EFFECT OF EXOGENOUS PHYTASE ADDITION TO DIETS ON PHYTATE PHOSPHORUS DIGESTIBILITY IN DAIRY COWS

By

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To the faculty of Washington State University:

The members of the committee appointed to examine the thesis of DILIP KUMAR GARIKIPATI find it satisfactory and recommend that it be accepted.

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ABSTRACT

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The effect of adding exogenous phytase to diets of lactating cows on phosphorus digestibility was evaluated. Cows (n=16) were randomly assigned among four treatments in a 4X4 Latin square design with four periods of 28 days. Dietary treatments were: barley without phytase, barley with supplemental phytase (427 IU/kg total mixed ration, TMR), corn without phytase, and corn with supplemental phytase (427 IU/kg TMR). Phytate P in the TMR comprised about 50% of the total dietary P (0.46%). The concentration of phytate P was 10% greater ($P < 0.05$) in barley than in corn (0.22 vs 0.2%). Samples of feed, blood, milk and feces were collected during each 28 d period. Dry matter intake and efficiency of milk production were not affected by exogenous phytase or grain type. Milk yield and composition were unaffected by dietary phytase and grain source. The concentration of inorganic P was higher ($P < 0.05$) in serum of cows fed exogenous phytase (5.8 vs 6.5 mg/dL in cows fed barley diets, and 5.5 vs 6.0 mg/dL in cows fed corn diets). Using lignin as an internal marker, digestibility of phytate P was increased ($P < 0.05$) by the exogenous dietary phytase and total P digestibility tended ($P < 0.1$) to be increased. There was no effect of grain source on P digestibility and

excretion of the total fecal P. Fecal excretion of phytate P was decreased ($P < 0.05$) in cows fed exogenous phytase. The addition of phytase to diets of lactating cows increased P digestibility and decreased phytate P excretion. In conclusion, incorporation of exogenous phytase into diets of lactating cows may have a role in P management on dairy farms.

Key words = Phytase, Phytate P, Barley, Corn, Cattle

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Phosphorus (P) is an essential dietary nutrient and is generally the most costly mineral to supplement in animal diets. Chandler (1996) indicated that P accounts for more than 50% of the cost of typical vitamin-mineral mixes used on dairy farms. However, dairy cattle in the U.S. often are fed diets containing P levels that are well above NRC (2001) recommended levels (Knowlton et al., 2004). The reasons for the high intakes of P by dairy cattle include: 1) lactating dairy cattle are fed diets consisting of about 50% cereal grains and byproducts, all of which contain relatively high P levels; 2) the P levels in many forages has increased due to heavy application of P to soils; 3) unanswered research questions concerning intestinal absorption of P from feedstuffs; and, 4) possible benefits from P intakes that are greater than those recommended by NRC. A major result from high P intakes of cattle is that P excretion is unnecessarily increased because total P excretion is highly correlated with P intake (Rotz et al., 2002; Valk et al., 2002).

Phosphorus is one of the key polluting nutrients from animal agriculture. It is an important contributor to both water and soil pollution. Phosphorus pollution arises because animals are often concentrated onto limited acreage. Ruminants in the U.S. produce 483 billion kg of manure each year (Coelho, 1999). Most of this manure will be applied as fertilizer to crops in an attempt to reduce the cost of crop production by decreasing the need for commercial fertilizers and to utilize the nutrients in the manure. Repeated application of livestock manures to soils has resulted in P saturation, allowing for P runoff into surface waters or leaching into ground waters (Coelho, 1999).

Phosphorus is a limiting nutrient to algae in surface waters and P runoffs into waters lead to profuse growth of algae. Excess algal growth lead to physical blockage of sunlight to plants underwater and the algae utilize nutrients and oxygen in the water. Eventually, the plants die and decompose, utilizing more oxygen for decomposition and releasing toxins. This process is called eutrophication. Eutrophication kills aquatic life and makes water unsuitable for consumption and recreational purposes (Sharpley et al., 1994; Sharpley and Tunney, 2000).

Dietary P management is a key strategy in reducing P accumulation in dairy farms (Kuipers et al., 1999; Valk et al., 2000). Total P excretion could be reduced if a greater percentage of dietary P was retained in the animal's body or secreted in the milk. The digestibility of P in cereal grains and their byproducts generally are lower than the digestibility of P in inorganic supplements (NRC, 2001), thus, total dietary P is increased to compensate for lower availability of P from grains. If the availability of P in cereal grains and their byproducts was increased, then total dietary P would be reduced, which would correspondingly decrease fecal P excretion. A major reason for the lower P availability from grains is that most of the P in grains is present as phytate, which must be hydrolyzed before intestinal absorption of P can occur. Although total hydrolysis of phytate along the entire gastrointestinal tract is nearly complete in cattle (Morse et al., 1992b), the extent of phytate hydrolysis in the rumen is critical for the P to be in an absorbable form when it reaches the P absorption sites in the intestine. Accordingly, a discussion of phytic acid and its hydrolysis is given below.

Phytic acid and Phytase

Phytic acid, *myo*-inositol 1,2,3,4,5,6 hexa*ki*sphosphate typically represents 65-85% of total P in seeds (Reddy et al., 1982). Phytic acid consists of a sugar molecule, called myo-inositol, with covalently linked phosphate groups. Phytate is the mixed salt of phytic acid and several important mineral cations such as Ca, Fe, Zn and K. Deposition of phytate in plants occurs during seed development (Lott, 1984; Morris and Ellis, 1976; O' Dell et al., 1972; O'Dell et al., 1972). Phytate concentration is low in forages compared to cereal grains (Clark et al., 1986).

The phytic acid in cereals is not uniformly distributed within the kernel but associated with specific morphological components in the seeds (Ravindran, 1999). Corn has 88% of its phytic acid distributed in the germ layer, 3.2% in the endosperm and the remainder in the hull. In contrast, wheat has 87.1% phytic acid distributed in the aleurone, 12.9% in the germ and 2.2% in the endosperm (O'Dell et al., 1972). In barley, phytic acid is distributed similarly to that of wheat (O'Dell et al., 1972) and is in the form of Ca-Mg salt (Harland and Oberleas, 1999). In soybeans, phytic acid is deposited in protein bodies as complexes of chelated minerals (Prattley and Stanley, 1982). Phytic acid P concentrations in corn, barley, and wheat are reported as 0.24, 0.27 and 0.32% of grain DM, respectively (Cheryan, 1980).

Phosphorus associated with phytic acid is not available for intestinal absorption unless the inorganic form of P is hydrolyzed from inositol ring by action of phytase (McCance and Widdowson, 1935; Nelson, 1967; Pointillart et al., 1987; Pointillart, 1991; Morse et al., 1992b). The phytase enzyme occurs in two forms: 3-phytase, which is present in plants and initiates dephosphorylation of phytate at the 3-position; and 6-phytase, which occurs in microorganisms and initiates dephosphorylation at the 6-position (Nayini and Markakis, 1986). The phytate-degrading enzymes also have been divided into two types based upon their optimal pH. These are the acid phytate-degrading enzymes with a pH optimum around 5.0, and the alkaline phytate-degrading enzymes with a pH optimum around 8.0 (Konietzny and Greiner, 2002). Most of the phytate-degrading enzymes belong to acid type. Patwardhan (1937) showed that the ruminal intestine has some ability to break phosphate from phytic acid (reviewed by Sansinena, 1999). Several studies have demonstrated phytase secretion from the mucosa of small intestines of rats (Pileggi et al., 1955), guinea pigs (Davies et al., 1970), rabbits and calves (Bitar and Reinhold, 1972) and humans (Courtois and Pe'rez, 1949).

