Project No. : 05

Effect of Phytase on Phytate P Utilization by Turkeys

David R. Ledoux
Assistant Professor - Principal Investigator
University of Missouri-Columbia

Jeffre D. Firman
Assistant Professor - Co-investigator
University of Missouri-Columbia

Jonathan N. Broomhead
Undergraduate Student Assistant
University of Missouri

Grant Number: 14-08-0001-G-2029-01
U.S. Geological Survey
U.S. Department of the Interior

Missouri Water Resources Research Center
University of Missouri-Columbia
E-1511 Engineering Building East
Columbia, Missouri
65211

"The activities on which this report is based were financed in part by the Department of the Interior, U.S. Geological Survey, through the Missouri Water Resources Research Center. The contents of this publication do not necessarily reflect the views and polocies of the Department of the Interior, nor does mention of trade names or commercial products constitute thier endorsement by the united States Government."

An *in vitro* procedure for studying enzymic dephosphorylation of phytate in maize-soya bean feeds for turkey poults

ABSTRACT

An *in vitro* method was developed for poultry to predict inorganic phosphorus release from maize-soya bean feeds containing supplemental phytase (E.C. 3.1.3.8), and to quantify the effect of acid phosphatase (E.C. 3.1.3.2.), fungal protease (E.C. 3.4.23.6) and *Aspergillus niger* cellulase (E.C. 3.2.1.4.) on phytate dephosphorylation. Pepsin and pancreatin digestion periods were preceded by a 30 min preincubation at pH 5.25 to simulate digestion in the crop of poultry. Pancreatin digestion was carried out in dialysis tubings, with a ratio of about 1:25 (v/v) between the digesta and dialyzing medium, to simulate gradient absorption from the duodenum. The feed/water ratio was kept within physiological limits and a constant feed weight to digestive enzymes was maintained. There was a linear response to increasing dosages of phytase up to 1000 FTU/kg feed, and to increasing phosphate concentration in feeds. *In vivo* validation was performed with growing turkeys (1-3 wk) fed diets containing 12 g/kg of calcium; 0, 500, 1000 FTU/kg of phytase in a factorial arrangement with 0, 1, 2, 3 g/kg of supplemental phosphate (from KH_2PO_4). After a simple transformation (variable/in vitro phosphorus = *f* (in vitro phosphorus)) amounts of phosphorus hydrolyzed from feed samples by *in vitro* digestions correlated with the 3 week body weight gains ($R= 0.986$ $P<0.0001$), toe ash ($R=0.952$ $P<0.0001$), feed intake ($R=0.994$ $P<0.0001$) and feed efficiency ($R=0.992$ $P<0.0001$). The dephosphorylating ability of phytase *in vitro* was significantly enhanced ($P< 0.05$) by the addition of acid phosphatase. Fungal acid protease and *Aspergillus niger* cellulase also enhanced the dephosphorylation process *in vitro*.

INTRODUCTION

Salts of phytic acid (*myo*-inositol hexakis-dihydrogenphosphate, phytate) comprise about two-thirds of the phosphorus in cereal grains and oil seed meals, the two major components of poultry feeds. Phytate phosphorus is poorly available to simple-stomached animals. Insoluble protein-metal-phytate complexes are formed below the isoelectric pH of proteins, reducing calcium, magnesium, iron and zinc absorption from the intestinal tract of animals and humans (Reddy *et al.* 1989). Physiological pH values alter solubilities of phytate complexes with calcium, phosphorus and protein (Champagne & Phillippy, 1989) as well as influence the velocity of reactions catalyzed by enzymes of digestive, feed or food origin. Phytate, as well as different *myo*-inositol phosphate esters, products of enzymatic hydrolysis of phytate, vary in their inhibitory effect on pepsin, α-amylases, and

show different chelating capacity toward nutrients. As a result digestibility of proteins, starch and lipids is reduced (Reddy et al. 1989).

Phytase, myo-inositol hexaphosphate phosphohydrolase (E.C. 3.1.3.8 and E.C. 3.1.3.24) catalyzes the stepwise hydrolysis of inorganic orthophosphate from the myo-inositol backbone of phytates (Irving & Cosgrove, 1974). Phytase preparations derived from moulds and added to diets fed to simple-stomached animals have improved retention of phytate phosphorus and calcium, and decreased the content of phosphorus in animal manures (Nelson et al. 1971; Simons et al. 1990; Lei et al. 1993; Ketaren et al. 1993; Schöner et al. 1993). Phytase added as a feed ingredient is subjected to the environmental conditions present in the intestinal tract (eg. pH changes, proteolysis). In the intestine, phytase acts on complexes of phytate with other plant tissue components, which are parts of the complex matrix of digested food in the intestinal lumen. An assay involving standardized physiological conditions is required therefore for studying the action of an exogenous phytase on phosphorus release from feeds.

Lease (1967) proposed an in vitro procedure, comprising pepsin and pancreatin digestion, to determine availability of Zn in oilseed meals. The dialyzability of ^{65}Zn from the pH 6.8 pancreatin digest had little agreement with the in vivo uptake of ^{65}Zn by tissues. An in vitro method simulating human digestion and absorption of non-heme dietary Fe from complex meals (Miller et al.,1981) generated results that correlated well with in vivo data from humans (Schricker et al. 1981). Wolters et al. (1993) used a continuous in vitro method based on hollow-fibre dialyzing unit for estimation of the bioavailability of minerals in foods. This procedure correlated well with the method of Miller et al. (1981) for zinc, copper and iron estimation, but not for calcium and magnesium. Bedford & Classen (1993) designed an in vitro assay for predicting the effects of xylanase addition to rye-based feeds for poultry. The growth promoting ability of a commercial xylanase preparation was related to in vitro viscosity of the diet, which underwent pepsin and pancreatin digestions.

A valid in vitro method for the prediction of phosphorus availability in practical diets fed to turkeys does not exist. Thus, the purpose of this research was to design an in vitro method to determine phosphorus availability from maize-soya bean meal feeds containing commercial phytase and to validate the procedure with an in vivo experiment with turkey poults. Available phosphorus is meant throughout this work as the sum of inorganic phosphorus (endogenous or supplemental), organic non-phytate phosphorus, and a part of phytate phosphorus which is available, or is made available e.g. by enzymatic release (Sanders et al., 1992). For the in vitro studies the term available phosphorus is synonymous with "dialyzable" e.g. inorganic phosphorus concentration in the dialyzate, while for in vivo studies it refers to an ability of

promoting growth, or to increase the percentage of toe ash. Factors that may influence feed dephosphorylation (acid phosphatase and tissue-degrading activities, different calcium concentrations in the diet) were also tested in vitro.

MATERIALS AND METHODS

Animals. Two hundred forty newly hatched (1 d old) Nicholas Large White turkey hens were obtained from a commercial hatchery (Cargill, Inc., California, MO). Birds were housed in stainless steel battery brooders with wire-mesh floors, with temperature maintained at 32±1°C in the first week. Every 7 d the temperature was decreased by 2°C. Lighting was continuous and feed and water were provided ad libitum. On day 7, poults were weighed (mean weight:110 g) and assigned randomly to dietary treatments. On day 21, poults were weighed individually and feed consumption determined per pen. The poults were then killed by asphyxiation with CO_2, followed by cervical dislocation. Toe samples were obtained by carefully and uniformly severing the middle toe from each foot between the second and third tarsal bones.

Diet Composition and Experimental Design.

The composition of diets is given in Table 1. Turkeys were fed a starter maize-soya bean meal diet that met or exceeded all nutrient requirements (NRC, 1984) for one week, and experimental diets for another two weeks. Experimental diets were formulated from a basal diet E that contained 4.63±0.02 g/kg of total phosphorus (by analysis), 1.9 g/kg of available phosphorus (by calculation), and 12 g/kg of calcium (by calculation). Diet E was supplemented with four levels of supplemental inorganic phosphorus (0, 1, 2, or 3 g/kg) from reagent grade KH_2PO_4 (Sigma Chemicals Co, St. Louis, MO), and three levels of phytase 0, 500, 1000 FTU/kg (from Natuphos® 5000, Royal Gist Brocades NV, Agro Business Group, Ruswuk, The Netherlands) in a 4 x 3 factorial arrangement. Each treatment was replicated four times with five birds per pen. Diets were formulated to contain similar levels of protein (280 g/kg), energy (11.732 MJ/kg), and calcium (12 g/kg). The crude fat and fiber content of diets NRC and E were 54.1, 4.40 g/kg and 62.7, 4.86 g/kg respectively. Phytase activity was determined before mixing the phytase preparation with other feed ingredients. One unit of phytase activity (FTU) was defined as the amount of enzyme which frees 1 μM of inorganic phosphorus from 1.5 mM sodium phytate per minute at pH 5.5 and 37°C. The protein and energy contribution from the enzyme addition were considered insignificant.

Assays.

Duplicate samples of feed were digested by wet-ash procedure, which was validated by including standard reference material 1572 (Citrus leaves) from the National Institute of Standards and

Technology. Phosphorus concentration was determined colorimetrically by the molybdo-vanadate method (AOAC, 1970). Toe samples were dried at 100°C for 24 h and ashed in an electric muffle furnace at 600°C for 24 h.

Measurement of intestinal pH. Intestinal pH was measured in the crop, gizzard and duodenum of four 3 wk old turkeys fed a starter (NRC) diet. After killing, the intestine was removed and the pH of intestinal contents was read directly, using a pencil-thin, gel-filled combination electrode (Fisher Scientific, St. Louis, MO, 13-620-290). pH values (mean ± SD) found were 5.28±0.29, 2.71±0.54, 5.98±0.06 for the crop, gizzard and duodenum, respectively. Thus for the in vitro procedure feed samples were adjusted to pH values of 5.25, 2.50 and 6.00 for the three incubation periods simulating the three sections of the intestinal tract.

In Vitro Digestions and Measurements: Samples of diets were collected during feed preparation and refrigerated. Plastic syringes (5 ml) were prepared by cutting off their Lauer-locks to form a plastic tube equipped with piston. A 1 g (± 0.001 g) sample of maize-soya bean meal feed, ground through a 1 mm screen, was weighed into each of syringe. The feed sample was hydrated with double distilled water and HCl solution so that the concentration of 0.03 M HCl in a final volume of 1.5 mL was obtained. When enzyme solution was applied, the double distilled water was partly substituted for the enzyme solution. The contents of each tube were vortexed, the tubes were sealed with parafilm and incubated in a water bath at 40°C for 30 minutes. Then 0.5 mL of 1.5 M HCl was added to each tube along with 3000 units of pepsin, mixed well, vortexed, sealed with parafilm and reincubated for 45 minutes at the same temperature. During pepsin digestion samples were vortexed twice. At the end of this period, 0.65 ml of 1 M $NaHCO_3$ containing 3.7 mg/mL pancreatin (8 x USP) was added dropwise with constant stirring into each tube. The slurry was transferred quantitatively to segments of dialysis tubing (molecular weight cut-off 12000-14000, diameter 16.0 mm, Sigma Chemical Co., St. Louis, MO) by means of the piston. Segments were placed in 250 mL Erlenmeyer flasks containing 100 mL of 0.1 M NaCl in a 0.05 M succinate buffer (pH 6.0) and incubated in a shaking water bath at 41.1°C (the temperature of dialysate was 40°C). A ratio of about 25:1 (vol/vol) between the dialyzing medium and segments contents was maintained. Samples of the dialysate were withdrawn at specified time intervals and inorganic phosphate released was determined (Lowry & Lopez, 1946). Samples of diets were analyzed by the in vitro procedure in triplicate.

Evaluation of the in vitro procedure - effect of phosphorus and phytase concentrations. Samples of basal diet E were mixed with graded levels of KH_2PO_4 to give 0.5 to 4.0 g P/kg diet in 0.5 g/kg increments, or with Natuphos® 5000 to have phytase activity of 0, 250, 500, 1000 and 2000 FTU/kg, and investigated by the in vitro method for the amount of inorganic phosphate released.

The influence of calcium concentration in feeds on the yield of phytate hydrolysis. The calcium:supplemental phosphorus ratio is a well known factor contributing to the availability of phytate phosphorus, as confirmed in several studies with poultry and pigs. We examined the sensitivity of the in vitro procedure to different calcium levels by decreasing the calcium content in the diet from 12 to 6 g/kg, with different levels of phytase activity: 0, 500, and 1000 FTU/kg. Phytase was applied in solution to a 1 g sample of maize-soya bean meal containing 12 or 6 g/kg of calcium, subjected to the in vitro procedure and analyzed for the amount of inorganic phosphorus released.

Enzymes and Enzyme Activity Measurements. The microbial phytase (E.C. 3.1.3.8) used in this study (NATUPHOS® 5000, a commercial preparation with the phytase activity of 6250 FTU/g (declared by the producer) was provided by Royal Gist Brocades, Agro Business Group, Ruswuk, The Netherlands. A crude phytase preparation from Aspergillus ficuum, P-9792, Sigma Chemical Co, St. Louis, MO) was also tested in vitro. Phytase activity was determined at 40°C using 2 mM of sodium phytate in 100 mM acetate buffer (pH 4.5) as a substrate. The incubation mixture contained 3 ml of substrate and 0.5 ml of enzyme solution; the incubation time was 60 minutes. The reaction was terminated by adding 0.5 ml aliquot of the reaction mixture to 4 ml of acetate-acid-molybdate reagent, and the liberated phosphorus was determined spectrophotometrically (Heinonen and Lahti, 1981). One unit of phytase activity (**PhytU**) was defined as 1 µMe of inorganic phosphorus that was liberated in one minute under the above conditions. Natuphos® 5000 had the activity of 250 PhytU/g, while the preparation purchased from Sigma Chemical Co. - 51 PhytU/g. Pepsin (P-6887) and pancreatin (P-7545, activity: 8 x USP) were purchased from Sigma Chemical Co., St. Louis, MO. Aspergillus niger mycelium which contained intracellular as well as bound to cell wall phytase and acid phosphatase was obtained from Department of Food Biotechnology, University of Agriculture in Krakow, Poland. Fungal acid protease (E.C. 3.4.23.6), type XIII, from Aspergillus saitoi, (P-2143, Sigma Chemical Co., St. Louis, MO,) had an activity of 0.8 units/mg (1 unit is defined as the amount which hydrolyze hemoglobin to produce color equivalent to 1.0 µMe tyrosine per minute at pH 2.8 at 37°C). Fungal cellulase (E.C. 3.2.1.4.) from Aspergillus niger (C-1184, Sigma Chemical Co.) had an activity of 0.49 units/mg (1 unit liberates 1.0 µM of glucose from cellulose in one hour at pH 5.0 at 37°C). Acid phosphatase (P-3752, Sigma Chemical Co, St. Louis, MO) was found to be free of phytase activity. The activity of acid phosphatase (E.C. 3.1.3.2.) was assayed at 40°C using 5.5 mM disodium p-nitrophenylphosphate in 100 mM acetate buffer (pH 4.5). Final volume of the solution containing the enzyme and the substrate was 1.05 ml. After 30 minutes of incubation, the reaction was terminated by the addition of 5 ml of 40 mM NaOH, and the amount of p-nitrophenol released was determined spectrophotometrically at 405 nm. One unit of acid phosphatase activity (**AcPU**) was equal to 1 $\mu M \cdot min^{-1}$ of p-nitrophenol liberated.

Statistical analyses. Data were analyzed by the General Linear Models Procedure of SAS (1985). In the experiment with turkeys the variances for body weight gains, feed intake, toe ash and feed efficiency were found to be heterogenous (Bartlett's test). An attempt to homogenize the variances by following the Taylor's power procedure (Fry, 1993) did not produce any increase in the sensitivity of differences detection. ANOVA and regression analysis were performed therefore on non-transformed data and individual standard error of means were determined for each treatment (Baker, 1986). Means were compared using LSD test ($P<0.05$). For fitting the in vivo responses to in vitro dialyzable phosphorus different models (linear, logarithmic, and "Hanes' transformation") were compared by calculating coefficients of determination as well as the F values for the lack of fit (Fry, 1993). "Hanes' transformation" : variable/in vitro P = f (in vitro P) was inspired by Hanes' plot used for determining the kinetic parameters of an enzymatically catalyzed reaction (Dixon and Webb, 1964).

RESULTS

Evaluation of the in vitro procedure - effect of phosphorus and phytase concentrations. A linear release of phosphorus was observed over the phosphorus concentration range investigated (Figure 1A). The phosphate recovery ranged from 93 to 113% (mean=104, SD=4.39). Phosphorus release in response to the increasing levels of phytase was linear up to 1000 FTU/kg ($R^2=0.987$, $P<0.0001$). Higher activities (1500, 2000 FTU/kg) produced responses which were better described by a quadratic equation (for 2000 FTU/kg: $R^2=0.985$, $P < 0.0001$). The response across all concentration can be described by the kinetics of an enzymatically catalyzed reaction - the quantity of product formation as a function of an enzyme concentration (Figure 1B).

Turkey Growth Response Trial. A significant phytase x phosphorus interaction was observed for each of the parameters studied except feed efficiency (Table 2). In treatments without added phosphate, feed intake, body weight gain and toe ash responded with significant increases to graded levels of phytase. The increase in feed intake and body gains of turkeys fed 1 g/kg of supplemental phosphorus were attenuated by the addition of 1000 FTU of phytase to the diets. Such a decrease however, was not observed in the percentage of ash in the toes. Further increase in phosphorus supplementation (2 g/kg) suppressed the increase in feed intake caused by phytase addition. No significant changes in feed intake, gain or toe ash, which could be attributed to phytase, were observed with 3 g/kg of supplemental phosphorus.

