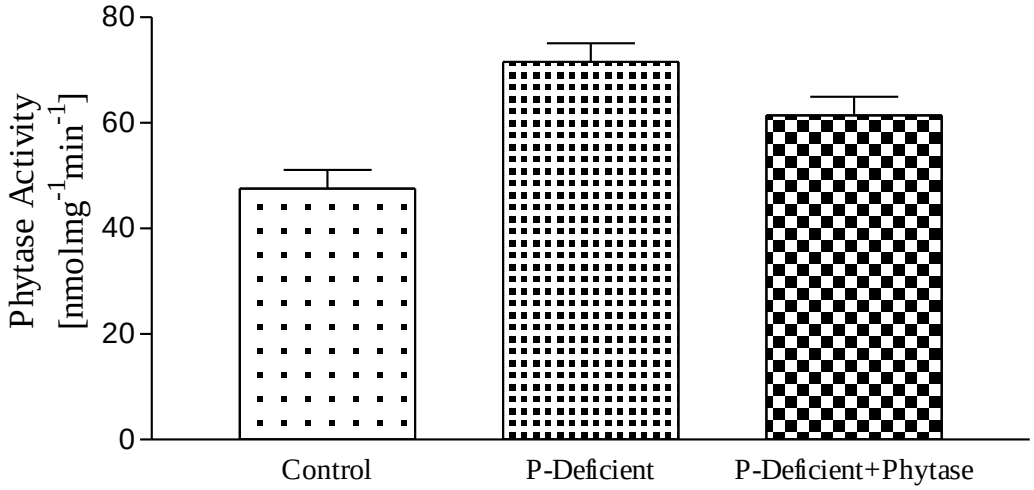

Figure 2. Effect of inorganic P and exogenous phytase on intestinal phytase activity during the growing period of broiler chicks. The bars represent the mean ± SEM of 4 determinations of phytase activity; mucosa from three birds was pooled in triplicates.

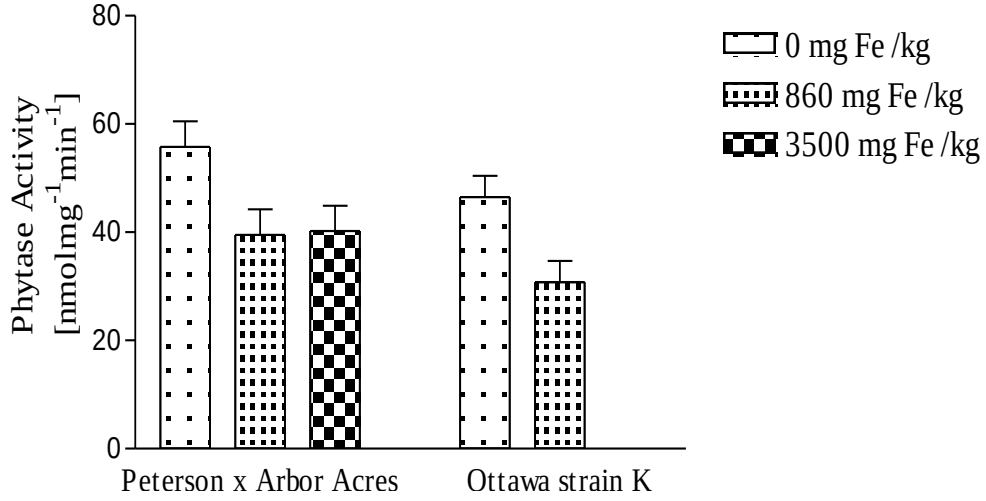

Figure 3. Effect of iron addition as ferrous sulfate to diet of two strains of birds. The bars represent the mean ± SEM of the 6 determination of phytase activity on individual bird bases.

reported in the literature varies from 5.5-6.5 (Maenz and Classen, 1998), 6.8 (Kriger, 1938), 7.1 (Biehl and Baker, 1997), 7.2 (Davies and Motzok, 1972), 7.4 (Davies et al., 1970), 7.8 (Maddaiah et al., 1969), 8.2 (Anders and Hill, 1967) to 8.3 (Bitar and Reinhold, 1972).