Phytase is present in some cereal grains such as barley, rye and wheat. In cereals, phytase activity is mainly associated with the aleurone layer (Gabard and Jones, 1986). In wheat kernels, phytase is distributed among the aleurone layer (34%), endosperm (34%), and scutellum (15%) (Peers, 1953). In barley, phytase is chiefly around the protein bodies of the aleurone layer (Tronier et al., 1971). Corn contains little or no phytase activity (Eeckhout and de Paepe, 1994). Analysis of untreated wheat and corn resulted in 465 and 31 units of phytase per kg, respectively (Sukria and Liebert, 2001). Heat treatment at

100° C for 10 min resulted in loss of all phytase activity. Similarly high temperatures (> 70° C) caused partial or total inactivation of native phytase (Pointillart, 1993). The optimal conditions for extracted barley phytase activity from barley seedlings (4 days old) was reported to be between 40 and 55° C with a pH of 5-6 (Bergman et al., 2000; Greiner et al., 2000). Raun et al. (1956) reported that the optimum pH for rumen microbial phytase was 5.5. In the rumen the pH is usually between 6.0 and 7.0, which allows hydrolysis of phytate to occur but not at an optimum rate.

Metal cations, such as Ca, Mg, and Zn, form insoluble complexes with phytate, which decrease the enzymatic rate of hydrolysis by phytase (Wise, 1983). For example, a high calcium diet increased excretion of phytate in feces of rats (Pileggi et al., 1955; Nahapetian and Young, 1980). Phytate digestibility in chicks is greatly improved by decreasing the calcium concentration in the diet (Mohammed et al., 1991). Vitamin D may indirectly improve utilization of phytate P digestion by increasing absorption of the hydrolyzed P (Nelson, 1967; Mohammed et al., 1991).

Most microorganisms produce only intracellular phytase although extracellular phytase was observed in filamentous fungi (Konietzny and Greiner, 2002). In the rumen, phytase is produced by ruminal microflora, comprised mostly of bacteria and protozoa (Raun et al., 1956). *Selenomonas ruminantium* is one of the prominent and functionally diverse bacteria present in the rumen and can account for up to 51% of the total viable bacterial counts in rumen (Caldwell and Bryant, 1966). Yanke et al. (1998) found that a large number of bacterial strains, mainly *Selenomonas ruminantium* and with substantial

percentage of *Megasphaera elsdenii*, were positive for phytase activity. They (Yanke et al., 1998) stated that phytase activity is mostly associated with bacteria and little phytase activity is associated with the protozoa, feed particle, and fungal fraction of rumen fluid. *Klebsiella* and *Corynebacterium* are among the populations of bacteria with phytase activity (Suzuki and Ushida, 2000). Suzuki and Ushida (2000) also stated that the phospho hydrolyzing activity of protozoal populations was approximately seven times greater than the initial velocity in releasing P of bacterial populations. The key differences between the two studies (Suzuki and Ushida, 2000; Yanke et al., 1998) are the bacterial sources and the sources of feed. Suzuki and Ushida (2000) results were based on ovine fed timothy hay and a commercial concentrate, whereas Yanke et al. (1998) used bovines that were fed a mixture of barley grain and alfalfa hay. Also, Yanke et al. (1998) observed that the phytase activity increased in response to higher levels of phytate in feed.

The phytase activity in *Selenomonas ruminantium* is associated with the outer membrane (D'Silva et al., 2000). Soil microorganisms such as *Bacillus sps* and *Enterobacter* produce extracellular phytase, which is significant in making phytate P available to the plants (Konietzny and Greiner, 2002).

Ruminants are able to digest phytate P because rumen microorganisms synthesize the phytase enzyme. This enzyme breaks the phosphate groups from the inositol, making the P available for absorption in the small intestine (Reid and Franklin, 1947; Raun et al., Nelson et al., 1976; Morse et al., 1992b). More than 98% of dietary phytate was reported

to be hydrolyzed in the whole gastrointestinal tract of dairy cattle fed 50% grain and 50% corn silage (Clark et al., 1986). Similarly, more than 90% of phytate P was hydrolyzed between 6 and 8 h of incubation with rumen microflora when phytate containing concentrate feeds were incubated (Morse et al., 1992b). In steers, phytate hydrolysis was reported to have been complete, based on fecal phytate recovery (Nelson et al., 1976). However, Sansinena (1999) found up to 47% of phytic acid escaped hydrolysis in the rumen. Thus, the percentage of phytate P that is hydrolyzed in the rumen is not definitively known.

Reid and Franklin (1947) found no phytate P in rumen, abomasum, small intestine, large intestine and rectum of sheep fed 57 to 74% of their total P in the phytate form. Reid and franklin (1947) also measured hydrolysis in vitro and found complete phytate P hydrolysis in vitro. However, Mathur (1953) reported 35 to 51% of the total phytate fed to dairy cows was excreted through feces. Mathur (1953) fed 52 to 54% of total P in the form of phytate P in the diets of cattle. Tillman and Brethour (1958) compared calcium phytate and calcium carbonate monocalcium phosphate as sources of additional P in 18 month-old wethers. The reported digestibility of P bound to phyate was 91.8% (Tillman and Brethour, 1958). Clark et al. (1986) fed Holstein cows diets of 50% grain and 50% corn silage with supplemental inorganic Ca during first 18 weeks of lactation and reported 98% of digestibility phytate P. Nelson et al. (1976) conducted two studies involving steers of 9 to 10 months of age to determine phytate P hydrolysis. Steers were starved for 18 h and slaughtered, only traces of phytate P was recovered from the feces and no phytate was recovered from rumen, abomasum, small and large intestine. Morse et

al. (1992b) observed greater than 90% of disappearance of phytate between 6 and 8 h of in vitro incubation of wheat middling, rice bran, hominy, soybean meal, and dried distiller's grains. They also reported greater than 99% of phytate P hydrolysis in vivo in cattle based on total fecal collection. Lambert (1998) found at least 95% phytate P digestibility in calves fed a corn based diet. He also observed no difference in P or phytate P excretion by addition of phytase enzyme in calves. Using a mobile bag technique in dairy cattle, Lowry (2003) reported 95% phytate P hydrolysis of a TMR after 14 h of incubation.

Monogastrics lacking microbial populations have a very little intestinal phytase activity, thus ingested phytate is not efficiently hydrolyzed (Bosch et al., 1998). As a result, most phytate ingested by nonruminants is excreted in feces unless exogenous dietary phytase is supplied. Inclusion of exogenous phytase in nonruminant diets increases utilization of dietary phytate and reduces needed supplementation of inorganic P (Bosch et al., 1998). Phytase incorporation (500 to 1000 IU/kg of diet) in nonruminant diets reduced total P excretion by 30-50% (Lei et al., 1993; Yi and Kornegay, 1996a; Kemme et al., 1997; Liu et al., 1997).

Factors affecting phytase activity

Microbial phytases have high affinities for phytic acid, whereas plant phytases and some fungal phytases have lower affinities and degrade inositol phosphates at lower rates. Most phytases have an optimal pH in the range of 4.5-6.0 and a temperature range of 45 to

60°C. Outside the optimal range of pH and temperatures the action of phytase is reduced (Lei and Porres, 2003). Phytases lose their enzymatic activity with exposure to high temperatures and show different sensitivities to pepsin and trypsin proteolysis (Lei and Porres, 2003).