Similarly, the effect of phosphorus on growth and bone mineralization of turkey poults was more profound at low levels of phytase supplementation. Feeds which did not contain supplemental phytase promoted better feed intake, body gains and toe ash due to increasing concentration of inorganic phosphorus. At 500 FTU/kg of

Effects of tissue degrading activities on the rate of phytate dephosphorylation. Results of the experiment are presented in Table 4. Phytase activity 500 FTU/kg not only significantly increased the amount of phosphorus released from maize soya bean meal, but had also a profound effect on the amount of dialyzable protein and the concentration of reducing sugars in the dialyzate. Further increase in phytase activity (1000 FTU/kg) however, did not significantly influence the extent of proteolysis or saccharification. The addition of fungal acid protease (5000 U/kg) along with 500 FTU/kg of phytase had a positive effect on the extent of feed proteolysis, slightly increased the concentration of reducing sugars and increased dephosphorylation by 15 %. The extent of maize soya bean meal feed dephosphorylation was further enhanced by the addition of fungal cellulase (5000 U/kg) to a mixture of the two previously studied enzymes. The amount of reducing sugars in the dialyzate increased significantly as a result of cellulolytic saccharification. It could be concluded that phytase activity of 1000 FTU/kg can be reduced by one half without negatively influencing the dephosphorylation rate, when appropriate tissue-degrading activities are present during the reaction.

Intracellular phytate-degrading enzymes hydrolyze phytates in feeds as determined by the in vitro procedure. The 10 g/kg feed supplementation with a dried Aspergillus niger mycelium resulted in a response (91.98 mM P/kg) similar to 1000 FTU/kg, whereas 50 g/kg supplementation caused complete conversion (152.0 mM P/kg) of total into inorganic phosphorus. The release of phosphorus from the basal diet (20.5 mM/kg) was not changed significantly (20.49mM/kg) when supplemented with 10 g/kg of autoclaved mycelium (data not shown).

The influence of calcium concentration in feeds on the yield of phytate hydrolysis. A decrease in calcium content of the diet from 12 to 6 g/kg, resulted in a significant increase in the amount of inorganic phosphorus released from maize-soya bean meal diets at each level of added phytase (0, 500, and 1000 FTU/kg, Figure 3).

DISCUSSION

The enzymatic activity of phytase is measured in vitro using a pure substrate and standardized conditions. Differences exist in phytase assays (pH from 2.5 to 5.5; substrate concentration from 1.5 mM sodium phytate to 42 mM of Mg-K phytate; temperature 37 or 40°C) reported in the literature. This impacts enzyme activity - e.g. 600 "Sigma units" used by Edwards (1993) in studies with broilers as the highest dosage equals 140-150 FTU. There is a need therefore for a standardized assay capable of predicting the efficacy of different feed enzymes under conditions similar to those applied in practice.

phytase addition, toe ash was improved by 1 or 2 g/kg of added phosphorus, but no further increase was observed with 3 g/kg of. With 1000 FTU/kg of supplemented phytase in the diets there was a significantly higher percentage of ash in the toes resulting from 1 g/kg of phosphorus addition. No further increase however, was caused by higher levels of phosphorus supplementation. Feed efficiency was not affected by increasing levels of phytase in poults fed diets supplemented with 0, 1 or 3 g/kg of phosphorus. However, in birds receiving 2 g/kg of inorganic phosphorus in the diet, feed efficiency was improved by the addition of 500 or 1000 FTU of phytase. In general, toe ash, feed intake, and body weight gain showed asymptotic responses both to phytase and phosphorus. The rate of fall-off from the asymptotes can be related to the degree to which phosphorus or phytase was lacking in the diets.

In vitro dialyzable phosphorus A significant phytase x phosphate interaction ($P<0.0001$) existed for in vitro dialyzable inorganic phosphorus. There was a linear increase in release of phosphorus from feeds containing graded levels of phosphate. Phytase addition also resulted in significant increases in phosphorus release from diets by the in vitro assay. At 0, 1, and 3 g/kg of added phosphorus these increases were significant, while at 2 g/kg the increase caused by 1000 FTU of phytase was not significantly higher than with 500 FTU/kg.

Different regression models (linear, logarithmic, and "Hanes' transformation") were tested to determine the ability of the in vitro method to predict in vivo responses (Table 3). The linear model accounted for 71 % of the variation in body weight gains observed in the in vivo study. The logarithmic model explained 88 % of the response in gain, while "Hanes transformation" predicted 97 % of the variability in body weight gains without significant lack of fit. Linear regression for other parameters accounted for 68 % of response in toe ash, 57 % of that for feed intake and 59 % for feed efficiency. Generally, "Hanes transformation" produced very good model for body weight gain, feed intake (covering 99 % of the observed variability), with a good fit. It allowed also to predict well feed efficiency and toe ash, but here the lack of fit was significant.

The effect of acid phosphatase activity in commercial phytases on the rate of dephosphorylation Acid phosphatase activity when applied to feed at two different concentrations (R=20, R=40) did not significantly change the amount of phosphorus released (Figure 2). Apparently acid phosphatase was not able to hydrolyze phytate in the absence of phytase. In a mixture with 1000 FTU/kg of phytase, acid phosphatase addition (R=20) resulted in a significant increase in the amount of dialyzable phosphorus released from maize-soya bean feed. Further enrichment however (R=40, R=80) did not result in any further increase in dephosphorylation.

The *in vitro* method described in this paper simulates digestive conditions in the crop, gizzard and duodenum. The crop pH of turkeys fed maize soya bean meal was 5.28, which is similar to optimal pH for phytase, therefore in the crop simulation pH was held at 5.25. The feed/water ratio, which may affect solubility of substrate and velocity of reactions, was kept within physiological limits and a constant feed weight to digestive enzymes was maintained. Simulation of digestive conditions in the small intestine was considered to be the most critical step for *in vitro* methods used to predict the absorption of minerals (Hunt *et al.*, 1987). Because phytase is inhibited by phosphate, and pancreatic enzymes can undergo end-product inhibition, a discrimination between low- and high molecular weight soluble compounds was made under conditions simulating gradient absorption from the duodenum (dialysis). In contrast to previously reported procedures, the initial pH and the osmolarity of the dialyzate was standardized; a ratio of about 1:25 (v/v) was maintained between the digesta and the dialyzing medium. Gastrointestinal transit times, which influence the extent of digestion and absorption, depend on many factors which are difficult to reproduce *in vitro*. Golian & Maurice (1992) reported a clearance time of 2.5 to 3 hour for 2-3 wk old broiler chicks and 3.5 to 4 h for poults aged 4-6 weeks. It is hard to determine however, how that time should be divided among different parts of the intestine. In our procedure the pepsin digestion time and pepsin concentration reported by Bedford & Classen (1993) were adopted. The time of pancreatin digestion (240 minutes) was chosen on the basis of the best correlation found between the dialyzable phosphorus and body weight gains of turkeys.

The amount of inorganic phosphorus released by the *in vitro* assay increased linearly with increasing concentrations of inorganic phosphate in feed samples. Similarly, the changes in inorganic phosphorus concentration of the dialyzate resulted from different phytase activities in feeds suggest that the assay is accurate enough for monitoring effects of different phytase levels on inorganic phosphate release from feeds.

Turkeys have a high requirement both for calcium (12 g/kg) and available phosphorus 6 g/kg (NRC, 1984). The ratio of calcium /available phosphorus is a major factor contributing to the retention of phytate phosphorus (Sanders *et al.*, 1992), and is especially important in diets deficient in phosphorus (Vandepopuliere *at al.*, 1961). Mohammed *et al.* (1991) reported, that with reduced inorganic phosphorus and increased cholecalciferol levels in broiler diets, an increase in phytate phosphorus utilization up to 65 % was observed. The calcium to phosphorus ratio also influences the extent of phytate hydrolysis by phytase (Schöner *et al.* 1993). In the present experiment turkeys were fed 12 g/kg of calcium. However, the birds' requirement for phosphorus (NRC, 1984) would only be met in the treatment with 3 g/kg of supplemental phosphorus and 1000 FTU/kg of phytase (assuming 1000 FTU/kg from Natuphos® 5000 to be equivalent to 1

g/kg of inorganic phosphorus; Vogt, 1992). All the other treatments were phosphorus deficient but met the calcium requirement. Increasing the levels of phosphorus in the diet not only increased the amount of available phosphorus, but also improved the calcium/phosphorus ratio. Phytase would dephosphorylate phytate, increasing available phosphorus and improving calcium/phosphorus ratio, in spite of a possible increase in calcium utilization resulting from phytase action. The question arises therefore whether these two phenomena are synergistic. The amounts of dialyzable phosphorus determined by the in vitro assay seem to support such a hypothesis.

Body weight gains were slightly better correlated with in vitro phosphorus release (R=0.847) than was toe ash (R=0.817). The in vitro procedure was better in predicting responses at lower levels of phytase and phosphorus supplementation, while at highest levels the linear model applied to the in vitro measurements overestimated the actual in vivo responses. Fritz et al. (1969) reported that the toe ash percentage and the weight gain of broiler chicks aged 3 weeks were linearly correlated with the logarithm of the phosphorus content of the diet. When the responses obtained using the in vitro method were linearized by logarithmic transformation, the correlations with body weight gains and toe ash were 0.940 and 0.882, respectively ($P< 0.0001$). The best linearization method found however, was the "Hanes transformation", which resulted in the best fit with all the in vivo data. This raises the intriguing possibility that Monods' equation (Fry, 1993) known as a very good model for the estimation of microbial growth, when microbes growing on nutrient deficient media are then supplemented with graded concentrations of the deficient nutrient, can be applied to higher organisms provided that a good analytical method for determining nutrient availability from feed is available. This will be addressed in more detail elsewhere.

Vandepopuliere at al. (1961) found that increased utilization of phytate phosphorus lead to improved growth rather than increased bone ash, similar to data in this experiment. It can be concluded that for diets rich in phytase and supplemental phosphate with adequate calcium and cholecalciferol levels (96.4 µg/kg diet), the phosphorus released by means of enzyme action is utilized for growth rather then for bone mineralization. The growth promoting character of phosphorus released by the action of phytase can possibly be partially attributed to the increased concentration of myo-inositol, the final product of phytate dephosphorylation, which is believed to stimulate growth of chicks, turkeys, rats, and mice (Combs, 1992).

With broiler chicks receiving graded levels of phytase and supplemental phosphate, Denbow et al. (1993) reported increased feed intake and body weight gains. Similar responses were also observed in young turkeys fed supplemental phytase and phosphate (Ravindran et al. 1993). In contrast, we did not observe any

significant changes in feed intake responses at 2 and 3 g/kg levels of inorganic phosphate supplementation. The amounts of phosphorus released as determined by the in vitro procedure were moderately (R = 0.766) correlated with feed intakes. No effect of varying phosphorus and calcium concentrations on feed efficiency was found by Sanders et al. (1992). Results of the present study seem to confirm this observation but only in treatments supplemented with inorganic phosphate. Phytase addition improved feed efficiency at 2 g/kg of phosphorus supplementation, the increases at the other levels failed to be significant. On the other hand the increases in feed efficiency were correlated (see Table 3) with the amounts of phosphorus released by the in vitro procedure.

Acid phosphatase activity can accelerate phytate dephosphorylation by phytase with sodium phytate as the substrate (Żyła, 1993) or with rapeseed phytates (Żyła & Koreleski, 1993). The present study indicates that this effect occurs for maize-soya bean meal feed subjected to simulated intestinal conditions. These findings demonstrate the importance of acid phosphatase activity in commercial phytase preparations intended for use in the feed industry.

The described in vitro procedure was applied to measure the extent of feed proteolysis and saccharification. Effect of enzymic phytate degradation on the extent of feed proteolysis observed in this study confirms that the removal of phytate from soy proteins improves its digestibility (Ritter et al., 1987). Ketaren et al. (1993) credited phytase for "proteolytic activity", as it increased live-weight gain, protein retention, and daily protein deposition in pigs. Acid proteinase activity was found in aleurone grains of plant seeds (Yatsu & Jacks, 1968), along with phytates, and thus can be suspected to play a role in phytate dephosphorylation during seed germination. We found that exogenous fungal acid protease (5000 units/kg) when applied to a maize-soya bean meal feed along with 500 FTU/kg of phytase increases proteolysis yield and enhances dephosphorylation. This effect was magnified as a result of coapplication of Aspergillus niger cellulase (5000 units/kg) to the mixture of phytase and acid protease enzymes. The positive influence of cellulolytic activity on the enzymic hydrolysis of phytic acid in soya bean meal has been reported by Han (1988). In contrast to our findings, however, he did not observe any positive influence of bromelain (plant protease) on dephosphorylation. Soya bean meal which was subjected by Han to a single incubation at pH 5.4 created certainly a different substrate than the full feed formulation which underwent conditions employed in our studies. The intracellular phytate-degrading enzymes from the waste Aspergillus niger mycelium left after citric acid fermentation have been characterized previously (Żyła, et al. 1989). In this study they were applied to the maize-soya bean meal feed as a supplement at two different concentrations, and the in vitro method was found useful in quantifying activities of enzymes which are either intracellular or bound to cell walls.

Interaction of dietary phosphorus with calcium in maize-soya bean meal can be expected to take place not only during or after absorption of these minerals, but also before absorption, in the gastrointestinal tract. Saio et al. (1967) indicated that the 11S protein in soya bean can bind more phytate in the presence of calcium. High levels of dietary calcium are thought to inhibit phosphorus absorption by forming insoluble calcium-phosphorus complexes in the intestine (Guyton, 1986). High dietary calcium may form insoluble complexes with phytate, which are resistant to an enzyme action (Fisher, 1993). The effect of calcium concentration in the diet on dialyzable phosphorus release was demonstrated in the present work. Reducing the calcium concentration from 12 to 6 g/kg caused a significant increase in the amount of phosphorus freed from basal maize-soya bean meal diet, and significantly increased the amount of phosphorus released by phytase, both at 500 and 1000 FTU/kg.

In conclusion it can be postulated that the in vitro procedure described here simulated the digestive conditions of the intestinal tract of turkeys. It predicted phosphorus bioavailability in maize-soya bean meal feeds containing different concentrations of inorganic phosphorus and (or) phytase. It allowed for measuring of the phosphorus release, extent of proteolysis, and carbohydrate digestion resulting from phytase, acid phosphatase, fungal acid protease and cellulase addition to feeds. Enzyme preparations can be added to feeds in solution for the measurement of theoretical (100 %) values of hydrolysis, or mixed with other ingredients during feed preparation before in vitro analysis. The procedure was shown to be sensitive to calcium concentration in feeds. Finally, the described in vitro method is accurate, reasonably rapid, cheap, simple and robust. It is expected to be easily modified for different species, types of feeds, and nutrients.

LITERATURE CITED

Association of Official Analytical Chemists. (1970) 7. Animal feeds. Phosphorus. 7.095 -7.098. Photometric method. p. 125. In: Official methods of analysis. Association of Official Analytical Chemists, Washington, DC.

Baker, D. H. (1986). Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. J. Nutr. 116, 2339-2349.

Bedford, M.R. & Classen, H.L. (1993). An in vitro assay for prediction of broiler intestinal viscosity and growth when fed rye-based diets in the presence of exogenous enzymes. Poultry Sci. 72, 137-143.

Combs, G.F. (1992). In: The Vitamins. Fundamental Aspects in Nutrition and Health. Academic Press Inc. San Diego. CA. pp.411-415.

Denbow, D. M., Ravindran, V., Kornegay, E.T., Self, B. B. & Hulet, R. M. (1993). Improved availability of phosphorus in soya bean meal for broiler chicks by supplemental phytase. Poultry Sci. 72 (Suppl. 1), 250 (abs.)

Dixon, M. and Webb, E.C. (1964) In: Enzymes. Enzyme Kinetics. Michaelis Theory. Academic Press Inc., New York. pp. 63-70.

Edwards, H.M. (1993). Dietary 1,25-dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. J. Nutr. 123, 567-577.

Fisher, H. (1993). Low-calcium diets enhance phytate-phosphorus availability. Nutr. Rev. 50, 170-171.

Fritz, J. C., Roberts, T., Boehne, J. W. & Hove, E. L. (1969). Factors affecting the chik's requirement for phosphorus. Poultry Sci. 48, 307-320.

Fry, J.C. (1993). In: Biological Data Analysis. A Practical Approach. IRL Press. Oxford, New York, Tokyo. pp. 14-21.

Golian, A., & Maurice, D. V. (1992). Dietary poultry fat and gastrointestinal transit time of feed and fat utilization in broiler chikens. Poultry Sci. 71, 1357-1363.

Guyton, A.C. (1986) Medical Physiology., 7-th ed., p. 937. Philadelphia, PA: W.B. Saunders and Company.

Han, Y.W. (1988). Removal of phytic acid from soybean and cottonseed meals. J. Agric. Food Chem. 36, 1181-1183.

Heinonen, J. K., Lahti, R. J. (1981). A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal. Biochem. 113, 313-317.

Hunt, J.R., Johnson, P.E. & Swan, B. (1987). Dietary conditions influencing relative zinc availability from foods to the rat and correlations with in vitro measurements. J. Nutr.117, 1913-1923.