This wide range may be ascribed to variations in the assay systems used. Phytase activity depends on [Mg^{2+}] and [Zn^{2+}] with maximal IPA at about 20 and 2 mmol/L of $MgCl_2$ and $ZnCl_2$, respectively. Inhibition of IPA occurred when the assay system contained 2.5 and 5.0 mM $ZnCl_2$ (Maenz and Classen, 1998). Zinc phytate precipitated when [Zn^{2+}] exceeded 0.625 mM (Bitar and Reinhold, 1972). Intestinal alkaline phosphatase is inhibited by L-phenylalanine in rat and chicks (Bitar and Reinhold, 1972; Rao and Ramakrishnan, 1985; Iqbal et al. 1994). In this study, phytase activity was not affected by the addition of L-phenylalanine. It was concluded that IPA resulted from an enzyme other than alkaline phosphatase. The observation of inhibition in chicks may be reconciled by the fact that at pH 8.3 Bitar and Rienhold (1972) measured alkaline phosphatase rather than phytase. This is also supported by the linear inhibition noted with the inhibitor cocktail that is specific for other phosphatases. It is well documented that phytase activity is greatest in the proximal region of the intestine (Kriger, 1938; Davies and Flett, 1978; Iqbal et al., 1994; Maenz and Classen, 1998) and our results confirmed the earlier observations. In the proximal region of the

duodenum, the broiler strain had double the activity when compared to the distal region. However, Marounek et al. (2010) concluded that phytase activity was present in all sections of the digestive tract of laying hens in particular in the cecum area.

A gender effect was not evident in immature broilers sampled individually. McGuaig et al. (1972) reported differences in phytase activity in broilers according to strain and sex of the birds. They reported that females of each strain tested had slightly higher activity than males. In this study, no significant effects of age or gender in broilers were detected from 1 d to 8 weeks. This could be due to the fact that phytase was determined based on intestinal extracts from individual birds. Steenbock et al. (1958) reported high variation for IPA of individual extracts but when they used a large number of animals they detected differences between treatments. Laying pullets had IPA that was comparable to immature broilers but mature cockerels had about 50% of the IPA observed in comparable pullets. The results show that IPA is regulated by phosphorus requirements of the bird as evidenced by higher IPA in rapidly growing broilers and laying pullets that have increased P turnover in comparison to low IPA in mature cockerels with little P turnover (Ravindran et al., 1995; Marounek et al., 2010). This was also supported by our results on the effect of dietary P on IPA. Mature laying hens have a higher P requirement and phytate P utilization than males (Maddaiah et al., 1963) and the gender and age differences reported by McGuaig et al. (1972) in chicks may be due to the thyroactive casein used in the diet. Phytate utilization in animals has been ascribed phytase generated by gut microflora (Wise and Gilburt, 1982). Similar phytate P utilization in chicks after caecectomy or megadoses of dietary antibiotics (Biehl and Baker, 1997) and similar phytate breakdown in conventional or germfree rats (Miyazawa et al., 1996) question the role of gut microflora. We detected high IPA in chicks sampled at hatch, prior to extensive microbial colonization, and it was comparable to IPA activity in growing broilers and mature pullets. Our results together with research cited question the role of microflora as the sole source of IPA in chickens.

Phytate P retention depends on P status of the animal and substantial degradation of phytate P was observed in rats (Moore and Veum, 1983) and chickens (Ballam et al., 1984) fed diets low in P. This response could be due to an increase in IPA. In the present study, feeding low P diets in the starter or grower period stimulated IPA. Lowering the TP level of the starter diet resulted in a significant increase in phytase activity. Our results are in agreement with earlier reports (Davies and Motzak, 1970; McGuaig et al., 1972) on the effects of dietary P on IPA. On the other hand, Maddaiah et al. (1964) failed to show any significant difference as a result of lowering the P level by 0.4% in 4 week-old chicks or mature hens.

In summary, birds adapt to low P level in the diets by producing higher intestinal phytase activity and thus higher capacity to utilize the phytate P and release more P. It is concluded that chickens have innate regulated intestinal phytase that is essential in maintaining the P status.

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