Several dietary factors reduce or enhance the intestinal phytase efficacy. High dietary Ca or a high ratio of Ca: P interfere with P absorption from the intestines and reduces the effectiveness of phytase activity (Sandberg et al., 1993; Lei et al., 1994). Moderate to high levels of inorganic P can also inhibit the activity of phytase by reducing the phytase production in the ruminal bacteria (Lei and Porres, 2003). Supplemental organic acids, such as citric acid or lactic acid enhance solubility of dietary P and enhance phytase efficacy (Han et al., 1998; Maenz et al., 1999; Jongbloed et al., 2000). Adding exogenous phytase with other hydrolytic enzymes, such as acid phosphatase, protease and cellulose enhances phytate digestibility by dephosphorylation of phytate (Zyla et al., 1995). Fermentation of soybean meal with *Aspergillus usamii* improved P availability and zinc availability in chicks by degradation of phytate in the soybean meal by phytase from the fungi (Matsui, 2002).

Phytate: anti nutritive properties

Phytic acid is the acid form of the anion, phytate (Harland and Oberleas, 1999). Chelates of phytate are formed by the following minerals, Co, Cu, Fe, Ca, Mg, Mn, Ni, Se, and Zn (Harland and Oberleas, 1999). Binding of phytic acid to these minerals makes them unavailable for absorption by animals. In humans, large intakes of foods rich in phytate

can cause nutritional deficiencies of several of these minerals (Maga, 1982; Torre et al., 1991). Addition of phytase to diets improves the nutritive value of plant based foods by enhancing protein and mineral digestibility through phytate hydrolysis during digestion by hydrolysis of amino acids bound to phytic acid (Sandberg et al., 1996). Dutton and Fontenot (1967) found in wethers that the form of dietary P (inorganic or organic) had no effect on absorption and retention of Mg and Ca.

Function of P in the body

Phosphorus is a critical element with numerous and diverse functions in the body, including cell membrane structure (phospholipids), energy transfer (ATP), structure of DNA, and as an important constituent of bone (Satter et al., 2002). Within the body of ruminants, 80% of the P is in skeletal tissue. Bone P functions as an important P reservoir when body requirements temporarily exceed dietary intake (Minson, 1990). The remaining 20% of the body P is in soft tissues. Phosphorus is ubiquitous in the body's soft tissues where it is essential for a broad range of enzymatic reactions, especially those concerned with energy metabolism and transfer. Phosphorus is an important component in metabolism of carbohydrate, amino acid, fat, muscle, and nervous tissue; also for normal blood chemistry, and many coenzymes in the body (Ekelund, 2003). Phosphorus is also essential for transfer of genetic information (i.e. DNA and RNA), and is a vital component of the various buffering systems in the body (Karn, 2001). Another important use of P within ruminants is the maintenance and reproduction of the ruminal microorganisms (Breves and Schröder, 1991; NRC, 1996).

Clearly P plays a vital role in the growth, reproduction and lactation of animals. Because of the critical function of P in the body, it is important that homeostasis be maintained. Homeostasis of P involves bone resorption, salivary secretion, intestinal absorption, and urinary excretion (Challa et al., 1989).

Phosphorus bioavailability

The bioavailability of a particular mineral element is defined as the amount of that mineral that is absorbed and utilized by an animal (Gueguen, 1999). Even though many feedstuffs contain relatively high P concentrations, the bioavailability of P is often low. However, bioavailability of P for animals differs among feedstuffs. Phosphorus in high moisture corn and sorghum is more bioavailable than P in dry corn and sorghum for pigs (Allee, 1979). Wheat bran and wheat middling have a higher percent bioavailable P because these feed ingredients have natural phytase (Cromwell, 1999). The NRC (2001) specifies that the coefficient for absorption of P from forage and concentrates are 64% and 70%, with P availability of organic sources ranging from 30 to 90%. Maenz et al. (1999) observed that the true absorption of P in corn silage fed to nonlactating cows ranged from 85 to 94%. Brintrup et al. (1993) reported a 67% apparent P absorption coefficient when cows were fed a diet with 0.41% P concentration. Morse et al. (1992a) found a 74% apparent P absorption coefficient when cows were fed with 0.31, 0.41, and 0.56% P concentrations in the diet. Similarly Wu et al. (2000) found a 70% apparent absorption coefficient of P in dairy cows fed with 0.40% P. Tillman and Brethour (1958)

found a 75% true absorption coefficient when cows fed calcium phosphate and 90% phosphoric acid, similarly sheep fed monosodium phosphate had a 90% true absorption of P.

Phosphorus absorption

Phosphorus absorption occurs mainly in the phosphate form from the small intestine, especially the duodenum and jejunum, in ruminants (Care, 1994; Khorasani et al., 1997). Only small amounts of P are absorbed from the rumen, omasum, and abomasum in ruminants (NRC, 2001). The amount of P absorbed by the animal depends on the source of the P, the amount of P intake, the calcium to P ratio, intestinal pH, disease and parasites, environment, the age of the animal, and dietary levels of calcium, iron, aluminum, manganese, potassium, magnesium and fat (Ekelund, 2003). Efficiency of absorption of P in sheep was reduced by 18% with increasing amounts of calcium (NRC, 2001). However, several studies suggested that the Ca: P ratio in the diet is not critical unless the ratio is greater than 7:1 or less than 1:1 (Call et al., 1978; McDowell, 1992). Phosphorus absorption is in direct relationship to the supply of potential absorbable P in the lumen of the small intestine (NRC, 2001). Phosphorus absorption occurs via two mechanisms, active and passive (NRC, 2001). Active absorption depends on vitamin D and is operative when the animals are fed low P containing diets. Passive absorption predominates when normal to large amounts of potentially absorbable P are consumed. Passive absorption of P is related directly to the amount of P in the lumen of small intestine and to the concentration of blood P (NRC, 2001). According to Braithwaite

(1983) there is an inverse relationship between P intake and its absorption coefficient. When circulating serum concentrations of P are within normal range (4-8 mg/dL), P absorption rate may be reduced by saturation or inhibition in absorptive mechanism (Braithwaite, 1983). Morse et al. (1992a) also observed an inverse relationship between P intake and P absorption when the cows were fed increasing amounts of P in diets. A decrease in P absorption with increased concentrations of dietary P was also reported by Challa et al. (1989), Brintrup et al. (1993), Spiekers et al. (1993), and Wu et al. (2000).

Khorasani et al. (1997) observed that net absorption of P ranged from 71 to 85 g/d in eight lactating dairy cows fed four different diets. They also found that the relationship between P intake and total absorption was curvilinear, suggesting that forage level had a greater effect on total P absorption than P intake. Most organic P in diet is hydrolyzed by the microbes into inorganic P forms. The remaining organic P, which is not been hydrolyzed in the rumen, is solubilized by low pH of the abomasum (Breves and Schröder, 1991; Care, 1994).

Phosphorus absorption from the intestine depends largely upon the nature of the feed, the % P in the diet, and the P requirement of the animal (Gueguen, 1999). Although some P absorption occurs from rumen, it is low compared to P absorption from small intestine (Breves and Schröder, 1991; Care, 1994). When the supply of P exceeds requirement, the efficiency of absorption is reduced (Morse et al., 1992a; Care, 1994). A 75% efficiency of P absorption was reported in cattle fed diets supplemented with dicalcium phosphate (Tillman and Brethour, 1958; Challa and Braithwaite, 1988), whereas a 90% absorption

efficiency occurred with supplementation of phosphoric acid and monosodium phosphate (Tillman and Brethour, 1958).

Inorganic P moves across the brush border membrane in the cells lining the small intestine by the way of active co-transport mechanism with Na^+. The driving force for the accumulation of P in the cells of the small intestines is the movement of the Na^+ ion against the electrochemical gradient. The Na^+ gradient and subsequent accumulation of P in the cell is brought about by the Na^+, K^+ adenosine triphosphatase (ATPase) pump (Murer and Hildmann, 1981) as reviewed by Guyton (2002).