Irving, G. C. & Cosgrove, D. J. (1974). Inositol phosphate phosphatases of microbial origin. Some properties of the partially purified phosphatases of Aspergillus ficuum NRRL 3135. Australian J. Biolog. Sci. 27, 361-368.

Ketaren, P.P., Batterham, E.S.,Dettman, E.B. & Farrell, D.J. (1993). Phosphorus studies in pigs. 3. Effect of phytase supplementation on the digestibility and availability of phosphorus in soya-bean meal for grower pigs. Br. J. Nutr. 70, 289-311.

Lease, J. G. (1967). Availability to the chick of zinc-phytate complexes isolated from oil seed meals by an in vitro digestion method. J. Nutr. 93, 523-532.

Lei, X., Ku, P.K., Miller, E. R., Ullrey, D.E. & Yokoyama, M.T. (1993). Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J. Nutr. 123, 117-1123.

Lowry, O. H. & Lopez, J. A. (1946). The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem. 162, 421-428.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31, 426-428.

Miller, D. D., Schricker, B. R., Rasmussen, R. R. & Van Campen, D. (1981). An in vitro method for estimation of iron availability from meals. Am. J. Clin. Nutr. 34, 2248-2256.

Mohammed, A., Gibney, M. J. & Taylor, T. G. (1991). The effects of dietary levels of inorganic phosphorus, calcium and cholecalciferol on the digestibility of phytate-P by the chick. Br. J. Nutr. 66, 251-259.

National Research Council, (1984). Nutrient Requirement of Poultry. 8th ed. National Academy Press, Washington, DC.

Nelson, T. S., Shieh, T. R., Wodzinski, R. J. & Ware. J.H. (1971). Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. 101, 1289-1294.

Ravindran, V., Denbow, D. M., Kornegay, E. T., Self, B. B. & v Hulet, R.M. (1993). Supplemental phytase improves availability of phosphorus in soybean meal for turkey poults. Poultry Sci. 72, (Suppl. 1): 73 (abs.)

Reddy, N. R., Pierson, M. D. Sathe, S. K. & Salunkhe, D. K. (1989) Phytates in Cereals and Legumes. CRC Press,Inc., Boca Raton, FL.

Ritter, M.A., Morr, C.V., & Thomas, R. L. (1987) In vitro digestibility of phytate-reduced and phenolics-reduced soy protein isolates. J. Food Sci. 52, 325, SAS Institute, (1985). SAS® User's Guide: Statistics. SAS Institute Inc., Cary, NC.

Saio, K., Koyama, E., & Watanabe, T. 1969. Protein-calcium-phytic acid relationship in soybean. III. Effect of phytic acid on coagulative reaction in tofu-making, Agr. Biol. Chem. 32, 448-

Sanders, A. M., Edwards, H. M. & Rowland, G. N. (1992) Calcium and phosphorus requirements of the very young turkey as determined by response surface analysis. Br. J. Nutr 67, 421-435.

Schöner von, F. J., Hoppe, P.P., Schwarz, G. & Wiesche, H. (1993). Vergleich von mikrobieller Phytase und anorganischen Phosphat bei Masthahnenküken: Wirkungen auf die Mastleistungen und die Mineralstoffretention bei variierter Calcium-Versorgung. J. Anim. Physiol. Anim. Nutr. 69, 235-244.

Schricker, B. R., Miller, D. D., Rasmussen, R.R & Van Campen, D. (1981) A comparison of in vivo and in vitro methods for determining availability of iron from meals. Am. J. Clin. Nutr. 34,: 2257-2263.

Simons, P.C.M., Versteegh, H. A. J., Jongbloed, A. W., Kemme, P. A. Slump, P., Bos, K. D., Wolters, M. G. E., Beudeker, R. F. & Verschoor, G. J. (1990). Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64, 525-540.

Vandepopuliere, J.M., Ammerman, C.B., & Harms, R.H. (1961) The relationship of calcium-phosphorus ratios to the utilization of plant and inorganic phosphorus by the chick. Poultry Sci. 40, 951-957.

Vogt, H. (1992). Einsatz von Phytase im Broilermastfutter mit unterschiedenem Phosphorgehalt: 2 Versuch. Archive für Geflügelkunde 56, 222-226.

Wolters, M. G. E., Schreuder, H. A. W., Heuvel, van den, G.,Lonkhuijsen, Van, H. J., Hermus, R. J. J. & Voragen, A. G. J. (1993). A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in foods: application to breads varying in phytic acid content. Br. J. Nutr. 69, 849-861.

Yatsu, L.Y. & Jacks, T.J. (1968). Association of lysosomal activity with aleurone grains in plant seeds. Arch. Biochem. Biophys. 124, 466-471.

Żyła, K. (1993). The role of acid phosphatase activity during enzymic dephosphorylation of phytates by Aspergillus niger phytase. World J. Microb. Biotech. 9, 117-119.

Żyła, K. & Koreleski, J. (1993). In-vitro and in-vivo dephosphorylation of rapeseed meal by means of phytate-degrading enzymes derived from Aspergillus niger. J. Sci. Food Agric. 61, 1-6.

Żyła, K., Koreleski, J. & Kujawski, M. (1989). Dephosphorylation of phytate compounds by means of acid phosphatase from Aspergillus niger. J. Sci. Food Agric. 49, 315-324.

ACKNOWLEDGMENTS

This study was supported in part by the Department of the Interior, U.S. Geological Survey, through the Missouri Water Resources Research Center. The authors would like to thank the "Gist Brocades" company (The Netherlands) for supplying the Natuphos® 5000 preparation used throughout the study. Author K. Zyla expresses his appreciation to the Miller Fund (administered by the Department of Animal Sciences, University of Missouri) for financial support.

This is a contribution from the Missouri Agricultural Experiment Station. Journal Series Number 12083.

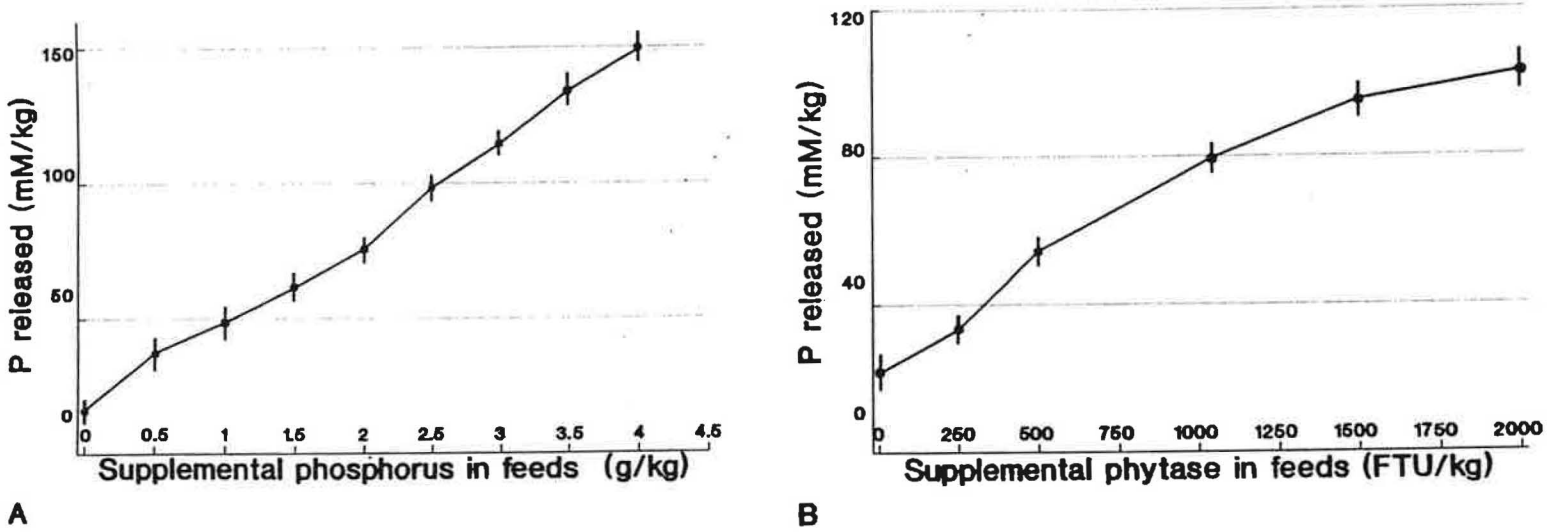
Description of the Figures

Figure 1: Effect of phosphorus (A) and phytase (B) concentrations in feeds on the phosphorus release determined by in vitro procedure*.

*Phosphorus release in response to the increasing levels of phosphorus was linear $R^{2} = 0.99$; $P < 0.00001$). For phytase the response was linear up to 1000 FTU/kg ($R^2=0.987$, $P <0.0001$). A quadratic equation better characterized the response over the entire activity range ($R^2=0.985$, $P < 0.0001$).

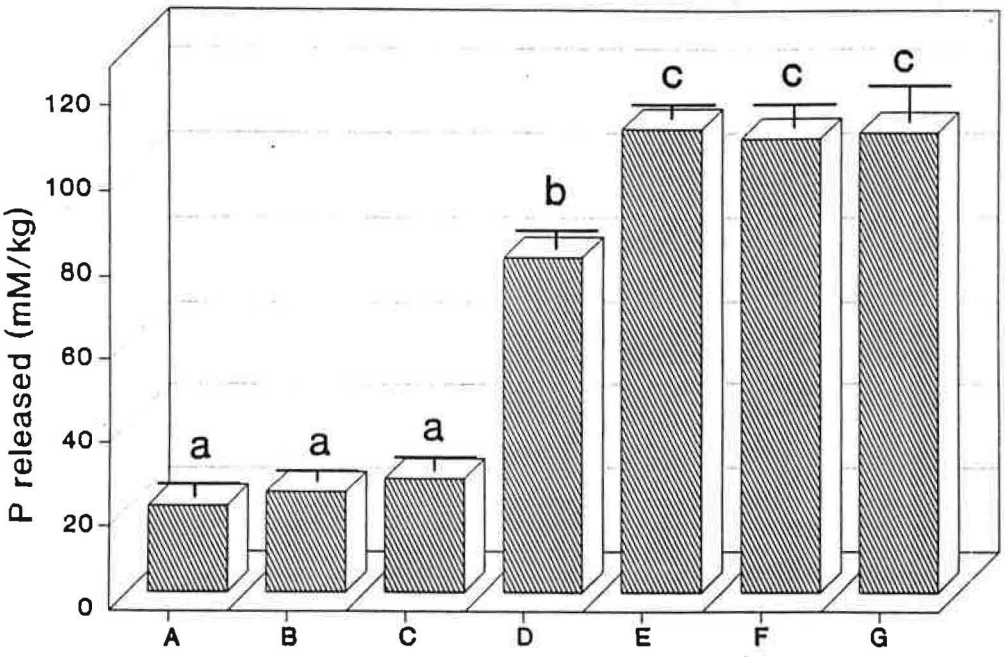
Figure 2: The influence of acid phosphatase (E.C. 3.1.3.2.) on the dephosphorylation of maize-soybean meal feed by phytase*.
*The acid phosphatase/phytase ratio (14.6 AcPU/PhytU) characteristic for Natuphos® 5000 was regarded as one unit of enrichment (R). Treatments: A-control (no enzyme), B-acid phosphatase (R=20), C-acid phosphatase (R=40), D-phytase 1000 FTU/kg, E-phytase 1000 FTU/kg and acid phosphatase (R=20), F-phytase 1000 FTU/kg and acid phosphatase (R=40), G-phytase 1000 FTU/kg and acid phosphatase (R=80). T-test was applied to determine significance between differences, $P<0.05$.

Figure 3: The influence of a calcium concentration on the amount of inorganic phosphorus released from maize-soya bean meal feed by phytase.
T-test was applied to determine significance between differences, $P<0.05$.



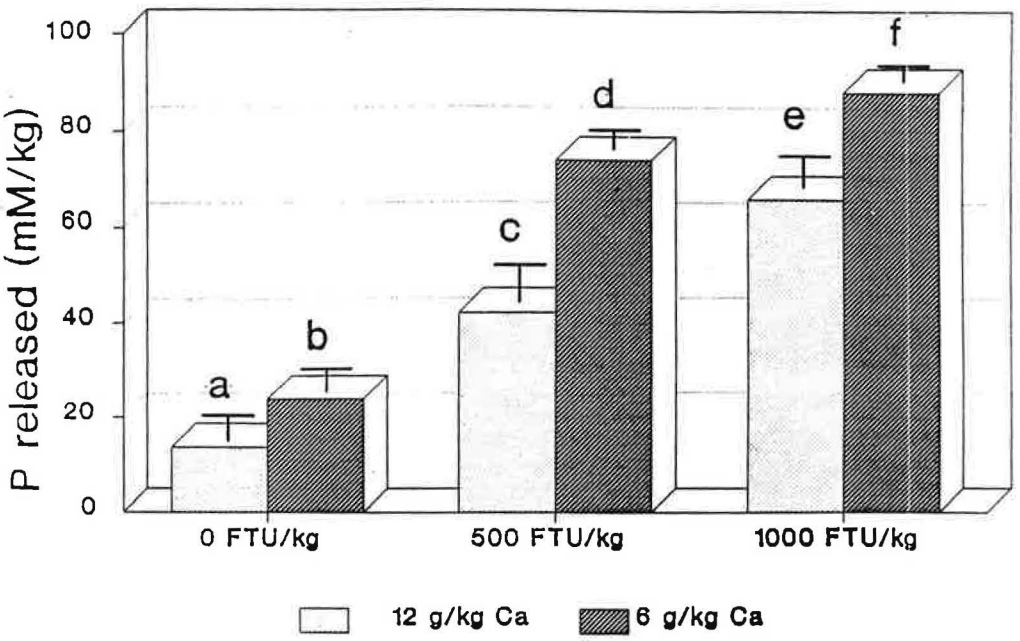
(a) An in vitro procedure for studying enzymic dephosphorylation of phytate in maize-soya bean feeds for turkey poults, Zyla, K., et al.

(b) Figure 1.



(a) An in vitro procedure for studying enzymic dephosphorylation of phytate in maize-soya bean feeds for turkey poults, Zyla, K., et al.

(b) Figure 2.



(a) An in vitro procedure for studying enzymic dephosphorylation of phytate in maize-soya bean feeds for turkey poults, Zyla, K., et al.

(b) Figure 3.

(TABLE 1, continued)

Metabolizable energy[¶]	MJ/kg	11,732	11,732
Crude fat[¶]	g/kg	54.1	62.7
Fiber[¶]	g/kg	4.40	4.86

---

[*]The NRC diet provided all nutrients at concentrations recommended (NRC, 1984); diet E all the nutrients with the exception of phosphorus.

[†]Trace mineral premix provided: 222 mg MnO_2; 209mg ZnO; 654 mg $FeSO_4 \cdot 7H_2O$; 9 mg Cu_2O; 1.9 mg ethylenediamine dihydroiodide; 160 mg $CaCO_3$ per kg diet.

[‡]Supplied 0.2 mg of selenium per kilogram of diet

[§]Vitamin premix (mg/kg): all-trans-retinyl acetate, 3.03; cholecalciferol, 96.4μg; all-rac-α-tocopheryl acetate, 14; niacin, 55;calcium panthotenate, 16; riboflavin, 6.6; vitamin B-6, 2.2; menadione sodium bisulfite, 1.7; folic acid, 1.4; thiamin mononitrate, 1.1; biotin, 0.2; vitamin B-12, 11μg, ethoxyquin, 83.

[ff]Twelve diets were formulated by appropriate mixing of 985 g/kg of diet E with 15 g/kg of a premix containing 0, 1, 2, or 3 g/kg KH_2PO_4 (reagent grade, Sigma Chemical Co. St. Louis MO), in a 4 x 3 factorial arrangement with three levels of phytase (0, 500, 1000 FTU/kg)

[¶]by calculation (Feed Formulation, The Brill Corporation, Norcross, GA)

Table 2: Effect of dietary phosphorus and phytase activity on performance, bone mineralization of turkey poults, and in vitro dialyzable phosphorus*.

Phytase	Phosphorus added to diets g/kg			
FTU/kg	0	1	2	3
		Mean (SE)		
Feed Intake† (g)				
0	264_{A}^{a}(7)	393_{B}^{a}(9)	442_{C}^{a}(6)	441_{C}^{a}(14)
500	373_{A}^{b}(12)	430_{B}^{b}(9)	445_{BC}^{a}(4)	454_{C}^{a}(1)
1000	408_{A}^{c}(13)	440_{B}^{b}(4)	441_{B}^{a}(11)	445_{B}^{a}(7)
Gain† (g)				
0	164_{A}^{a}(8)	289_{B}^{a}(9)	328_{C}^{a}(6)	346_{C}^{a}(9)
500	254_{A}^{b}(9)	325_{B}^{b}(11)	348_{BC}^{b}(5)	359_{C}^{a}(11)
1000	279_{A}^{c}(7)	336_{B}^{b}(4)	361_{C}^{b}(7)	372_{C}^{a}(6)
Feed Efficiency (Gain/Feed)‡				
0	0.62_{A}^{a}(0.03)	0.73_{B}^{a}(0.02)	0.74_{B}^{a}(0.02)	0.78_{B}^{a}(0.01)
500	0.68_{A}^{a}(0.02)	0.76_{B}^{a}(0.02)	0.78_{B}^{ab}(0.02)	0.79_{B}^{a}(0.02)
1000	0.68_{A}^{a}(0.01)	0.77_{B}^{a}(0.02)	0.82_{C}^{b}(0.02)	0.82_{C}^{a}(0.01)

(Continued on next page)

(Table 2, continued)

Toe ash[†] (%)

0	6.41_{A}^{a}(0.11)	9.41_{B}^{a}(0.20)	13.84_{C}^{a}(0.15)	14.98_{D}^{a}(0.33)
500	8.54_{A}^{b}(0.26)	12.62_{B}^{b}(0.11)	14.91_{C}^{b}(0.18)	14.45_{C}^{a}(0.23)
1000	9.66_{A}^{c}(0.38)	13.91_{B}^{c}(0.43)	14.61_{B}^{b}(0.22)	14.64_{B}^{a}(0.19)

In Vitro Phosphorus[†§] (mM/kg)

0	13.55_{A}^{a}	53.23_{B}^{a}	70.65_{C}^{a}	103.87_{D}^{a}
500	42.26_{A}^{b}	68.39_{B}^{b}	110.97_{C}^{b}	140.32_{D}^{b}
1000	65.16_{A}^{c}	81.94_{B}^{c}	118.71_{C}^{b}	162.58_{D}^{c}

Pooled SEM: 6.96

---

[*]Each value in the table represents the mean of five pens values (±SEM). Values of the same variable with no common superscripts within the same phosphorus level are significantly different. Values of the same variable with no common subscripts within the same phytase level are significantly different. LSD-test, $P<0.05$.