Hormones play an important role in regulating P absorption in ruminants. Parathyroid (PTH) both inhibits and activates P transport. At low concentrations of P in intestinal lumen, PTH binds to the basolateral membrane receptor of small intestine and initiates phospholipase C. When concentrations of P in the intestinal lumen are high, PTH binds to the basolateral membrane and activates adenylate cyclase. Phospholipase C and adenylate cyclase activate phosphorylation of the Na^+ P co-transporter. Phosphorus transportation can be inhibited by PTH related peptide by activating the same receptors located in the basolateral membrane that activates phospholipase C and adenylate cyclase (Guyton, 2002).

In large intestine of cattle, phytate P is partly degraded by the phytase activity from intact bacterial cells but the P released may not be absorbed by the ruminant because little or no P is absorbed from the large intestine (Pfeffer et al., 1970).

Blood inorganic P

Plasma concentrations of inorganic P (Pi) in the blood reflect the cow's recent P intake but not necessarily her P nutritional status (Read et al., 1986). Diurnal variations in plasma Pi concentrations are related to feed intake with increased Pi concentrations occurring for 2 h after feeding (Forar et al., 1982). Forar et al. (1982) also observed an increase in plasma Pi with decreased milk yields. Plasma Pi concentrations are usually maintained between 4.0 and 8.0 mg/100mL (Underwood and Suttle, 1999). About 1 to 2 g of inorganic phosphate in blood plasma circulates in a 600 kg animal (NRC, 2001). Erythocytes have greater concentrations of P than plasma; whole blood contains 6 to 8 times as much P as plasma (NRC, 2001). Due to the persistent loss of P in the milk, plasma Pi concentrations were lower during lactation than during pregnancy in primiparous cows (Ternouth and Coates, 1997).

Plasma Pi may have more value as an indicator of dietary P levels than as a P status indicator because age, physiological stage of production, and length of time fed a P deficient diet, all affect the animal's P status, and thus have modulating effects on blood Pi levels (Karn, 2001). For example, serum Pi was lower in dairy cows fed a diet with 0.38% P than with 0.48% P but were within normal ranges (Wu and Satter, 2000). Others also have reported that serum Pi increased with an increase in the dietary concentration of P (Forar et al., 1982; Knowlton and Herbein, 2002). Plasma Pi was 30% lower in cattle receiving only 67% of the P recommended by the Dutch (Valk et al., 2002). Dietary

deficiencies of P significantly decrease plasma Pi concentrations (Brintrup et al., 1993). Forar et al. (1982) observed a diurnal variation in plasma Pi; they reported an 8% higher concentration of Pi during day time than in night. Higher serum P concentrations were found in lambs fed supplemented phytase (6.16 mg/dL) and organic P (6.99 mg/dL) than those without having additional phytase (5.12 mg/dL) (Shanklin, 2001).

Milk P

Bovine milk contains about 0.09% P, depending upon the protein and fat content (NRC, 2001) that is, milk P ranges from 0.083 to 0.1 percent (Flynn and Power, 1985; Wu et al., 2000). Inorganic P in milk was higher in first lactation cows than multiparous cows (Forar et al., 1982). Flynn and Power (1985) and Wu et al. (2000) observed a decrease in milk inorganic P during summer. Phosphorus in cow's milk is distributed as: 20% esterified to casein, 40% as colloidal inorganic calcium phosphate, 30% as phosphate ions in solution, and only 10% associated with lipid fraction (NRC, 2001). Therefore, an adjustment of dietary P may not significantly affect the milk fat and protein percentages. Milk yield did not differ in cows fed a wide range of P concentrations. No difference in milk yields were found in cows fed dietary P of 0.33 and 0.39% (Brintrup et al., 1993), 0.34, 0.51, and 0.69% (De Boer et al., 1981), 0.35 and 0.44% (Brodison et al., 1989), 0.38 and 0.48% (Wu and Satter, 2000), and 0.4 and 0.49% (Wu et al., 2000). Reducing dietary P from 0.48 to 0.38% for 2 years in dairy cows did not impair milk production or reproductive performance (Wu and Satter, 2000). Morse et al. (1992a) observed a 13.4% increase in milk production when cows were fed a high P diet compared with a low P

diet, however, the % P in milk was not affected by dietary P intake (Forar et al., 1982). Guyton (2002) showed that supplemental phytic acid had no effect on milk P secretion. Knowlton and Herbein (2002) observed a decrease in total milk P secretion as a percentage of P intake with an increase in dietary P content, but found no effect of dietary P concentration on milk P concentration. About 26.7% of dietary P is secreted in the milk (Morse et al., 1992a)

The NRC (2001) specifies that a cow producing 54.4 kg of milk per day needs 0.38% dietary P or 114 g of P per day, but a cow producing 25 kg of milk per day requires 0.32% dietary P or 65 g of P per day. Diets within the range of 0.33 to 0.37% dietary P are sufficient for lactating dairy cows (Wu and Satter, 2000; Wu et al., 2001).

Phosphorus homeostasis

A fundamental understanding of how body P concentrations are controlled is important not only in knowing what constitutes adequate P intakes, but also in understanding the importance of P concentrations in various body fluids and how they might be useful as possible indicators of P status (Karn, 2001). In contrast to carnivores and omnivores in which P is lost primarily in the urine, P losses in the herbivore occur primarily via feces (Karn, 2001). Phosphorus homeostasis in ruminants is achieved through salivary recycling and endogenous fecal excretion (NRC, 2001). In cattle fed low P diets, body P levels probably are controlled by reducing endogenous fecal P losses through a combination of reduced salivary P flow and increased P absorption (Coates and Ternouth,

1992). Urinary P losses normally are relatively small and Challa et al. (1989) reported that urinary P excretion only becomes quantitatively important when plasma P concentrations exceed a renal threshold value of between 60 and 90 mg/L.

Saliva

Salivary P concentration is related to plasma P concentration and salivary P volume is related to dry matter intake (Karn, 2001). Total salivary P secretion is a product of salivary volume and P concentration. The mean daily inflow of salivary P to rumen is about 58 g in cows (Nikolic et al., 1978) as reviewed by Durand and Kawashima (1980). The amount of P secreted in saliva decreases as dietary P is reduced (Nel and Moir, 1974). Salivary secretions of P constitute about 80% of the endogenous P recycled to the gastrointestinal tract, depending upon the dry matter intake, usually combined with P intake, and fiber content of the diet (Care, 1994) as reviewed by Valk et al. (2002). Salivary P is present as inorganic P (orthophosphate), a form that is highly available to the rumen microbes (Valk et al., 2002), and is absorbed in the small intestine along with dietary P (Horst, 1986). Khorsani et al. (1993) found that cows fed triticale silage, which was high in neutral detergent fiber (NDF), secreted more salivary P into the rumen than cows fed alfalfa and barley silages, which were lower in NDF. Higher salivary P concentrations were found in lambs receiving organic P with phytase enzyme (0.68 mg/ml) than those without enzyme (0.5 mg/ml), presumably because of more available P in lambs fed diets with phytase (Shanklin, 2001). Endogenous fecal P losses result almost entirely from unabsorbed salivary P.

Depending on ruminal pH, P in saliva is present as $H_2PO_4^-$, HPO_4^{2-}, or PO_4^{3-}. This explains differences observed in absorption efficiency among salivary P (75 to 85%), inorganic P supplements (80 to 90%) and P in feed (50 to 60%) (Yano et al., 1991). Challa et al. (1989) found that P absorption from saliva ranged from 75 to 80% in calves. The concentration of P in the saliva is 4 to 5 times that of plasma Pi (NRC, 2001).

Salivary P recycling is a major P conservation mechanism for ruminants and supplies much of the P to ruminal microbes. Salivary P secretion accounts for as much as 40% of the total P entering the rumen. The mean composition of mixed saliva and parotid saliva of cows is 26 and 23 mEq phosphate/L (Bailey and Balch, 1961). Salivary P concentration in lactating cattle ranged from 4.3 to 8.6 mmol/L, and salivary P concentration in nonlactating cattle ranged from 8.2 to 12.1 mmol/L (Valk et al., 2002). Salivary P concentration was reported to be elevated by P supplementation (Clark, 1953; Gartner et al., 1982). Salivary P levels were 25 and 37 mg P/100ml for heifers fed low (0.12%) and adequate (0.2%) P diets, respectively (Karn, 2001). Salivary P increased as P absorption increased and was directly related to serum concentration (Breves and Schröder, 1991; Challa and Braithwaite, 1988; Challa et al., 1989) as reviewed by Karn (2001). Valk et al. (2000) reported that lower salivary P concentrations are induced by lower plasma P concentrations. As P intakes decrease, endogenous P from saliva increases as a percent of total P in the rumen. However, total P in ruminal fluid decreases as dietary P decreases, i.e. salivary P decreases in total amount. Decreases in salivary P probably occur because plasma inorganic P decreases. The increase in endogenous P as a

percent of total P in the rumen means that there is a net movement of endogenous P from the body to the rumen, hence, supporting the hypothesis that ruminal microbial population demands are greater than the animal's P demand (Preston and Pfander, 1964). In cows, the daily salivary secretion ranges from 30 to 60 g (Breves and Schröder, 1991). Salivary P may be the main P source for ruminal microbes especially when insoluble phosphates are consumed in the diet (Durand and Kawashima, 1980). Recycling of P through saliva is substantial in ruminants and may exceed fecal excretion by 5 to 10 fold (Tamminga, 1996).

Ruminal fluid concentrations

Ruminal P consists of feed and salivary P, with salivary P contributing up to 50% of the total amount (Valk et al., 2000). The suggested lower concentration of P to maintain normal microbial growth in the rumen is 100 mg/L of ruminal fluid (Durand and Kawashima, 1980). In several studies in which low P diets were fed to cattle, P concentrations in ruminal fluid remained well above 100 mg of P/L of ruminal fluid (Witt and Owens, 1983). Inorganic P levels in ruminal fluid were not sufficient under all conditions to distinguish between levels of P supplement being fed, although it was sensitive enough to differentiate between supplemented and unsupplemented cows (Karn, 2001).

Evans and Davis (1996) found that P concentrations were 198, 417 and 543 mg P/L of ruminal fluid when diets containing 0.04, 0.16 and 0.54% P were fed to Jersey steers.

Concentrations of P in ruminal fluid tended to plateau just above 0.16% dietary P or 31 mg of P/kg body weight. Ruminal P concentration was 264, 379, and 434 mg/L of ruminal fluid when steers were fed 0.066, 0.123, and 0.173% P in their diets, respectively (Witt and Owens, 1983). Adult ruminant animals recycle endogenous P via saliva into rumen and secrete P through ruminal wall to maintain P near 200 mg/L of ruminal contents even when P intakes are temporarily low (Witt and Owens, 1983).

Urinary P

In contrast to nonruminants in which excess absorbed and endogenous P are excreted in the urine, ruminants excrete only minor amounts of P in urine (McDowell, 1992). Ruminants have a well-developed capacity for conserving P and relatively little is excreted in urine even with large intakes of P (Wu et al., 2001). For example, a 600 kg cow excretes only 1.2 g of urinary P per day (NRC, 2001). According to Wu et al. (2001) 2.5 mg of P/dL of urine is the threshold level and any values above 2.5 mg P/dL should be considered as a reliable sign of P adequacy. Although the kidney is not a major excretory route for P in the bovine (Horst, 1986), there is some variance in urinary P excretion with increased dietary P concentration (Morse et al., 1992a). Urinary P decreased from 3.5 g/d to 1.1 g/d in calves from 4 to 10 months of age (Estermann et al., 2002). Challa et al. (1989) suggested that urinary P should not be considered significant until serum P concentration exceeds 6-9 mg/dL. Urinary P output in cows fed 0.34% P was 0.32 g/d, which increased to 1.28 and 3.89 g/d with feeding 0.52 and 0.67% P in the

diet (Knowlton et al., 2000). Only 0.9% of the total dietary P is normally excreted in the urine of cows (Morse et al., 1992a).

Fecal P

The alimentary tract is the most important route for P excretion in ruminants (Durand and Kawashima, 1980). Fecal P is a combination of unabsorbed dietary P and unabsorbed endogenous P (McDowell, 1992). The total endogenous fecal P may constitute more than 67% of the total fecal P in cattle and sheep (Coates and Ternouth, 1992; Scott et al., 1995). Morse et al. (1992a) reported that 60.5% of dietary P is excreted in feces of dairy cows. In cattle about 44% of the P in the manure is in organic form, whereas in pigs it is 49%. Phytate P represents 17% of the total fecal P in cattle and 39% in pigs (He and Honeycutt, 2001). Diets of lower dry matter digestibility result in higher fecal P (Valk et al., 2000). Phosphorus loss in feces depends on the DMI of the animals (Brintrup et al., 1993; Spiekers et al., 1993).

The total yearly excretion of P by a dairy cow producing 9000 kg of milk and consuming a diet consisting of 0.4% P was estimated to be 18.2 kg (Van Horn et al., 1994). When the P in the ration was increased to 0.6%, P excretion increased to 31.8 kg per cow. Cows fed 0.32% dietary P excreted less P in the feces and had higher digestibility of P than cows fed 0.43%, but there was no difference in P retention between cows fed the different P levels (Ekelund, 2003). The decrease in absorption efficiency with high P intake may be due to the homeostatic mechanism or as a result of progressive saturation

of absorption mechanism of P. Fecal excretion from early lactation cows fed 0.34% P was 42.5 g/d, and it increased to 86.5 and 113 g/d when the cows were fed 0.52 and 0.67% dietary P, respectively (Knowlton et al., 2000). Cows fed diets with 0.56% P excreted 100.4 g P/d compared to cows fed diets with 0.3% P who excreted only 60 g P/d in feces.

As previously stated, fecal P is composed of unabsorbed dietary P and unabsorbed endogenous P (Spiekers et al., 1993). The undigested feed P is that P which is not available for absorption. The unabsorbed endogenous P includes sloughed cells from digestive tract and salivary P secreted to maintain P homeostasis. Salivary P is excreted via the feces if excess P is available in the small intestine (Guyton, 2002). Inevitable fecal loss of P in ruminants is a function of total fecal DM excretion (Preston and Pfander, 1964). Accordingly, the Agricultural and Food Research Council (A.F.R.C., 1991) hypothesized that inevitable fecal loss of P is determined mainly by DMI (dry matter intake) and not by live body weight. The total fecal P excretion (dietary P not absorbed and endogenous P) averages about 1 g/kg DMI. Microbial debris, purines and pyrimidines of nucleic acid contribute to half of the inevitable P loss in the animal (NRC, 2001).

Ruminal microbes

Ruminal microbes have a P requirement apart from the animal's requirement that must be first met for optimum ruminal microbial activity (Bryant et al., 1959). Ruminal bacteria,

protozoa, and fungi need P to maintain metabolism and growth (Komisarczuk-Bony and Durand, 1991); and total P content of rumen microorganisms ranges from 2 to 6% of the dry matter (Valk et al., 2000). Within ruminal bacteria, 80% of total P is contained in nucleic acids and 10% in phospholipids (van Nevel and Demeyer, 1977) as reviewed by Durand and Kawashima (1980). The mean N to P ratio in ruminal microbes is 5.3. The P content of DNA is 10.03% and that of RNA is 9.64% (Durand and Kawashima, 1980). In vitro studies suggest that 4 g P/kg digestible organic matter or 100 mg P/L of ruminal fluid is adequate for growth of ruminal bacteria (Durand and Kawashima, 1980). This conclusion agrees with others that maximum microbial degradative and synthetic activities can be maintained if ruminal inorganic P levels are at least 75-100 mg/L (Komisarczuk et al., 1987). Chicco et al. (1965) observed that rumen microorganisms need 60 mg or more of P per L of ruminal fluid for cellulose digestion. For optimum plant cell degradation and microbial protein synthesis within the rumen, the available P should be at least 5 g/kg fermented organic matter, supplied via the diet and saliva (Komisarczuk-Bony and Durand, 1991). Microorganisms are largely dependent on dietary P for their P requirement and the host animal is affected first under a marginal P deficiency (Durand and Kawashima, 1980).