[†]Probabilities associated with main effects (ANOVA) were: phosphorus (df=3) 0.0001; phytase (df=2) 0.0001; phosphorus x phytase (df=6) 0.0001.

[‡]Probabilities associated with main effects (ANOVA) were: phosphorus (df=3) 0.0001; phytase (df=2) 0.0001; phosphorus x phytase (df=6) 0.5932

[§]Analysis performed in triplicates.

Table 3: Different regression models tested for fitting the in vitro results and the data from in vivo experiment with turkeys --

Parameter	Model*	R^2	Fit F†	Estimates ±SE (P<0.005) Intercept	Slope
Body	LIN	0.712	14.40	208.86±20.09	39.20±6.81
weight	LOG	0.884	2.92	241.85±6.339	200.14±14.03
gain (g)	HAN	0.973	1.91	0.001852±0.0002	0.002330±0.00005
Toe	LIN	0.677	40.0	7.2344±1.13	1.912±0.38
ash	LOG	0.778	26.0	8.9963±0.64	9.337±1.42
(%)	HAN	0.906	23.2	0.062167±0.007	0.054057±0.002
Feed	LIN	0.566	14.63	329.27±23.0	31.97±7.80
intake	LOG	0.829	3.31	351.40±7.91	176.6±17.5
(g)	HAN	0.988	1.00	0.00072±0.00009	0.002046±0.00003
Feed	LIN	0.587	2.47	0.6408±0.019	0.0404±0.006
efficiency	LOG	0.626	1.64	0.6795±0.011	0.1933±0.024
gain:feed	HAN	0.984	2.13	0.3522±0.057	1.1685±0.019

*-Models: LIN- linear, LOG- logarithmic, HAN- transformation: variable/in vitro P = f (in vitro P), analogous to Hanes' plot used in enzyme kinetic to determine the Michaelis-Menten constant.

†-F (Lack of fit Mean Square /Pure error Mean Square). $F_{0.05}(10,48)=2.03$; $F_{0.1}(10,48)=2.71$.

Table 4: Effect of fungal acid protease (E.C. 3.4.23.6) and Aspergillus niger cellulase (E.C. 3.2.1.4) on the rate of phytate dephosphorylation in maize-soya bean meal feed by a phytase preparation (Natuphos® 5000)*.

Treatment	P released (mM/kg)	Reducing sugars (mM glucose/100 g)	Lowry protein (g/kg)
Basal diet (no enzyme)	$23.3^{a} \pm 1.49$	$96.9^{a} \pm 4.5$	$41.5^{a} \pm 0.73$
Phytase 500 FTU/kg	$78.6^{b} \pm 2.25$	$114.3^{ab} \pm 5.9$	$48.6^{b} \pm 1.87$
Phytase 1000 FTU/kg	$92.0^{c} \pm 2.03$	$121.0^{ab} \pm 11.0$	$49.3^{b} \pm 1.43$
Phytase 500 FTU/kg + fungal acid protease 5U/g	$90.2^{c} \pm 2.86$	$126.8^{b} \pm 9.6$	$55.6^{c} \pm 1.08$
Phytase 500 FTU/kg + fungal acid protease 5U/g + A. niger cellulase 5U/g	$102.5^{d} \pm 1.66$	$181.3^{c} \pm 8.9$	$59.2^{d} \pm 1.88$

*Means ± standard error of samples run in triplicates are reported. T-test was applied to determine the significance of differences between means ($P<0.05$).

Enzymic Dephosphorylation of Corn-Soybean Meal Feed Simulated Intestinal Conditions of the Turkey.

ABSTRACT

A crude laboratory phytase preparation, a commercial phytase, and a commercial experimental preparation were used in vitro to study the extent of corn-soybean feed ($P_{tot.}$: 4.83± 0.06 g/kg) dephosphorylation under simulated intestinal conditions of the turkey. The phytases had different specific activities and varying side activities of acid protease, acid phosphatase, and pectinase, and showed different abilities for feed dephosphorylation. Acid phosphatase was shown to be a key activity in feed dephosphorylation, while fungal acid protease enhanced dephosphorylation by stimulating gastric digestion. An "enzymic cocktail" that completely dephosphorylated phytates present in corn-soybean feed also contained pectinase and citric acid. Complete dephosphorylation was accompanied by 12 to 29 % increase in dialyzable protein, and 45 to 81 % increase in the concentration of reducing sugars liberated from feed. There is a potential for improved digestibility of phytate phosphorus, protein, and carbohydrates when the "cocktail" is used as a feed additive for monogastric animals.

INTRODUCTION

Phytates are known to be a principal storage form of phosphorus in plant seeds, and, because of their strong chelating properties, decrease bioavailability of minerals (Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+}) from the gastrointestinal tract of monogastric animals and humans (Kratzer and Vohra, 1986; Reddy, 1989). The action of plant (EC 3.1.3.26) or microbial phytases (EC 3.1.3.8.) on phytates may deprive food and feed components of antinutritional properties, increase available phosphorus, and phosphorus retention by simple-stomached animals, as well as increase retention of calcium and micronutrients. Phytate dephosphorylation by phytase has been studied both outside (enzymic pretreatment of feed, Rojas and Scott, 1968) and inside the intestinal tract of animals (Nelson et al. 1971; Simons et al. 1990; Ketaren et al. 1993; Lei et al. 1993; Schöner et al. 1993). Soaking, germination and natural lactic acid fermentation of grains, foods, and feedstuffs were proposed as an alternative way of phytate hydrolysis by taking advantage of the intristic phytases present in plant seeds or biosynthesized by contaminating bacteria (Chompreeda and Fields, 1984; Larsen, 1993).

In the presence of exogenous phytase, the extent of phytate hydrolysis in the intestine of poultry (expressed as phosphorus digestibility, availability or utilizability) has been reported

to be no more than 55 % (Simons et al. 1990; Schöner et al. 1993). This amount of phytate degradation allows for the substitution of phytase at no more than 1-1.2 g/kg of inorganic phosphorus in practical diets for pigs and poultry (Vogt, 1992; Yi et al. 1994). Phytase may have limited access to phytate because of phytate occlusion by starch, lipids, and protein. Metal ions may stabilize complexes of phytate with other tissue components, whereas conditions of the intestinal tract (pH changes and proteolysis) might be detrimental to the stability of phytase. In consequence, phytate dephosphorylation *in vivo* is incomplete.

Han (1988) studied the impact of proteinase (bromelain), cellulase, and hemicellulase on soybean meal phytate dephosphorylation by *Aspergillus ficuum* phytase. Using a single 3-h incubation at 37°C and one unit of each enzyme per 1g of the substrate (3.3 % w/v) Han (1988) found that cellulase and hemicellulase improve the rate of phytate hydrolysis, whereas proteinase was not effective. Acid phosphatase in *Aspergillus niger* phytase accelerated the hydrolysis of a sodium phytate solution (Żyła, 1993) and rapeseed phytates (Żyła and Koreleski, 1993). This activity had a vital role in a corn-soybean meal (CSM) feed dephosphorylation by a commercial phytase under simulated intestinal conditions of the turkey (Żyła et al. 1994). The positive role of acid phosphatase (pH optimum 2.5, biosynthesized by *Aspergillus ficuum*) in the hydrolysis of phytate and lower phosphate esters of *myo*-inositol has been reported (Ullah and Phillippy,1994).

The objective of this investigation was to design a "*cocktail*" of several enzymes to completely dephosphorylate phytate under simulated intestinal conditions of the turkey.

MATERIAL AND METHODS

Reagents:
Dodecasodium phytate, p-nitrophenylophosphate and soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Co. Dinitrosalicylic acid was purchased from Aldrich. All other chemicals used were of analytical grade. Feed ingredients were obtained from commercial suppliers.

Enzymes:
Microbial phytases (E.C. 3.1.3.8) used were the following: a commercial preparation (phytase, C), a laboratory preparation (phytase, L), and a commercial experimental phytase (CE). Phytase L was prepared from a waste *Aspergillus niger* mycelium and was in a liquid form. *Aspergillus niger* mycelium was obtained from the Department of Food Biotechnology, University of Agriculture in Krakow, Poland. The mycelium was washed free from medium, dried, and milled on a laboratory grinder. A weighed portion of mycelium powder was wetted with cold 0.3 M acetic acid solution (pH 3.0) and homogenized. The 50 g/l suspension was

centrifuged (3000 g, 2°C, 0.5 h) and the supernatant was mixed with a protease inhibitor solution (20 g/l) prepared from potato juice (Żyła et al., 1989). The resulting solution was concentrated 10 fold at 45°C in a laboratory vacuum evaporator and mixed with glycerol (20 g/l) before storage (4°C). Fungal acid protease (E.C. 3.4.23.6) type XIII, from Aspergillus saitoi, acid phosphatase (E.C. 3.1.3.2.) from potato, Aspergillus niger pectinase (E.C. 3.2.1.15), pepsin, and pancreatin (activity: 8 x USP) were purchased from Sigma Chemical Co., St. Louis, MO. The supplier of the experimental phytase also provided experimental preparations of Aspergillus niger acid phosphatase, fungal acid protease, and pectinase, which were tested in analogous fashion as "Sigma" enzymes, and denoted by EXP subscripts throughout this work.

Experimental diet composition
The detailed composition of corn-soybean meal (CSM) diet used in the experiments is given in Table 1.

Assays
Total phosphorus in feed was determined colorimetrically by the molybdo-vanadate method (AOAC, 1970) in duplicate samples which were digested by a wet-ash procedure. The procedure was validated by including standard reference material 1572 (Citrus leaves) from the National Institute of Standards and Technology. The protein content of different phytases was determined by the method of Lowry et al. (1951).

In vitro digestions and measurements
The in vitro procedure of Żyła et al. (1994), designed for simulating the intestinal tract of the turkey was used. A 1 g (± 0.001 g) sample of CSM, ground through a 1 mm screen, was weighed into a 5 ml plastic syringe. The syringes were prepared by cutting off their Luer-lock tips. The feed sample was hydrated with double distilled water and HCl solution so that a concentration of 0.03 M HCl in a final volume of 1.5 ml was obtained. When enzyme solution was applied, water was partly (or completely) substituted for by the enzyme solution. The contents of each tube were vortexed, the tubes were sealed with parafilm and incubated in a water bath at 40°C for 30 min. Then 0.5 ml of 1.5 M HCl and 3000 units of pepsin were added to each tube, mixed well, vortexed, sealed with parafilm and reincubated for 45 min at the same temperature. At the end of this period, 0.65 ml of 1 M $NaHCO_3$ containing 3.7 mg/ml pancreatin was added dropwise with constant stirring into each tube. The slurry was transferred quantitatively to segments of dialysis tubing (molecular weight cut-off 12000-14000, diameter 16.0 mm, Sigma Chemical Co., St. Louis, MO) by means of the syringe piston. Segments were placed in 250 ml Erlenmeyer flasks containing 100 ml of 0.1 M NaCl in a 0.05 M succinate buffer (pH 6.0) and incubated in a shaking water bath at 41.1°C (temperature of dialysate was 40°C). A ratio of about 25:1 (vol/vol) between the dialyzing medium and segment

contents was maintained. Samples of the dialysate were withdrawn after 240 min for determining inorganic phosphate (Lowry and Lopez, 1946) reducing sugars (Miller, 1959), and protein (Lowry et al., 1951). Samples were analyzed by the *in vitro* procedure in triplicate.

Enzyme Activity Measurements

Phytase activity was determined at 40°C using 2 mM of sodium phytate in 100 mM acetate buffer (pH 4.5) as a substrate. The incubation mixture contained 3 ml of substrate and 0.5 ml of enzyme solution; incubation time was 60 min. The reaction was terminated by adding a 0.5 ml aliquot of the reaction mixture to 4 ml of acetate-acid-molybdate reagent; liberated phosphorus was determined spectrophotometrically (Heinonen and Lahti, 1981). One unit of phytase activity (**PhytU**) was defined as the amount of enzyme required to liberate 1 µM of inorganic phosphorus in one minute under the above conditions.

The activity of acid phosphatase (E.C. 3.1.3.2.) was assayed at 40°C using 5.5 mM disodium *p*-nitrophenylphosphate in 100 mM acetate buffer (pH 4.5). The final volume of the solution containing the enzyme and the substrate was 1.05 ml. After 10 min of incubation, the reaction was terminated by the addition of 5 ml of 40 mM NaOH, and the amount of *p*-nitrophenol released was determined spectrophotometrically at 405 nm. One unit of acid phosphatase activity (**AcPU**) was equal to 1 $\mu M \cdot min^{-1}$ of *p*-nitrophenol liberated under the described conditions.

Acid protease (E.C. 3.4.23.6.) activity was assayed in 0.1 M borate-acetate-phosphate buffer pH 2.8 at 40°C. One ml of enzyme solution was added to 5 ml of 2.5% (w/v) hemoglobin (pH adjusted to 2.8), and the incubation proceeded for 30 min. The reaction was stopped by the addition of 10 ml of 110 mM TCA solution; the contents were filtered, and the liberated amino acids and peptides were determined with Folin-Ciocalteu phenol reagent. One unit of acid protease (**ACPRU**) was defined as the amount of enzyme which hydrolyzed hemoglobin to produce color equivalent to 1.0 μM tyrosine per min at pH 2.8 and 40°C).

The activity of pectinase (E.C. 3.2.1.15.) was determined in 0.05 M acetate buffer pH 4.5 at 40°C. 1.4 ml of 0.678 % (w/v) polygalacturonic acid solution was mixed with 0.1 ml of enzyme solution and incubated for 10 min. The reaction was terminated by the addition of 2 ml of dinitrosalycylic acid reagent, and the reducing sugars released were determined by the method mentioned above. For the colorimetric measurement of carbohydrates with the dinitrosalicilic acid reagent, known amounts of glucose (0.25 mg) were added to samples, blanks and standards as described by Slominski et al. (1993). One unit of pectinase (polygalacturonase) activity (**PECU**) was the amount of enzyme which released 1.0 μM of reducing sugars measured as D-galacturonic acid from polygalacturonic acid per min at pH 4.5

and 40°C.

RESULTS AND DISCUSSION

Side Activities Present in Phytase Preparations. Enzyme preparations for the food industry are characterized in terms of main as well as secondary (side) activities (Godfrey and Reichelt, 1983). Secondary activities are of essential importance when complete conversion of complex substrates is required, or when substrates are occluded by other tissue components. Enzymatic saccharification of wheat starch by Aspergillus niger glucoamylase is a good example of such a process (Konieczny-Janda and Richter, 1992).

In the present study three different preparations of phytase (main activity) were used to achieve complete dephosphorylation of a feed based on CSM. Preliminary characteristics of these preparations revealed different purities of main activity as well as different levels at which phytase was accompanied by secondary (acid phosphatase, acid protease and pectinase) activities (Table 2). Commercial phytase (C) was of a high purity and had low side activities. Laboratory-made phytase (L) had low specific phytase activity, but was high in acid phosphatase. Phytase CE had a level of acid phosphatase similar to phytase C and protease activity comparable to that found in phytase L. Phytase CE had the highest pectinase activity among the phytases.

The Efficacy of Phytases in Feed Dephosphorylation. In the first experiment, equivalent activities of phytase ranging from 0 to 1387 PhytU/kg from C, L, and CE respectively, were applied to 1-g samples of feed and digested in vitro (Żyła et al., 1994). Amounts of dialyzable phosphorus released from feeds are depicted in Figure 1. Although there were significant differences in dephosphorylation rate among phytases, there were no linear responses to increasing phytase concentrations, and dephosphorylation was not complete. This indicates that phytase activity alone is not able to completely dephosphorylate phytates in corn-soybean feed. The same conclusion was reported in feeding trials with turkey poults (Ledoux et al., 1994). Plateaus occur in the response curves when phytase level in feed exceeds 700-750 units/kg, which is in good agreement with in vivo studies (Simons et al.,1990; Yi et al. 1994). Body weight gain, bone mineralization, phosphorus utilization and digestibility responses to increasing phytase concentration in diets high in phytate phosphorus, level off after a saturating concentration of enzyme in feed is reached. Dephosphorylation rates with phytases C, L, and CE differed significantly at each level of enzyme dosage, and a significant negative relationship between the specific activity of the phytases and their ability to release phosphorus from CSM was observed (Figure 1, Table 2). The negative influence of phytase purification upon the dephosphorylation of rapeseed phytates has been reported

previously (Żyła and Koreleski, 1993).