Effects of P deficiency

Phosphorus is required for many physiological functions and there are numerous signs of P deficiency in animals. If P deficient diets are fed for an extended period, feed intake decreases. Insufficient intake of P can also affect the metabolic activity of cells in the

body, which influence the satiety center, causing a reduction in feed intake (McDowell, 1992). Feeding P at 0.31% of dietary DM over two or three lactations decreased bone P concentration but the decrease was not enough to affect bone strength (Wu et al., 2001). During short periods of insufficient dietary P intake, the P deficiency can be overcome by P recycling and resorption of P from bone. Rickets is mainly observed in young animals and osteomalacia in mature animals consuming P deficient diets (McDowell, 1992). A characteristic symptom of P deficiency is pica, which usually takes the form of osteophagia (McDowell, 1992). In high yielding cattle there was no effect of P intake on reproductive performance (Brodison et al., 1989; Call et al., 1987).

Environmental effect of P

According to the Environmental Protection Agency (EPA), 50% of the river areas are affected by agricultural pollution (Parry, 1998). Phosphorus that contributes to the pollution of bodies of water originates from soil erosion and runoff, and waste runoff from livestock operations. Many agricultural producers apply manure to pasture and croplands to recycle the nutrients and limit commercial fertilizer usage. The rates of manure application to fields are typically determined by comparing N content of the manure and the N requirement of the crop to be grown (Shanklin, 2001). Because livestock manure is relatively rich in P when compared to the N content in manure and the P requirements of plants, excess P is often applied to the soil (Van Horn et al., 1996). As only limited amounts of P are utilized by the crops, there often is accumulation of P in the soils. When the soil P threshold is reached, excess P moves into ground water or

flows into surface water (Tamminga, 1996). Of all dietary mineral elements for dairy animals, P represents the greatest potential risk if excess is released into the environment contaminating surface waters and causing eutrophication (NRC, 2001). Although nitrogen is considered the most critical manure element environmentally in many regions, P in surface runoff in regions near critical lakes and streams is believed to augment excessive algae growth. Thus, total farm P balance is considered more critical than N (Van Horn et al., 1996). The whole farm P balance was 6.6 ton/yr in a study conducted on 41 dairy farms in western states with average herd size of 466 cows. Imported feed made up 85.4% of total P inputs, and exported animal products and manure (and compost) made up 53.1 and 45.9% respectively, of total P output (Spears et al., 2003).

Phosphorus concentrations in animal manures typically are many times greater than in soils. For example, P concentrations (dry matter basis) range from 4 to 7 mg P/g of dairy manure, compared to 0.08 to 1.56 mg P/g of benchmark soils, and 0.486 to 2.439 mg P/g of surface soils (0 to 5 cm) receiving long-term manure applications (Sharpley, 1996). Rainwater interacts with the surface applied manure, dissolving and extracting P. The dissolved P can be either leached into underlying soil, contributing to the pool of plant available soil P or, if rainfall exceeds the infiltration rate and slope and soil characteristics are favorable, transported as surface runoff (Sharpley, 1996).

Phosphorus loss in runoff from agricultural land pollutes and contributes to accelerated eutrophication, the main problem in surface waters. Impaired water quality restricts water use for fisheries, recreation, industry, and drinking (Commission of the European

Communities, 1992; Environmental Protection Agency, 1996) reviewed by Sharpley and Tunney (2000). Dissolved P, comprised mostly of orthophosphate, is immediately available for uptake by algae and aquatic plants and is therefore of particular concern for receiving water bodies. Dissolved P accounts for most of the P loss in runoff from where soil erosion is minimal, such as no-till fields or grasslands (Nash and Murdoch, 1997; Sharpley et al., 1994). Because P often is the limiting nutrient for algal growth, when P is no longer limiting (occurs when P enters surface water), excess growth of algae or 'bloom' occurs. Dissolved P is immediately available for uptake by algae and aquatic plants and is therefore of particular concern for water bodies.

Dissolved P originates from the thin layer on soil surfaces where animal manure is applied without incorporation. States such as Delaware, Maryland, and Virginia have passed laws requiring P based management practices for manure and fertilizers when soils exceed state defined upper limits of soil test P (Sims, 2000). Phosphorus loss on animal farms may not be only related to how much P is excreted in manure and applied to fields but also how easily the manure P is dissolve in rainwater and subject to potential runoff loss (Sharpley and Moyer, 2000). Excess P in surface water may be linked to *Pfiesteria* outbreak, which can be harmful to humans (Guyton, 2002). Plant uptake of P varies with crop type, crop yield, and soil type. Some crops, such as corn silage and alfalfa hay, remove only small amounts of the mineral nutrients from manure (15.9 and 18.6 kg P per acre, respectively) and multiple cropping systems can remove 22.7 kg of P per acre (Van Horn, 1991). The low uptake of manure P by crops can allow P to

accumulate in soils, which can eventually create P runoff and contaminate near by surface water.

Given the increased regulatory focus on P application, more attention has been given to the need to develop and implement strategies to improve P balance on farms while sustaining animal productivity. Phosphorus over supplementation (~20%) of P to the national dairy herd is costing $100 million annually and is contributing to unnecessarily high P levels in manure (Satter and Wu, 2001). In the United States, dairy diets are formulated to contain 20% more P than recommended by the NRC (2001) as reported by surveys conducted by Bertrand et al. (1999). The extra P is not needed because Wu et al. (2000) recorded no difference in animal performance parameters between cows fed 4.0 g P/kg and 4.9 g P/kg feed, whereas fecal excretion was reduced by 23% with 4.0 g P/kg diet. A reduction of 25 to 30% less manure P and saving of $10 to 15 per year per cow could occur with reduced P supplementation.

Nonruminants and exogenous phytases

Phytases can be derived from a number of sources including plants, animals and microorganisms. *Aspergillus sp.* have been commonly employed for commercial phytase production (Pandey et al., 2001). In ruminants, strains belonging to *Selenomonas ruminantium, Megeshaera elsdenii, Prevotella ruminicola, Mitsuokella multiacidus* and *Treponema sp.* have phytase activity (Yanke et al., 1998). Phytase is proposed as an

animal feed additive to enhance the value of plant material in animal feed by liberating orthophosphate (Mitchell et al., 1997).

Non ruminants cannot efficiently use ingested phytate P because they do not possess a sufficient microbial population in the upper intestine to produce adequate phytase activity to hydrolyze the feed phytase (Bosch et al., 1998). Phytates decrease the bioavailability of minerals and trace elements in nonruminants because the negative charges in phytate bind positively charged cations to form stable complexes. Dietary proteins and amino acids also form complexes with phytate (van Doorn et al., 2004). The use of exogenous dietary phytase enables nonruminants to more efficiently dietary P, reducing the need for supplemental P. The studies reviewed by Bosch et al. (1998) reported reductions in P content of manure varying from 5.5 to 62.4% following the inclusion of 167 to 1597 units of phytase/kg feed.

Low phytate cereal grains

Low phytic acid genotype barleys have been developed that reduce the inositol phosphate content of barley seeds by almost 45% (Dorsch et al., 2003). Phytate bound P comprised only about 20% of total P in "high available P grain" compared to 80% in typical grain. Several genetic strains that differ in various steps in synthesis of phytate have been developed through selection; additional strains have been developed through gene transfer. Several low phytate hybrids of corn and barley suitable for growing have been released commercially. Strains of corn that produce grain with less phytate, known as

"high available P grain" have been developed. Feeding low phytate corn (Pioneer X313) to beef cattle resulted in similar concentrations of serum Pi and carcass characteristics compared to beef cattle fed normal corn (Loza et al., 2002). An increase in P digestibility was observed in finishing pigs fed low phytate barley (Thacker et al., 2004, 2003; Veum et al., 2002).

In conclusion, P is an essential dietary nutrient for all animals. Much of the P ingested by animals is present as phytate P, which must be hydrolyzed prior to P absorption. Although most phytate is hydrolyzed in cattle prior to fecal excretion, phytate hydrolysis must occur in rumen to enable P to be absorbed from the small intestine. The use of exogenous phytase in diets of pigs and chicks to enhance P absorption has become a common practice. However, few studies have been reported on the addition of phytases to ruminant diets. If successful in increasing P digestibility, supplemental dietary phytase for ruminants could become part of an overall scheme to reduce P excretion.

CHAPTER 2

EFFECT OF EXOGENOUS PHYTASE ADDITION TO DIETS ON PHYTATE PHOSPHORUS DIGESTIBILITY IN DAIRY COWS

ABSTRACT

The effect of adding exogenous phytase to diets of lactating cows on phosphorus digestibility was evaluated. Cows (n=16) were randomly assigned among four treatments in a 4X4 Latin square design with four periods of 28 days. Dietary treatments were: barley without phytase, barley with supplemental phytase (427 IU/kg total mixed ration, TMR on DM basis), corn without phytase, and corn with supplemental phytase (427 IU/kg TMR on DM basis). Phytate P in both corn and barley TMRs comprised about 50% of the total dietary P (0.46%). The concentration of phytate P was 10% greater ($P < 0.05$) in barley than in corn (0.22 vs 0.20%). Samples of feed, blood, milk and feces were collected during each 28 d period. Dry matter intake and efficiency of milk production were not affected by exogenous phytase or grain type. Milk yield and composition also were unaffected by adding dietary phytase and grain source. The concentration of serum inorganic P (Pi) was higher ($P < 0.05$) in serum of cows fed exogenous phytase (5.8 vs 6.5 mg/dL in cows fed barley diets, and 5.5 vs 6.0 mg/dL in cows fed corn diets). Using lignin as an internal marker, digestibility of phytate P was increased ($P < 0.05$) by the exogenous dietary phytase and total P digestibility tended ($P < 0.1$) to be increased. There was no effect of grain source on P digestibility and excretion of the total fecal P. Fecal excretion of phytate P was decreased ($P < 0.05$) in cows fed exogenous phytase. In conclusion, incorporation of exogenous phytase into diets of lactating cows may have a role in P management on dairy farms.

Key words = Phytase, Phytate P, Barley, Corn, Cattle

INTRODUCTION

Phosphorus plays a very important biological role in livestock production and has more known functions than any other mineral nutrient (Lynch and Caffrey, 1997). In fact, P is involved in every cellular event in the body and in extracellular fluid while a vital role of P is as a structural element in the skeleton; P also plays important roles in many metabolic processes, lactogenesis and is important in the maintenance and growth of the ruminal microorganisms. These microbes have a total P content of 2 to 6%, dry matter basis (Valk et al., 2000).

In feedstuffs available to animals, phytic acid typically represents 65-85% of the total P in seeds (Reddy et al., 1982). Phosphorus in phytic acid cannot be absorbed by animals unless hydrolyzed by the enzyme phytase, which removes the associated phosphate groups. Nonruminants cannot utilize phytate present in the feeds unless their gastrointestinal bacteria produce a phytase enzyme (Nys et al., 1999). As a result, a large amount of phytate P is excreted in the feces of nonruminants. Accordingly, the addition of exogenous phytase improves phytate P digestibility in swine and poultry (Kornegay, 1999; Omogbenigun et al., 2003). In ruminants, phytase is secreted intracellularly by ruminal bacteria (Yanke et al., 1998). Phytate hydrolysis also occurs in the lower gastrointestinal tract of ruminants because of the microbial population. Thus the total tract hydrolysis of phytate is nearly complete (Reid and Franklin, 1947; Tillman and Brethour, 1958; Nelson et al., 1976; Clark et al., 1986).

However, for P to be absorbed from the small intestine (SI), phytate hydrolysis must occur in the rumen. Using *in vitro* ruminal techniques, Morse et al. (1992b) found 90% hydrolysis of phytate in selected feedstuffs after 6 to 8 h of *in vitro* incubation. Similarly, Punj et al. (1969) reported about 95% of phytate in cattle feeds were hydrolyzed after 36 h of *in vitro* incubation. These *in vitro* experiments do not consider ruminal flow of ingesta. For example, Sansenina (1999) found up to 47% of ingested phytic acid escaped ruminal hydrolysis.

Because of the large feed intakes in high-producing dairy cows, ruminal turnover rates are rapid, which limits the time for hydrolysis in the rumen. Also, because bacterial phytase is an intracellular enzyme, limited initial accessibility may slow the hydrolysis of phytate present in the aleurone layer of barley and the germ of corn (O'Dell et al., 1972). Barley has more phytate than corn (0.22 vs 0.2%) and addition of phytase to the diets may increase phytate P digestibility by increasing the amount of absorbable P to the animals. Thus, the objectives of the present study were:

1) To determine if addition of exogenous dietary phytase increases phytate and P digestibility in lactating cows; and
2) To compare phytate P digestibility of diets containing either corn or barley when fed to lactating cows.

MATERIALS AND METHODS

Lactating multiparous Holstein cows (n=16) were arranged in four replicates of a 4X4 Latin square design with four dietary treatments and four periods. The experimental protocol was approved by the WSU Institutional Animal Care and Use Committee. Treatment periods were 28 d with the first 21 d serving as an adaptation period and the final 7 d for data collection. Diets were formulated to meet the cow's nutrient requirements according to the NRC (2001) and fed as total mixed rations (TMR). The dietary treatments were: 26% barley (Baroness) with no enzyme; 26% barley plus 427 IU phytase/kg of TMR on DM basis; 26% corn with no enzyme; and 26% corn plus 427 IU phytase/kg of TMR on DM basis. All diets contained approximately 0.46% P, 18% CP, and 35% NDF, and 19% ADF. Ingredient and nutrient compositions of the diets are given in Tables 1 and 2. The phytase was obtained from ADM Animal Health and Nutrition Division, Des Moines, IA and is heat stable. One unit of phytase is defined as the amount of enzyme that liberates 1 μmol of phosphate/min from 0.0051 mol/L of sodium phosphate at 37° C and pH 5.5. The phytase was added to the concentrate mix, which was pelleted.

Experimental animals

At the start of the study, the cows averaged 210 ± 19 days-in-milk (DIM) and had a mean BW of 702 ± 79 kg. The cows were randomly assigned to one of the four dietary

treatment groups (4 cows/treatment/period). The cows were fed via individual Calan gates and had access to water and feed. At the end of each period the cows were switched to a different treatment diet. The cows were acclimated for 21 d to the diet and samples were collected during the final 7 d of the period. At the end of the experiment each cow had received all the four treatment diets.