Effect of Acid Phosphatase (4 AcPU/g) and Pectinase (0.05 % w/w) on CSM Dephosphorylation. The positive effect of acid phosphatase activity on the dephosphorylation of corn soybean meal phytates under simulated conditions of the turkey intestine has been observed earlier (Żyła et al. 1994). In the present study, fungal acid phosphatase$_{EXP}$ alone, or in combination with an Aspergillus niger pectinase$_{EXP}$ (0.05 % w/w) was evaluated for influence on phytase C efficacy. Acid phosphatase addition to the feed (4 AcPU/g) increased the amount of inorganic phosphorus released from feed over controls by 33 % , while acid phosphatase plus phytase C (462 PhytU/kg), resulted in a 10 % increase in dephosphorylation as compared to phytase C alone (Table 3). There were no significant differences in dephosphorylation when the experimental preparation of acid phosphatase (derived from mold) was substituted for the same amount of AcPU from potato acid phosphatase (purchased from Sigma; data not shown). Coapplication of acid phosphatase and pectinase, phytase C and acid phosphatase, phytase C and pectinase, and the combination of phytase C, acid phosphatase and pectinase enhanced hydrolysis of polysaccharides (Table 3). In contrast to phytase L, phytase C did not significantly change the concentration of reducing sugars liberated from feed. Proteolysis was adversely affected by phytase L probably due to protease inhibitors in potato juice used to prepare phytase L (Table 3). Furthermore, a negative correlation (R= -0.91) was found between mean values of in vitro dialyzable phosphorus (InVDP) and dialyzable protein in treatments containing phytase. This indicates that conditions in the intestine are more detrimental phytate hydrolysis by phytase than those in the gizzard.

The Impact of Fungal Acid Protease on Feed Dephosphorylation. Phytate was reported to form complexes with proteins which are resistant to pepsin digestion (Kratzer and Vohra, 1986). Strong pepsin inhibition in the presence of phytate at pH 2-3 has been also reported by Vaintraub and Bulmaga (1991). These low pH values favor dephosphorylation since Aspergillus sp. phytase and acid phosphatase are very active at Ph 2.5-3.5 (Ullah and Gibson, 1987; Żyła, 1990). Since pepsin in the stomach likely does not digest phytate-degrading enzymes, enhanced gastric proteolysis could contribute to releasing more phytate from its complexes with protein and possibly accelerate dephosphorylation. Acid protease activity was found in the aleurone grains of plant seeds (Yatsu and Jacks, 1968), along with phytates, and thus can be suspected to increase phytate dephosphorylation during seed germination.

In order to intensify in vitro gastric proteolysis, fungal acid protease (14 ACPRU/g) was applied to feed samples along with phytase (231-1387 PhytU/kg). In this study dephosphorylation was improved by adding acid protease to each level of phytase C

(Table 4). There were no significant effects of acid protease applied with 925 or 1387 PhytU/kg from preparation L or CE. No significant improvement in dephosphorylation resulting from acid protease addition was observed with 231 PhytU/kg of phytase L, but coapplication of the protease and 231 PhytU/kg of phytase CE increased InvDP. The changes in the concentration of protein and reducing carbohydrates in dialyzates was associated with the extent of dephosphorylation. The concentration of dialyzable protein increased when the amount of phytase C was increased up to 694 PhytU/kg, but declined when the activity of phytase L was increased (Figure 2). Ketaren et al. (1993) credited phytase for "proteolytic activity", as it increased live-weight gain, protein retention, and daily protein deposition in pigs. Acid protease increased the extent of proteolysis, which at 925 PhytU/kg was similar for both phytases. In contrast to our findings, Han (1988) did not observe any positive influence of bromelain (plant protease) on soybean meal dephosphorylation. Soybean meal was evaluated by Han (1988) with a single incubation at pH 5.4; this was different substrate from the feed formulation which underwent multiple digestions in the present studies.

Saccharification, in contrast to proteolysis, was promoted by phytase L. The response to acid protease was similar for phytase L and C (Figure 3). The efficacy of Aspergillus saitoi acid protease (Sigma preparation) in accelerating dephosphorylation of corn-soybean feed by phytase, was compared with an experimental preparation of Aspergillus acid protease. Although activity (14 ACPR/g, plus 694 PhytU/kg) was the same in both preparations, twice as much enzyme from the experimental preparation was needed to reach the same level of dephosphorylation as for the Sigma source (Table 5). Proteases are known to differ in their ability to hydrolyze proteins of different origin. The determination of protease activity using hemoglobin as a substrate does not appear to give a good estimate of ability to break down plant proteins.

Trypsin Inhibitor and Citric Acid Assist the Process of Dephosphorylation. Trypsin inhibitor was applied to feed samples (500 μg/g) to protect phytate-degrading enzymes from pancreatic digestion; it increased the rate of phytate hydrolysis by 694 PhytU of phytase C from 3.1353 to 3.4403 g/kg ($P<0.001$), but had no effect on phytase L or mixture of phytase C and fungal acid protease ($P>0.05$). Hazell and Johnson (1987) found citrate to be effective in promoting iron diffusibility from plant foods under simulated intestinal conditions. It seemed possible that such an action might promote phytate hydrolysis either by assisting the breakdown of metal-phytate complexes e.g. by binding calcium, or indirectly by protecting pancreatic lipase from inhibition caused by iron ions (Iwai and Tsujisaka, 1984). Indeed, the application of citric acid (24μM/g) was effective in enhancing CSM dephosphorylation with 694 PhytU of phytase L (3.8934 vs 4.3171g/kg), phytase C (3.1353 vs 3.5116 g/kg) or phytase CE

(3.5244 vs 3.8416 g/kg). This has practical importance. Gentesse et al. (1994) found that CSM diet supplemented with 3 % citric acid had significant increases in the metabolizability of protein, calcium and phosphorus when fed to broilers.

Simultaneous Coapplication of Enzymes to Feed - Enzyme Mixtures ("Cocktails"). The coapplication of 14 ACPRU/g of acid protease ("Sigma" preparation), 147 μl/g of Aspergillus pectinase, 24μM/g of citric acid monohydrate, and 694 PhytU/kg of phytase from preparation C, L, and CE, caused a release of 4.0371 ± 0.0445; 4.7043 ±0.0432; and 4.4242 ± 0.0779 mg phosphorus from 1g feed samples, respectively. Subsequent coapplication of acid phosphatase (42 AcPU/g) with phytase L or CE increased hydrolysis to 100 ± 1 % of total phosphorus content in CSM. This "enzymic cocktail", however, required a very high level (14.7 % v/w) of fungal pectinase addition to the feed. Dephosphorylation dropped with each decrease in the level of pectinase supplementation with both phytase L and CE. Taking into account that side pectinase activity differs substantially between these phytases (see Table 2), it seemed obvious that an activity other than pectinase was crucial for dephosphorylation. This hypothesis was further supported when different activities of phytase (0-925 PhytU/kg) were evaluated with the "cocktail" components described above. As much as 57 ± 1 % of the total phosphorus content was hydrolyzed when no phytase activity was added (Table 6), suggesting the presence of phytate-degrading enzymes in the crude pectinase preparation used. Subsequently, the pectinase preparation was found to have high activity of both phytase and acid phosphatase. Based on these results, a new "enzymic cocktail" was designed with phytase activity of 925 PhytU/kg, and acid phosphatase 75 or 100 AcPU/g. The activity of acid protease as well as the concentration of citric acid in the new "cocktail" were the same as before. In spite of the high pectinase activity in phytase CE it was not possible to achieve complete dephosphorylation of phytates in CSM without addition of Aspergillus niger pectinase to the "cocktail". However, with acid phosphatase activity 100 AcPU/g, 7.4 μl of pectinase per g of feed was sufficient for complete dephosphorylation (Table 7). With phytase C similar rate of dephosphorylation was reached with the pectinase level of 14.7 μl/g and with acid phosphatase 100 AcPU/g. Phytase L did not require any pectinase addition when 75 AcPU/g of acid phosphatase was added to bring the dephosphorylation rate to 100 %.

The enzymic "cocktail" comprising of phytase (925 PhytU/kg), acid phosphatase (75-100 AcPU/g), fungal acid protease pectinase (14 ACPRU/g), citric acid (24μM/g) and pectinase (0-14.7μl/g) could completely dephosphorylate feeds. It seemed obvious however, that there was a non-identified factor present in phytase L as well as in the pectinase which stimulated the process. Among the identified activities, phytate-degrading enzymes were of utmost importance. Attempts to lower acid phosphatase activity, when phytase CE and all the other

"cocktail" components were present, resulted in significant decreases in the dephosphorylation rate (Figure 4). Among tissue-degrading activities, fungal acid protease and pectinase proved to be significant in assuring the access of phytate degrading enzymes to their substrate. The extent of tissue degradation resulting from the application of the enzymic "cocktail" was measured as increase in concentration of reducing sugars and dialyzable protein. Increase in the concentration of reducing sugars ranged from 45 % with phytase L up to 81% with phytase C in the presence of all the "cocktail" components. The "cocktail" with phytase CE increased the concentration of dialyzable protein by 12 %, and with phytase L by 29 % (Figure 5). This is in agreement with the observation of Ritter et al (1987) suggesting that the removal of phytate from soy proteins improves its digestibility.

Although the results presented need to be confirmed in in vivo nutritional studies with turkey poults, there is a potential for improved digestibility of phytate phosphorus, protein and carbohydrates when the "cocktail" is used as feed additive for monogastric animals.

LITERATURE CITED

Association of Official Analytical Chemists. 7. Animal feeds. Phosphorus. 7.095 -7.098. Photometric method. p. 125. In: Official methods of analysis. **1970**, p. 125. Association of Official Analytical Chemists, Washington, DC.

Chompreeda, P.T., Fields, M.L. Effects of heat and fermentation on the extractability of minerals from soybean meal and corn meal blends. J.Food Sci. **1984,** 49, 566-568.

Gentesse, N., Bernier, J.F., Lefrançois, M.R. Effect of dietary citric acid on diet metabolizability, blood acid-base balance and abdominal fat deposition in broilers. Poultry Sci. **1994,** 73 (Suppl. 1), 34.

Godfrey, T., Reichelt, J. In: Industrial Enzymology. The Application of Enzymes in Industry. The Nature Press: New York. 558-569, **1983.**

Han, Y. W. Removal of phytic acid from soybean and cotton seed meals by Aspergillus ficuum phytase. J. Agric. Food Chem. **1988,** 36, 1181-1183.

Hazell, T., Johnson I.T. In vitro estimation of iron availability from a range of plant foods: influence of phytate, ascorbate and citrate. Br. J. Nutr. **1987,** 57, 223-233.

Heinonen, J. K., Lahti, R. J. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal. Biochem. **1981,** 113, 313-317.

Iwai, M., Tsujisaka, Y. Fungal lipase. Inhibition. In: Lipases; Borgström, B., Brockman, H.L.; Elsevier: Amsterdam, New York, Oxford, **1984.**

Ketaren, P.P., Batterham, E.S., Dettman, E.B., Farrell, D.J.

Phosphorus studies in pigs. 3. Effect of phytase supplementation on the digestibility and availability of phosphorus in soya-bean meal for grower pigs. Br. J. Nutr. **1993,** 70, 289-311.

Konieczny-Janda, G., Richter, G. Progress in enzymatic saccharification of wheat starch. Starch/Stärke **1992,** 43, 308-315.

Kratzer, F. H. Vohra, P. Role of phytic acid and other phosphates as chelating agents. In: Chelates in Nutrition, CRC Press Inc: Boca Raton, FL, 49-61, **1986.**

Larsen, T. Dephitynization of a rat diet. Consequences for mineral and trace elements absorption. Biol. Tr. El. Res. **1993,** 39, 55-71.

Ledoux, D.R., Zyla, K., and T.L. Veum. Effects of a commercial microbial phytase in turkey poults fed corn-soybean meal diets. In: 1994 Animal Sciences Progress Report, Agricultural Experiment Station, University of Missouri-Columbia, (Special Report 465), 90-94, **1994.**

Lei, X., Ku, P.K., Miller, E. R., Ullrey, D.E., Yokoyama, M.T. Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J. Nutr. **1993,** 123, 117-1123.

Lowry, O. H., Lopez, J. A. The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem. **1946,** 162, 421-428.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem **1951,** 193, 265-275.

Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. **1959,** 31, 426-428.

Nelson, T. S., Shieh, T. R., Wodzinski, R. J., Ware. J.H. Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. **1971,** 101, 1289-1294.

Rojas, S. W., Scott, M. L. Factors affecting the nutritive value of cottonseed meal as a protein source in chick diets. Poultry Sci. **1969,** 48, 819-835.

Reddy, N. R., Pierson, M. D., Sathe, S. K., Salunkhe, D. K. Nutritional Consequences of Phytates, In: Phytates in Cereals and Legumes. CRC Press,Inc., Boca Raton, FL. 81-102, **1989.**

Ritter, M.A., Morr, C.V., Thomas, R. L. In vitro digestibility of phytate-reduced and phenolics-reduced soy protein isolates. J. Food Sci. **1987,** 52, 325-

Schöner, F. J., Hoppe, P.P., Schwarz, G., Wiesche, H. Vergleich von mikrobieller Phytase und anorganischen Phosphat bei Masthahnenküken: Wirkungen auf die Mastleistungen und die Mineralstoffretention bei variierter Calcium-Versorgung. J. Anim. Physiol. Anim. Nutr. **1993,** 69, 235-244.

Simons, P.C.M., Versteegh, H. A. J., Jongbloed, A. W., Kemme, P. A. Slump, P., Bos, K. D., Wolters, M. G. E., Beudeker, R. F., Verschoor, G. J. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. **1990,** 64, 525-540.

Slominski, B.A., Guenter, W., Campbell, L.D. New approach to water-soluble carbohydrate determination as a tool for evaluation of plant cell wall degrading enzymes. J.Agric. Food Chem. **1993,** 41, 2304-2308.

Ullah, A.H.J., Gibson, D.M. Extracellular phytase (EC 3.1.3.8) from Aspergillus ficuum NRRL 3135: Purification and characterization. Prep. Biochem. **1987,** 17, 63-91.

Ullah, A.H.J., Phillippy B.Q. Substrate selectivity in Aspergillus ficuum phytase and acid phosphatase using myo-inositol phosphates. J. Agric. Food Chem, **1994,** 42, 423-425.

Vaintraub, I.A. and Bulmaga, V.P. Effect of phytate on the in vitro activity of digestive proteinases. J. Agric. Food Chem. **1991,** 39, 859-861.

Vogt,H. Einsatz von Phytase im Broilermastfutter mit unterschiedenem Phosphorgehalt: 2 Versuch. Arch. Geflügelk. **1992,** 56, 222-226.

Yatsu, L.Y., Jacks, T.J. Association of lysosomal activity with aleurone grains in plant seeds. Arch. Bioch. Biophys. **1968,** 124, 466-471.

Yi, Z., Kornegay, E.T., McGuirk, A. Replacement values of inorganic phosphorus by microbial phytase for pigs and poultry. J. Anim. Sci. **1994,** 72, Suppl 1. 330.

Żyła, K. Acid phosphatases purified from industrial waste mycelium of Aspergillus niger used to produce citric acid. Acta Biotechnol, **1990,** 10, 319-327.

Żyła, K. The role of acid phosphatase activity during enzymic dephosphorylation of phytates by Aspergillus niger phytase. World J. Microb. Biotechnol, **1993,** 9, 117-119.

Żyła, K., Ledoux, D.R., Garcia, A., T.L. Veum. An in vitro procedure for studying enzymic dephosphorylation of phytate in maize-soya bean feeds for turkey poults. Br J. Nutr. - in consideration. **(1994).**

Żyła, K., Koreleski, J., Kujawski, M. Dephosphorylation of phytate compounds by means of acid phosphatase from Aspergillus niger. J.Sci. Food Agric. **1989,** 49, 315-324.

Żyła, K. & Koreleski, J. In-vitro and in-vivo dephosphorylation of rapeseed meal by means of phytate-degrading enzymes derived from Aspergillus niger. J. Sci. Food Agric. **1993,** 61, 1-6.

NOTES:

This study was supported in part by the Department of the Interior, U.S. Geological Survey, through the Missouri Water Resources Research Center and by Finnfeeds International Ltd, Marlborough, U.K.. Author K. Żyła expresses his appreciation to the Miller Fund (administrated by the Department of Animal Sciences, University of Missouri) for financial support.

Presented in part at the 83-rd Annual Meeting of the Poultry Science Association, Starkville, Mississippi, August 7-12, 1994 (Abstract No. 267).

Contribution from Missouri Agricultural Experiment Station. Journal Series Number 12170.

Table 1: Composition and nutrient content of CSM diet

Ingredient	g/kg
Soybean meal (44% CP)	580.41
Corn (ground, shelled)	354.25
Corn oil	46.13
Calcium carbonate (38% Ca)	10.69
Salt (NaCl)	4.00
Methionine-hydroxy analog	2.19
Trace mineral premix	1.10
Selenium premix	0.52
Vitamin premix	0.52
Choline-HCl	0.19
Nutrient content (g/kg)	
Ca*	6.00
$P^*_{available}$	1.90
P_{total}	4.83
Protein*	288.70
Metabolizable energy* (MJ/kg)	12085.00
Crude fat*	62.7
Fiber*	4.88

*by calculation ("Feed Formulation", The Brill Corporation, Norcross, GA)

Table 2. Specific activity of phytase and levels of secondary (side) activities in preparations C, L, and CE:

Phytase	Protein content (mg/g)	Phytase activity (PhytU/g)	Acid phosphatase activity (AcPU/g)	Pectinase activity (PECU/g)	Acid protease activity (ACPRU/g)
C	128	4440	2401	15	49
L*	23	15	134	7	6
CE	221	375	395	5550	138

	Phytase specific activity (PhytU/mg)	AcPU/PhytU	PECU/PhytU	ACPRU/PhytU x 10^3
C	34.69	0.54	0.003	11
L	0.65	8.93	0.45	393
CE	1.70	1.05	14.80	368

*Phytase L was in a liquid form. The protein concentration and enzymes activities were expressed per ml.

Table 3. The influence of acid phosphatase$_{EXP}$ (4 AcPU/g) and pectinase$_{EXP}$ (0.05 % w/w) on InVDP, dialyzable protein and reducing sugars concentration in the dialyzates from CSM feed.

Treatment	InVDP g/kg	Dialyzable Protein mg/g	Reducing Sugars mg/100g
Control	$0.6296^{a}\pm0.02$	$43^{b}\pm0.6$	$199^{a}\pm5$
Acid phosphatase	$0.8401^{c}\pm0.01$	$42^{b}\pm0.6$	$222^{b}\pm4$
Pectinase	$0.7140^{b}\pm0.01$	$44^{bc}\pm0.8$	$216^{ab}\pm7$
Acid phosphatase + pectinase	$0.9380^{d}\pm0.01$	$47^{c}\pm0.7$	$248^{c}\pm11$
Phytase C	$2.5618^{e}\pm0.04$	$46^{c}\pm1.5$	$221^{b}\pm7$
Phytase C +acid phosphatase	$2.8431^{f}\pm0.01$	$45^{bc}\pm1.0$	$231^{b}\pm8$
Phytase C+ pectinase	$2.7299^{f}\pm0.02$	$47^{c}\pm0.6$	$239^{c}\pm4$
Phytase C+ acid phosphatase + pectinase	$2.8244^{f}\pm0.05$	$46^{c}\pm1.5$	$233^{b}\pm9$
Phytase L	$3.2946^{g}\pm0.06$	$38^{a}\pm1.4$	$231^{b}\pm9$

Means with different superscripts are significantly different ($P<0.05$, t-test).

Table 4. Dephosphorylation of CSM by phytases C, CE, and L in the presence of acid protease (Sigma, 14 ACPRU/g).

Phytase Dosage (PhytU/g)	C IvDP (g/kg)	p*	CE IvDP (g/kg)	p*	L IvDP (g/kg)	p*	SEM
231	2.4525	0.002	2.5133	0.02	2.5119	0.07	0.05
462	3.2472	0.02	3.5572	0.001	3.7521	0.001	0.05
694	3.5774	0.02	3.7893	0.005	4.0091	0.008	0.06
925	3.5167	0.003	3.9303	0.05	4.4392	0.08	0.06
1387	3.5831	0.002	4.1828	0.07	4.3040	0.716	0.08

*Probability that the IvDP mean for samples with acid protease (Table), and without fungal acid protease (see Figure 1) are not different (paired T-test).

Table 5. Effects of two acid proteases on the dephosphorylation of CSM supplemented with 694 PhytU, phytase CE.

Acid protease		Dialyzable P (g/kg):
activity[1]	Source	mean ± SE
14	SIGMA	3.7893[c] ± 0.0366
3.5	Experimental prep	3.5747[a] ± 0.0416
7	Experimental prep	3.6808[b] ± 0.0430
14	Experimental prep	3.6490[ab] ± 0.0398
28	Experimental prep	3.7738[c] ± 0.0259
42	Experimental prep	3.8456[c] ± 0.0434
70	Experimental prep	3.9757[d] ± 0.0905

[1](ACPR/g of feed).

Means with different superscripts are significantly different ($P<0.05$, t-test).

Table 6. Effects of a "dephosphorylating cocktail" (fungal acid protease$_{EXP}$ (42 ACPRU/g; Aspergillus niger pectinase, Sigma, 147 µl/g; acid phosphatase$_{EXP}$ (42 AcPU/g; citric acid, 24µM/g) on CSM dephosphorylation, in the presence of varied phytase (CE) activity.

Phytase activity FTU/kg	Conversion rate[1] (%)
0	57 ± 1
231	84 ± 1
347	91 ± 1
462	93 ± 2
694	100 ± 1
925	102 ± 2

[1]Percentage of total phosphorus content in CSM.

Table 7: Dephosphorylation of CSM ($P_{tot.}$: 4.83± 0.06 g/kg) phytase, 925 PhytU/kg (from different preparations); fungal acid $protease_{EXP}$, 42 ACPRU/g; citric acid, 24μM/g; with varied activities of acid $phosphatase_{EXP}$ and pectinase (Sigma) .

Phytase	Treatment		N	IvDP (g/kg) (mean ± SE)
	AcPU/g	Pectinase:μl/g		
CE	100	0	10	4.6393^{a} ± 0.043
CE	100	7.4	10	4.8551^{c} ± 0.040
CE	100	14.7	13	4.8324^{c} ± 0.033
C	75	7.4	10	4.7186^{ab} ± 0.047
C	100	7.4	8	4.7999^{bc} ± 0.051
C	75	14.7	8	4.8093^{bc} ± 0.093
C	100	14.7	8	4.8637^{c} ± 0.099
L	75	0	8	4.8655^{c} ± 0.060
L	75	7.4	14	4.8587^{c} ± 0.031

Means with different superscripts are significantly different ($P<0.05$, t-test).

Description of Figures

Figure 1: The influence of the phytase dosage, and the preparation type on the inorganic phosphorus released from 1 g CSM.

Figure 2: Dialyzable protein released from 1 g CSM as a result of increasing dosages of phytases C and L, with and without fungal acid protease (ACPR).

Figure 3: Reducing sugars released from 1 g CSM as a result of increasing dosages of phytases C and L, with and without fungal acid protease (ACPR).

Figure 4: The influence of acid phosphatase activity on the dephosphorylation of CSM in the presence of: 925 PhytU/kg (from preparation CE); fungal acid protease, 42 ACPRU/g; citric acid, 24μM/g; and pectinase 7.4 μl/g.

Figure 5: Dialyzable protein and reducing sugars released from CSM by phytases C, CE, and L, accompanied by an enzymic "cocktail": C+, CE+, L+. Ctrl denotes the values when no enzymes were added to the feed.

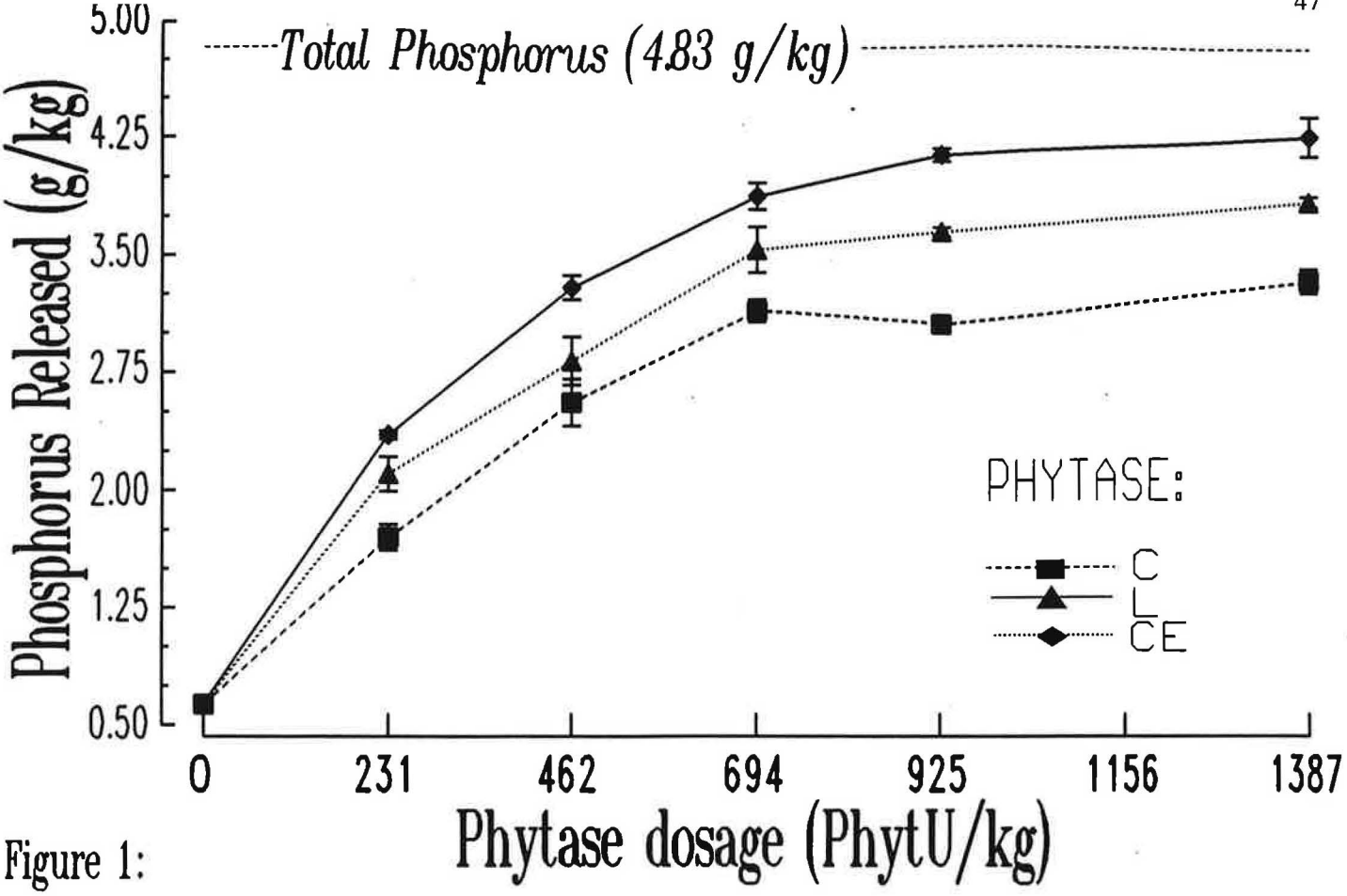

Total Phosphorus (4.83 g/kg)
Phosphorus Released (g/kg)
5.00
4.25
3.50
2.75
2.00
1.25
0.50
0
231
462
694
925
1156
1387
Phytase dosage (PhytU/kg)
PHYTASE:
C
L
CE

Figure 1:

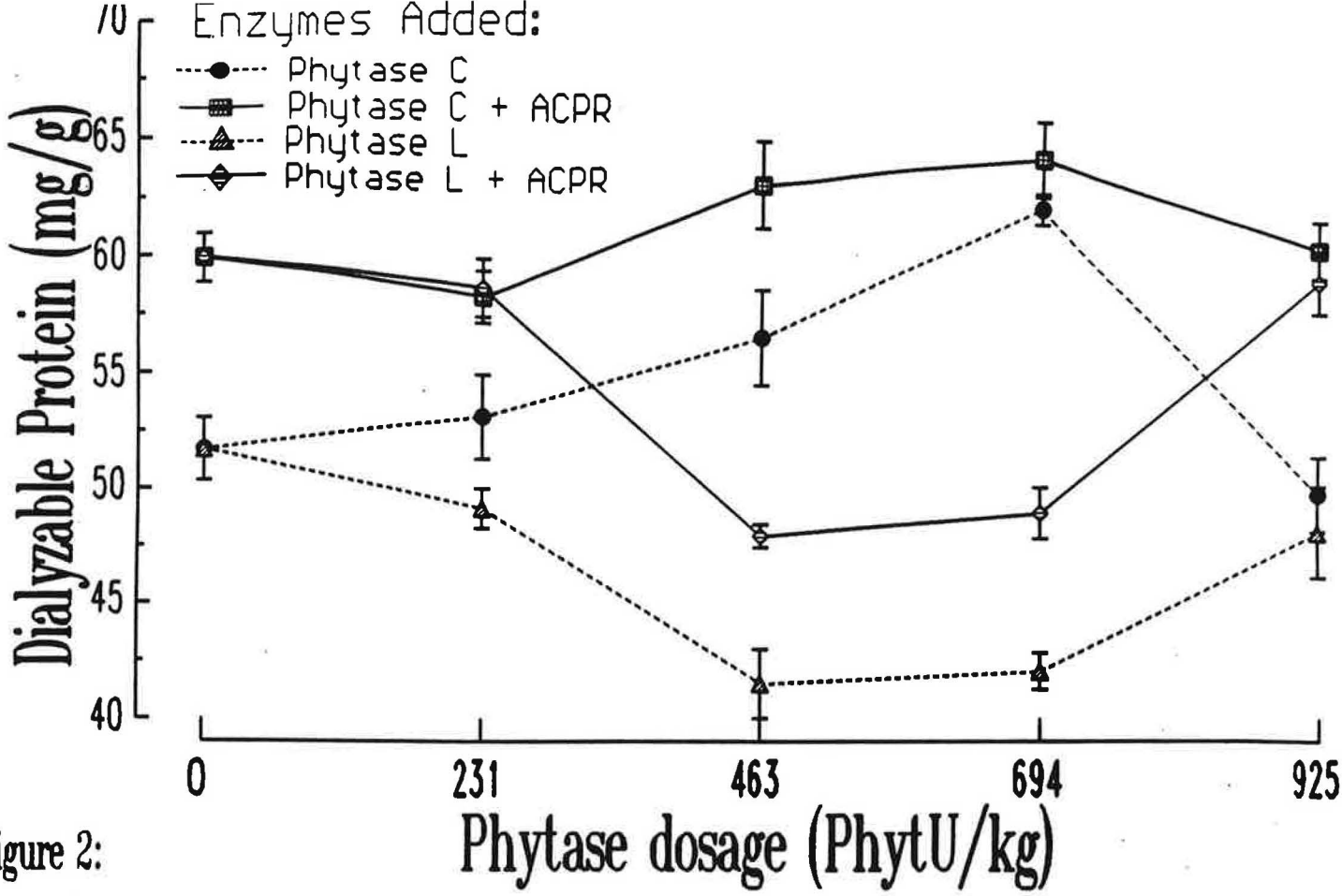

Enzymes Added:
Phytase C
Phytase C + ACPR
Phytase L
Phytase L + ACPR
Dialyzable Protein (mg/g)
70
65
60
55
50
45
40
0
231
463
694
925
Phytase dosage (PhytU/kg)

Figure 2:

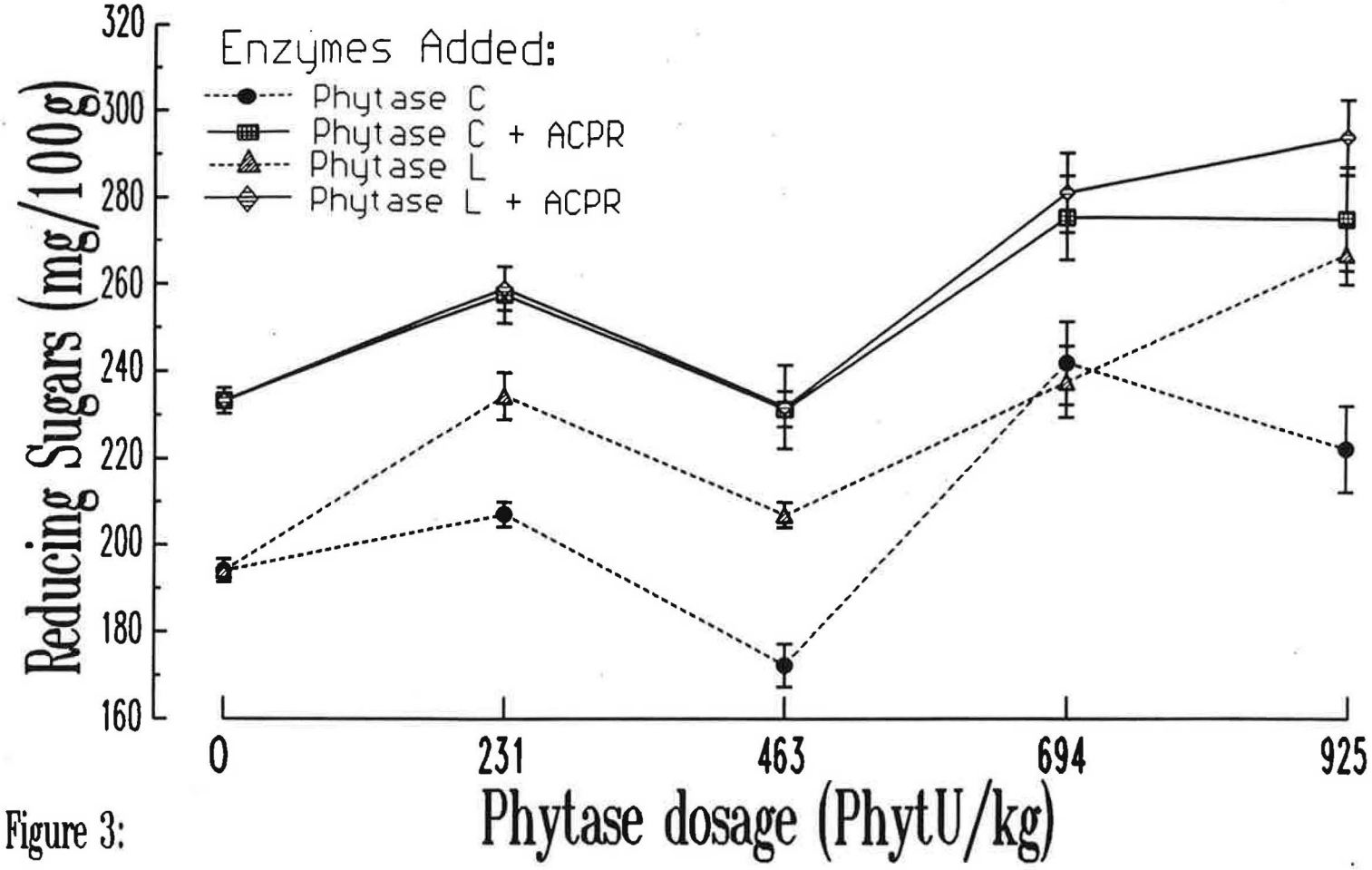

Enzymes Added:
Phytase C
Phytase C + ACPR
Phytase L
Phytase L + ACPR
Reducing Sugars (mg/100g)
320
300
280
260
240
220
200
180
160
0
231
463
694
925
Phytase dosage (PhytU/kg)

Figure 3:

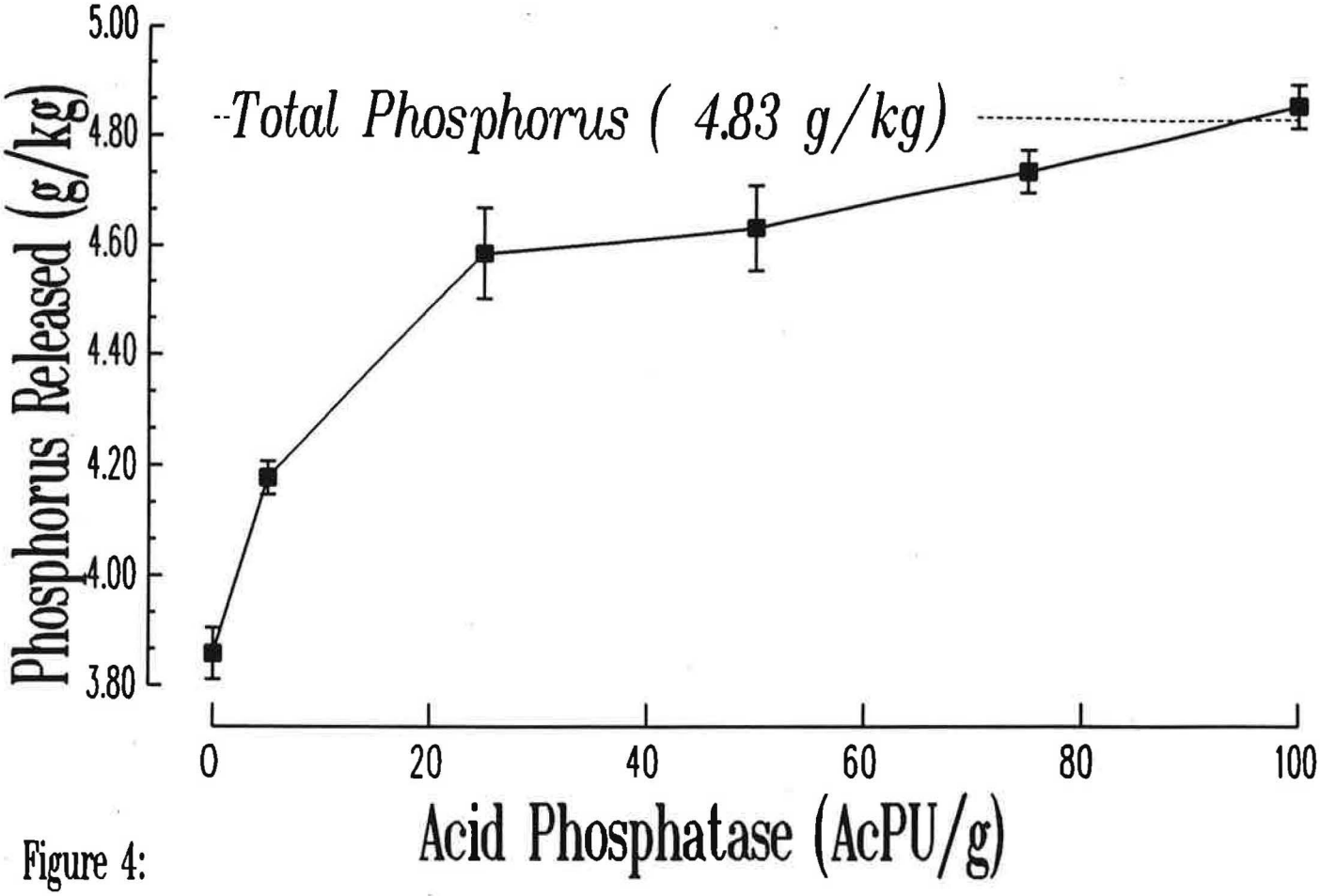

Total Phosphorus (4.83 g/kg)
Phosphorus Released (g/kg)
5.00
4.80
4.60
4.40
4.20
4.00
3.80
0
20
40
60
80
100
Acid Phosphatase (AcPU/g)

Figure 4:

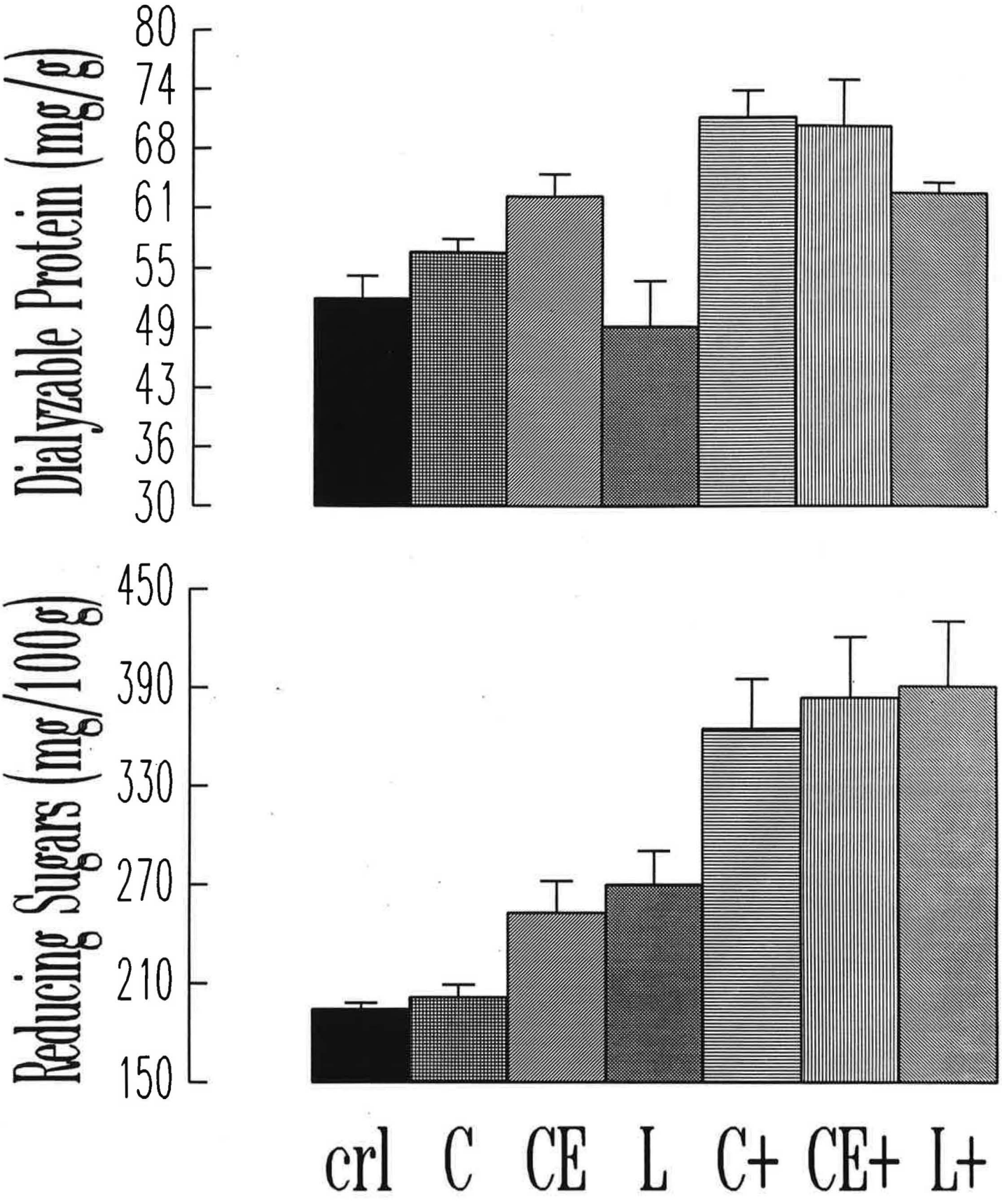

Dialyzable Protein (mg/g)
80
74
68
61
55
49
43
36
30
Reducing Sugars (mg/100g)
450
390
330
270
210
150
crl
C
CE
L
C+
CE+
L+

Figure 5

Partial substitution of phytase for inorganic phosphorus in the diets of turkey hens grown to market age[1]

Summary

Commercial phytase (Natuphos® 5000, 1000 units/kg), was fed to turkey hens grown to market age as a partial substitute for feed phosphate in corn-soybean meal diets. Dietary nutrients were adjusted every four weeks and maintained at the levels recommended by NRC (1984). Available P (aP) levels were either NRC recommended, or reduced by .15%. There was no effect of aP ($P > .05$) on turkey hen performance over the combined 15-wk period. Over that period, turkey hens fed phytase consumed more feed ($P < .05$), and had higher BW gains ($P < .05$) than those fed no phytase. Turkey hens fed NRC levels of aP had a higher ($P < .05$) percentage of toe ash compared to those fed lower levels of aP. Toe ash percentage was not affected by phytase ($P > .05$). Neither phytase nor aP had an effect ($P > .05$) on tibia breaking strength, percent breast meat, percent drumstick, or percent abdominal fat. Litter P and total P excreted were reduced ($P < .05$) in turkey hens fed lower levels of aP, but were not affected ($P > .05$) by phytase.

Description of Problem

Cereal grains, grain by-products and oilseed meals can supply 50-70% of the total phosphorus (P) requirement of the turkey. Up to 75 % of this P is bound up as phytate (myoinositol hexaphosphate), which is poorly available to poultry. The poor availability of phytate P results in a significant amount of P excreted in poultry manure. Cromwell [1] estimated that 6.8 million tons of poultry manure containing on the average 1.7% P (120,000 tons P) was produced annually. Poultry manure is usually disposed of by applying it to pastures and cropland. In areas of intensive poultry production, the amount applied can exceed the crop's P requirement for growth. When this occurs, P can pose a significant environmental problem.

The enzyme phytase sequentially degrades phytate to myo-inositol and orthophosphate. The efficacy of microbial phytase in improving phytate P utilization was first demonstrated by Nelson in 1968 [2]. Although the enzyme proved effective in subsequent studies [3] the treatment was not cost effective due to the high cost of the enzyme compared to that of inorganic sources of P. Recent advances in recombinant DNA technology have resulted in the production of genetically engineered microorganisms that efficiently produce phytase in large quantities [4]. Yi et al.

---

[1]Contribution from the Missouri Agricultural Experiment Station Journal Series No. 12216

[5] estimated that for the turkey the cost of replacing inorganic P by 500 units of phytase was 1.17 times the cost of inorganic P. This replacement value is expected to decrease as the costs of manure P disposal increase. In addition, these costs do not take into account benefits due to improved utilization of other minerals and nutrients, and increased nutrient density of diets due to phytase supplementation of diets.

There have been several reports demonstrating the efficacy of microbial phytase in improving phytate P utilization and reducing P excretion in broiler chicks and laying hens [6-10]. However, to date, there have been no reports on the efficacy of microbial phytase in turkey feeds. If commercially available microbial phytases are to be adopted as a replacement for phytase in either the broiler or turkey industry, their efficacy must be demonstrated under commercial type conditions. Therefore, the objectives of this experiment were to determine if: 1) 1000 units of a commercial phytase (Natuphos® 5000) could be used to replace .15% inorganic P in turkey hens grown to market weights; 2) addition of 1000 units of Natuphos® 5000 phytase to commercial turkey diets will result in reduced P excretion; 3) substitution of phytase for inorganic P will result in any effects on body composition.

Materials and Methods

Three hundred and twenty day-old female Nicholas Large White turkey poults were purchased from a commercial hatchery (Central Kansas Hatchery, Moundridge, Kansas) weighed, and allotted randomly to floor pens in a commercial type facility with natural ventilation, thermostatically controlled curtain sides, and overhead ventilation fans. The animal care and use protocol was reviewed and approved by the University of Missouri-Columbia Animal Care and Use Committee. A 2 X 2 factorial design was used with eight pen replicates of 10 seven-day-old poults assigned randomly to each of four dietary treatments for fourteen weeks. Poults were monitored daily for signs of morbidity and mortality. Dietary treatments were: 1) no phytase, NRC 1984 [11] recommended levels of P (control); 2) as treatment one, but phytase (Natuphos® 5000) was supplemented at the level of 1000 units/kg; 3) as treatment 1, but levels of P were reduced by .15%; 4) as treatment 3, but with phytase (Natuphos® 5000, 1000 units/kg). Dietary nutrients were adjusted every four weeks and, except for P in treatments 2 and 4, kept at the levels recommended by NRC (Table 1). From day 1 to 7 all poults were fed the control diet. The actual phytase activity in feeds was validated after feed preparation for each of the experimental periods.

At the end of weeks 4, 8, 12, and 15 of the experiment, poults were weighed individually and feed intake was determined for each pen. At 15 wk of age, 16 poults from each treatment (8 replicates of 2 poults each) were bled by wing vein and samples

Furthermore, P released from phytates is known to be utilized primarily for growth [21]. Although toe ash was lower in hens fed lower aP, there appeared to be adequate bone mineralization since there were no leg problems observed in these birds and toe ash values were all in the adequate range [22].

No phytase by aP interactions were observed for plasma inorganic P, plasma Ca, litter P, or total excreta P (Table 4). Plasma inorganic P was not affected by phytase or aP and averaged 7.99 mg/dL. Supplemental phytase reduced plasma Ca, whereas higher levels of aP increased plasma Ca. Both percent litter P and total P excreted were reduced in turkey hens fed lower levels of aP (1.64 vs 2.23 % and 736 vs 946 g; Table 4). There was no effect of phytase on percent litter P or total P excretion.

The failure of Natuphos® phytase to reduce P excretion in turkey hens fed the lower level of aP might seem to be surprising since increased P retention and reduced P excretion has been observed previously in broilers [7,8] and laying hens [9] fed rations supplemented with Natuphos® phytase. However, it is known that the efficacy of a microbial phytase is greater at low aP levels and is strongly dependent on Ca/aP ratios [7]. Results of recent studies with turkey poults conducted in our laboratory [23] indicated that in corn-soybean meal feeds containing 1.2 % Ca and 0-1000 units of phytase/kg, P digestibility increased with increasing aP up to .40%, reached a plateau, and then decreased at higher aP concentrations. In another study, P digestibility increased from 57% in poults fed 1.2% Ca, .39% aP, and 1000 phytase units/kg, to 64% in poults fed 1% Ca, .39% aP, and 1000 phytase units/kg [24].

It can be concluded therefore that either too high a P concentration or too wide a Ca/P ratio or both, prevented Natuphos phytase from improving bone mineralization and increasing P digestibility under commercial type conditions employed in this study.

Conclusions and Applications

1. Performance of turkey hens fed lower levels of aP suggests that the aP requirement of turkey hens for growth is lower than that currently recommended by NRC.
2. Natuphos® phytase improved feed intake and BW gains of turkey hens grown to market age.
3. Partial substitution of phytase for inorganic P did not influence body composition, toe ash, or bone breaking strength.
4. In turkey hens grown to market age, partial substitution of phytase for aP did not result in increased P retention from commercial corn-soybean meal diets.

stored for plasma calcium (Ca) and inorganic P determination. Plasma inorganic P was determined colorimetrically by the molybdo-vanadate method [12]. Plasma Ca was determined by flame atomic absorption spectrophotometry. Following blood sampling, the same 16 poults per treatment were killed by decapitation. Middle toes from both feet, and left tibiae were collected from all 16 poults for toe ash and tibia breaking strength determination. Toes were dried at 100°C for 24 h and ashed at 600°C overnight. Left tibiae were stripped of adhering tissue and tibia breaking strength of fresh tibiae was determined using an Instron Universal Testing Instrument (Model 1132, Instron Corp., Canton, MA) by measuring the force required to break the tibiae when placed on two supports 20 mm apart. Breast weight, drumstick weight and abdominal fat weight was determined on 16 poults per treatment. Prior to the start of the experiment all pens were cleaned and old litter removed. Cedar shavings, which was used as litter, was added to each pen during the experiment, quantified and recorded. At the end of the experiment, litter from each pen was turned several times, allowed to air dry, weighed, ground, and stored to determine litter P. Litter P was determined by first grinding the dry litter through a series of stainless steel screens with the final screen size being 1 mm. The ground litter was then digested by wet ashing with nitric and perchloric acids and analyzed for inorganic P [12]. The P content of cedar shavings was determined analogously and the value obtained was substracted from those of litter P.

Data were analyzed by the General Linear Model procedure of SAS® [13]. The model included main effects of phytase level, P level, and the interaction of phytase and P levels. Statistical significance was accepted at $P < .05$.

Results and Discussion

The effects of dietary phytase and available P (aP) levels on turkey hen performance for each experimental period, as well as for the combined 15 week period are presented in Table 2. There were no phytase by aP interactions observed for any of the performance variables. Turkeys hens fed phytase consumed more feed than those fed no phytase during weeks 5 to 8 (5.31 vs 5.13 kg), and over the combined 15-wk period (21.6 vs 21.1 kg). There was no effect of aP on feed intake. During weeks 1 to 4, 5 to 8, and over the combined 15-wk period, turkey hens fed phytase had higher BW gains than those fed no phytase. During weeks 9 to 12, hens fed NRC levels of aP had higher BW gains than those fed lower aP levels; however, aP levels had no effect on BW gains over the combined 15-wk period. Feed conversions by turkey hens were improved by phytase during weeks 1 to 4, and by NRC levels of aP during weeks 9 to 12. Neither phytase nor aP had any effect on feed conversions over the combined 15-wk period.

5. The failure of phytase to decrease P excretion may be due to the high dietary levels of aP and Ca fed in this study.

References and Notes

1. **Cromwell, G. L., 1991.** Phytase appears to reduce phosphorus in feed, manure. Feedstuffs 63(41):14.

2. **Nelson, T.S., T.R. Shieh, R.J. Wodzinski, and J.H. Hare,** 1968. The availability of phytate P in soybean meal before and after treatment with a mold phytase. Poultry Sci. 47:1842-1848.

3. **Nelson, T.S., T.R. Shieh, R.J. Wodzinski, and J.H. Ware,** 1971. Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. 101, 1289-1294.

4. **Conneely, O.M.,** 1992. From DNA to feed conversion:Using biotechnology to improve enzyme yields and livestock performance. Pages 57-65. Proc. Alltech's Eight Annual Symposium, Nicholasville, Kentucky.

5. **Yi, Z., E.T. Kornegay, V. Ravindran, and D.M. Denbow,** 1994. Improving corn and soybean meal P for broilers using Natuphos® phytase and calculation of replacement values of inorganic P by phytase. Poultry Sci. 73(Suppl. 1):89.

6. **Simons, P.C.M., H.A.J. Versteegh, A.W. Jongbloed, P.A. Kemme, P. Slump, K.D. Bos, M.G.E. Wolters, R.F. Beudeker, and G.J. Verschoor,** 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64, 525-540.

7. **Schöner, F.J., P.P. Hoppe, G. Schwarz, and H. Wiesche,** 1993. Vergleich von mikrobieller Phytase und anorganischen Phosphat bei Masthahnenküken: Wirkungen auf die Mastleistungen und die Mineralstoffretention bei variierter Calcium-Versorgung. J. Anim. Physiol. Anim. Nutr. 69, 235-244.

8. **Vogt, H.,** 1992b. Effect of supplemental phytase to broiler rations different in phosphorus content. Arch. Geflügelk. 56:222-226.

9. **Klis, J.D. van der, H.A.J. Versteegh, and C. Geerse,** 1992. The effect of dietary phytase and calcium level on the ileal absorption of phosphorus in laying hens. Proc. XIX World's Poultry Congress Vol. 3:458-459.

10. **Kornegay, E.T., D.M. Denbow, Z. Yi, and V. Ravindran,** 1994. Responses of broilers to graded levels of Natuphos® phytase added to corn-soybean meal-based diets containing three levels of available phosphorus. Poultry Sci. 73(Suppl. 1):77.

11. **National Research Council,** 1984, 1994. Nutrient Requirement of Poultry. 8th ed. National Academy Press, Washington, DC.

12. **Association of Official Analytical Chemists,** 1970. 7. Animal feeds. Phosphorus. 7.095 -7.098. Photometric method. p. 125. In: Official methods of analysis. Association of Official Analytical Chemists, Washington, DC.

13. **SAS Institute, 1984. SAS User's Guide: Statistics. SAS Institute, Inc., Cary, NC.**

14. **Potter, L.M.,** 1990. Phosphorus requirements for turkeys. Pages 1-12 in:Proc. 1989 Texasgulf Nutrition Symposium, Raleigh,

North Carolina.

15.**O'Dell, B.L. and A. de Boland,** 1976. Complexation of phytate with proteins and cations in corn germ and oilseed meals. J. Agric. Food Chem. 24:804-808.

16.**Kratzer, F. H. and P. Vohra,** 1986. Role of phytic acid and other phosphates as chelating agents. In: Chelates in Nutrition, CRC Press Inc: Boca Raton, FL, 49-61

17.**Zyla, K., D.R. Ledoux, A. Garcia, and T.L. Veum,** 1994. An in vitro procedure for studying enzymic dephosphorylation of phytate in maize-soya bean feeds for turkey poults. Br. J. Nutr. (accepted).

18.**Zyla, K., D.R. Ledoux, and T.L. Veum,** 1994. Complete enzymic dephosphorylation of corn-soybean meal feed under simulated intestinal conditions of the turkey. J. Agric. Food Chem. (submitted).

19.**Ketaren, P.P., E.S. Batterham, E.B. Dettman, and D.J. Farrell,** 1993. Phosphorus studies in pigs. 3. Effect of phytase supplementation on the digestibility and availability of phosphorus in soya-bean meal for grower pigs. Br. J. Nutr. 70, 289-311.

20.**Waldroup, P.W.,** 1990. Phosphorus in broiler nutrition. Pages 1-12 in:Proc. 1989 Texasgulf Nutrition Symposium, Raleigh, North Carolina.

21.**Vandepopuliere, J.M., Ammerman, C.B., and R.H. Harms,** 1961 The relationship of calcium-phosphorus ratios to the utilization of plant and inorganic phosphorus by the chick. Poultry Science 40, 951-957.

22.**Puls, R.,** 1988. Mineral levels in animal health:Diagnostic data. Sherpa International, British Columbia, Canada.

23.**Ledoux, D.R., K. Zyla, and T.L. Veum,** 1994. Effects of a commercial microbial phytase in turkey poults fed corn-soybean meal diets. In: 1994 Animal Sciences Progress Report, Agricultural Experiment Station, University of Missouri-Columbia (Special Report 465). Pages 90-94.

24.**Zyla, K. and D. R. Ledoux,** 1994. Growth performance and bone mineralization of turkey poults fed microbial phytase added to diets containing different calcium to phosphorus ratios. Poultry Sci. 73. (Suppl.1): 167.

ACKNOWLEDGEMENTS

This study was supported in part by the Department of the Interior, U.S. Geological Survey, through the Missouri Water Resources Research Center. The authors thank Gist-brocades, The Netherlands, and BASF Corporation for supplying the phytase used in this study. The technical assistance of Harold Huff, Department of Agricultural Engineering, is greatly appreciated.

Table 1. Composition of starting and growing feeds fed to turkey hens, % "As Is"

Ingredients	1 - 4 Wk		5 - 8 Wk		9 - 12 Wk		13 - 15 Wk	
	NRC	NRC-15%	NRC	NRC-15%	NRC	NRC-15%	NRC	NRC-15%
Corn	43.77	44.37	50.84	51.43	62.14	62.76	70.92	71.55
Soybean Meal 48%	49.37	49.26	44.19	44.09	33.50	33.35	24.18	24.03
Dicalcium Phosphate	2.32	1.52	1.82	1.02	1.47	0.67	1.33	0.53
Limestone	1.23	1.73	1.05	1.55	0.94	1.45	0.83	1.33
Corn Oil	1.47	1.27	1.35	1.15	1.04	0.84	1.08	0.88
Salt	0.40	0.40	0.35	0.35	0.40	0.40	0.40	0.40
Methionine MHA	0.21	0.21	0.09	0.09	0.05	0.05	0.06	0.06
Selenium Mix[A]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin Mix[B]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Trace Elements[C]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Choline	0.05	0.05	0.01	0.01	-	-	-	-
Lysine	-	-	-	-	0.23	0.25	0.97	0.99
Sand	0.98	0.99	0.10	0.11	0.03	0.03	0.03	0.03
Calculated Analysis								
ME Kcal/Kg	2800	2800	2900	2900	3000	3000	3100	3100
Crude Protein	28.00	28.00	26.00	26.00	22.00	22.00	19.00	19.00
Available Phosphorus	0.60	0.45	0.50	0.35	0.42	0.27	0.38	0.23
Calcium	1.20	1.20	1.00	1.00	0.85	0.85	0.75	0.75

[A]Supplied .2 mg of selenium per kilogram of diet.
[B]Supplied per kilogram of diet: 4000 IU Vitamin A, cholecalciferol 1750 ICU, 6.25 IU Vitamin E, .75 mg menadione, 7.5 mg d-pantothenic, 3 mg riboflavin, 1 mg Vitamin B_6, .625 mg folic acid,, .5 mg thiamine, .1 mg biotin, .005 mg Vitamin B_{12}.
[C]Supplied per kilogram of diet: 222 mg MnO_2, 209 mg ZnO, 654 mg $FeSO_4.7H_20$, 9 mg Cu_2O and 1.9 mg ethylenediamine dihydrodide.

Table 2. Effects of phytase and available phosphorus (aP) levels on performance of turkey hens

Treatment		Weeks				
Phytase	aP	1 to 4	5 to 8	9 to 12	13 to 15	1 to 15
(Units)[2]	----------Feed Intake (Kg)[1]----------					
0	NRC	1.21	5.07	7.46	7.16	20.91
0	NRC-.15%	1.21	5.19	7.51	7.40	21.31
1000	NRC	1.25	5.31	7.53	7.40	21.40
1000	NRC-.15%	1.23	5.31	7.83	7.36	21.73
Pooled SEM		.02	.05	.12	.12	.22
Source of Variation	----------Probability----------					
Phytase		.0852	.0027	.1209	.3936	.0303
aP		.6024	.2841	.1830	.4317	.1724
Phytase X aP		.7332	.2570	.3170	.2338	.6972
Treatment		----------Body Weight Gain (Kg)[1]----------				
0	NRC	.594	2.23	2.59	1.50	6.92
0	NRC-.15%	.587	2.28	2.46	1.73	7.06
1000	NRC	.628	2.34	2.60	1.68	7.24
1000	NRC-.15%	.626	2.37	2.51	1.66	7.16
Pooled SEM		.010	.02	.04	.06	.07
Source of Variation	----------Probability----------					
Phytase		.0012	.0006	.4844	.4035	.0040
aP		.6797	.1069	.0131	.1196	.6578
Phytase X aP		.8103	.6801	.6223	.0604	.1088
Treatment		----------Feed Conversion (Kg:Kg)[1]----------				
0	NRC	2.04	2.27	2.88	4.82	3.02
0	NRC-.15%	2.06	2.28	3.05	4.54	3.01
1000	NRC	1.99	2.27	2.90	4.30	2.97
1000	NRC-.15%	1.97	2.24	3.11	4.44	3.03
Pooled SEM		.03	.02	.05	.19	.03

Table 2. Effects of phytase and available phosphorus (aP) levels on performance of turkey hens (Cont.)

Treatment		Weeks				
Phytase	aP	1 to 4	5 to 8	9 to 12	13 to 15	1 to 15
Source of Variation	----------Probability----------					
Phytase		.0303	.4681	.3821	.7269	.5250
aP		.9841	.5378	.0005	.1170	.3221
Phytase X aP		.5710	.4519	.6276	.2765	.2554

[1]Data are means of eight replicate pens of ten hens each.
[2]One phytase unit is defined as the amount which liberated 1 micromole of inorganic phosphorus per minute from .0015 mole sodium phytate at 37°C and pH 5.5.

Table 3. Effects of phytase and available phosphorus (aP) levels on toe ash tibia breaking strength, and relative breast, drumstick, and abomasal fat weights of turkey hens[1]

Treatment						
Phytase	aP	TOE Ash	Tibia Breaking Strength	Breast Weight	Drumstick Weight	Abdominal Fat Weight
(Units)[2]	(%)	(%)	(Kg)	----------(%)----------		
0	NRC	14.97	76.6	18.56	4.99	1.36
0	NRC-.15%	14.14	74.9	19.71	4.86	1.22
1000	NRC	14.57	70.6	19.71	5.00	1.13
1000	NRC-.15%	14.24	72.6	20.26	4.81	1.23
Pooled SEM		.26	2.3	.52	.08	.08
Source of Variation		----------Probability----------				
Phytase		.5763	.0820	.0828	.7901	.1762
aP		.0335	.9536	.1561	.0515	.7919
Phytase X aP		.3485	.4226	.6830	.6994	.1504

[1]Data are means of eight replicates two hens each.
[2]One phytase unit is defined as the amount which liberates 1 micromole of inorganic phosphorus per minute from .0015 mole sodium phytate at 37°C and pH 5.5.

The lack of an effect of aP level on performance was surprising since hens fed the lower level of aP were consuming diets that would not have met their aP requirements based on NRC recommendations. This suggests that the turkey hen's requirement (for growth) is lower than that listed by NRC [11]. Potter [14] reported that the aP requirement for optimum body weight gain and bone mineralization of the young turkey (between 0 and 3 weeks) was .41% (confidence limits from .37% to .48%). This value is a level below that used in the present study. Since the turkey requirement for aP, (expressed as a percentage of the diet), decreases with age this suggests that, in the present study, turkey hens fed the lower aP level might have received an adequate supply of aP.

If it is assumed that hens were receiving an adequate supply of aP for optimizing body weight gains, then the improved performance observed for hens fed phytase must be the result of phytase effects other than those attributed to increased P availability resulting from phytate dephosphorylation. Phytate is known to form insoluble complexes with proteins [15] that are resistant to pepsin digestion. In addition, phytic acid has been shown to inhibit the activity of several digestive enzymes including, trypsin, pepsin, and α-amylase [16]. In in vitro studies conducted in our laboratory [17,18] corn-soybean meal rations supplemented with Natuphos® phytase were subjected to multiple digestions which simulated the intestinal tract conditions of the turkey. In addition to substantial increases in inorganic P released from feed, an increased release of protein and reducing sugars was observed. Thus, phytate dephosphorylation by phytase may have increased protein and carbohydrate utilization, leading to the improved performance. This hypothesis is supported by reports indicating improved nitrogen absorption in laying hens fed phytase [9]. Similarly, phytase supplementation resulted in an increased nitrogen and amino acid digestibility in pigs [19].

Turkey hens fed NRC levels of aP had a higher percentage toe ash compared to those fed lower levels of aP (Table 3). No phytase by aP interactions were observed for toe ash, tibia breaking strength, percent breast meat, percent drumstick, or percent abdominal fat (Table 3). Toe ash was not affected by phytase. Similarly, the effects of phytase or aP on tibia breaking strength, percent breast meat, percent drumstick, or percent abdominal fat were not significant and averaged 73.7 kg, 19.5 %, 4.91 %, and 1.23 %, respectively. Lower toe ash in hens fed lower levels of aP would indicate that hens on those treatments may not have received enough aP to meet their requirements for optimum bone ash. The fact that turkeys may have obtained their aP requirement for growth but not for bone mineralization is consistent with previous research indicating that P requirements for optimizing feed utilization and body weight gain is lower than that for optimizing bone ash [20].

Table 4. Effects of phytase and available phosphorus (aP) levels on plasma inorganic phosphorus, plasma calcium, and litter phosphorus of turkey hens

Treatment					
Phytase	aP	Plama Inorganic P[1]	Plasma Ca[1]	Litter P	Total Excreta P
(Units)	(%)	----------(mg/dL)----------		(%)	(g)
0	NRC	8.17	10.9	2.25	928
0	NRC-.15%	7.55	10.5	1.65	742
1000	NRC	8.01	10.5	2.21	963
1000	NRC-.15%	8.24	10.3	1.62	729
Pooled SEM		.22	.1	.03	19
Source of Variation					
Phytase		.2385	.0058	.3110	.5559
aP		.3761	.0061	.0001	.0001
Phytase X aP		.0593	.5150	.7655	.1907

[1]Data are means of eight replicates of two hens each
[2]Data are means of eight replicate pens

Table 1: Composition and nutrient content of basal diets*

Composition	Diet	
Ingredient	Diet: NRC	E
	g/kg	
Soya bean meal (44% CP)	481.18	563.43
Maize (ground, shelled)	405.17	341.98
Meat and bone meal	60.00	-
Maize oil	29.65	45.22
Dicalcium phosphate (18.5 % P; 20.0 %Ca)	9.97	-
Calcium carbonate (38% Ca)	6.29	26.15
Salt (NaCl)	3.00	4.00
Methionine-hydroxy analog	2.44	2.04
Trace mineral premix†	1.00	1.00
Selenium premix‡	0.50	0.50
Vitamin premix§	0.50	0.50
Choline-HCl	0.30	0.19
Variable ingredients††	0.00	15.00
Nutrient content		
Ca¶ g/kg	12.0	12.0
$P_{available}$¶ g/kg	6.0	1.9
P_{total} g/kg	8.39	4.63
Protein¶ g/kg	280.00	280.00

(Continued on next page)