Sampling

Cows were fed individually and milked twice daily with the milk yield recorded daily. Body weights were recorded initially and on day 21 of each trial period. The amount of diet offered and refused was recorded daily to calculate feed intake. Total mixed ration samples were collected weekly and composited (w/w) by each trial period for each treatment. Blood, milk, and fecal samples were collected from cows during each sampling period. Blood samples were collected from the coccygeal vein into non-heparinized vacutainers. Serum was harvested after centrifugation at 3000 X g for 15 min, and stored at -20° C until further analysis. Milk samples were collected in 30 ml plastic bottles and immediately taken to the laboratory where they were frozen until further analysis. Subsamples of milk also were sent to the regional Dairy Herd Improvement Association (DHIA) laboratory for analysis of major components. Fecal samples were collected from the rectum of cow in plastic cups, sealed immediately, and taken to the laboratory where they were dried at 60° C in a forced-air oven and stored until further analysis.

Laboratory analysis

Feed and feces were dried at 60° C for 48 h, then ground through a 1 mm screen in Wiley Mill (Arthur H. Thomas, Philadelphia, PA). A subsample of ground feed and feces were dried at 100° C for 24 h for calculation of absolute DM. Feed samples were analyzed in duplicates for CP, ADF, NDF, acid detergent lignin (ADL), acid insoluble ash (AIA), Ca, P, and phytate P. Fecal samples were analyzed for NDF, ADF, ADL, AIA, Ca, P, and phytate P. Blood and milk samples were analyzed for Ca and P. Crude protein was determined using a Leco ® 528 Protein Analyzer (AOAC, 1995, procedure no.990.03), NDF and ADF were determined using an Ankom ® Fiber analyzer (Ankom Technology, Macedon, NY; AOAC, 1995), and ADL was according to the AOAC (2001). Acid insoluble ash was determined in samples of feed and feces according to the method of van Keulen and Young (1977). Calcium was determined by atomic absorption spectrophotometry (Robinson, 1975), and P was by a colorimetric procedure (AOAC, 2001). Concentrations of phytate P in feed and fecal samples were determined by the ferric precipitation method of Raboy et al. (1984). A detailed description of this method is given below.

Ferric precipitation method

Duplicate samples (0.5-1.0 g) were placed in 50 ml centrifuge tubes and 20 ml of extraction reagent (0.4M HCl: 10% H_2SO_4) was added to each centrifuge tube, which was then covered with parafilm. The tubes were stirred overnight using a magnetic

stirrer. After the overnight stirring, tubes were centrifuged at 10,000 X g for 15 min at 4° C (Sorvall centrifuge equipped with a SA-600 rotor). The supernatant was filtered through Whatman #1 filter paper to remove the unwanted solid material from the sample. Ten milliliters of filtrate was transferred to a 30 ml corex tube to which was added 10 ml of deionized water and 5 ml of a ferric chloride solution (0.2M HCL: 5% Na_2SO_4: 15mM $FeCl_3$). The tubes were then incubated in a boiling water bath for 30 min during which the phytate precipitated as a ferric salt. The sample tubes were cooled by placing in an ice bath. The cooled tubes were centrifuged at 8000 X g for 10 min. The supernatant was discarded and the pellet containing ferric phytate was resuspended in 10 ml of 0.2M HCl by vigorous vortexing. The centrifuging and washing steps were repeated. The tubes were inverted to remove the supernatant and kept under the hood. Two milliliters of concentrated H_2SO_4 was added to each of the tubes and allowed to sit overnight in the hood. On the next day, 4 to 5 drops of 30% H_2O_2 was added to each tube, which were then placed in a heating block until the temperature of the block reached 200° C. The tubes were removed and allowed to cool for 15 min and 0.5 ml of 30% H_2O_2 was added to each of the samples, which were then placed in a heating block for 20-25 min. Samples were removed from the heating block and allowed to cool. This process was done until the sample in the tube became clear. Deionized water was added to bring the sample volume to 12.5 ml. Next, 100 µL of the diluted sample was added to 3.9 ml of deionized water to make a volume of 4 ml to which was added 4 ml of Chen's reagent (1 volume of 6N H_2SO_4, 1 volume 2.5% ammonium molybdate, 1 volume of 10% ascorbic acid and 2 volumes of deionized water) to make a final volume of 8 ml. The samples were allowed to sit for 2 h at room temperature and the absorbance measured at 820 nm using a

spectrophotometer. For the P standard curve, the solutions added are given in Table 3. Phytate phosphorus was calculated according to:

Phytic acid P, mg/g = μg P x 12.5 ml (volume diluted, digested sample) x 2 (we used 10 ml of the original 20 ml extract)/ 0.1 ml (amount taken from dilute, digested sample for analysis)/ 0.5 g (original sample weight)/ 1000 (convert from μg to mg).

Digestibility calculation

Total P and phytate P digestibility are calculated using both ADL and AIA as internal markers. Digestibility was calculated using the following formula

$$\text{Digestibility} = \%\ \frac{\text{marker in feed}}{\text{marker in feces}} \times \%\ \frac{\text{nutrient in feed}}{\text{nutrient in feces}}$$

Statistical analysis

Statistical analysis was performed using the General Linear Models procedure of SAS (SAS, 2001) using the following model:

$$Y_{ijkl} = \mu + G_i + E_j + (G*E)_{ij} + P_k + C_l + e_{ijkl}$$

μ = constant

G_i = effect due to grain treatment (barley or corn) (i= 1 or 2)

E_j = effect due to enzyme treatment (no phytase or added phytase) (j= 1 or 2)

$(G*E)_{ij}$= effect of interaction between grain and enzyme treatment

P_k= fixed effect due to period (k= 1 to 4)

C_l= effect due to cows in the groups (l= 1 to 4)

e_{ijkl} = residual error

Differences were declared significant at $P < 0.05$ and trends at $P < 0.1$.

RESULTS AND DISCUSSION

The treatment TMRs had similar chemical compositions (Table 2) and differed only in their content of corn, barley, and added phytase. The mean concentration of dietary P was 0.46%, which is greater than the minimum P recommended by the NRC (2001). The TMR ingredients of whole cottonseeds, wheat mill run, culled peas, and soybean meal contributed to the comparatively high dietary P. Barley had slightly more phytate P than corn (0.22 vs 0.20%) and the phytate is distributed differently within the kernel (O'Dell et al., 1972) .The ADL percentage differed ($P < 0.05$) between the barley based (3.16%) and corn based (3.47%) diets. The concentration of added phytase was 427 IU/kg TMR (dry matter basis), which was added to one of the barley and one of the corn based diets.

Intake and body weight

Dry matter intakes (DMI) of cows were similar for all treatments and averaged 28.6 ± 0.88 kg/d for the 16 wk of the study (Table 4). These results differ from those of Casper et al. (1990), Casper and Schingoethe (1989), Yang et al. (1997), and McCarthy (1989) who observed a lower DMI for cows fed barley based diets compared to corn based diets. De Visser et al. (1990) attributed the general variation in DMI of cows to differences in ruminal pH and concentrations of propionic and lactic acid. When fed to cattle, barley diets resulted in more propionic and lactic acid in rumen than did corn diets (Tamminga et al., 1990). The relatively high NDF level (35%) in the current study may have reduced differences in ruminal VFA concentrations among dietary treatments. Dry matter intakes

are reported to decline dietary NDF concentrations of more than 25% NDF (Allen, 2000). Body weights of the cattle did not differ among the treatments (Table 4). The initial mean BW of the cattle was 702 ± 79 kg and the final BW was 706 ± 77 kg.

Milk production and composition

There was no effect of dietary treatment on milk production or composition (Table 4). Average milk fat percentage for cows fed barley based diets was 3.37 ± 0.09 %, compared to 3.38 ± 0.09 % for corn based diets. Similarly, the mean values for milk protein in barley based diets were 3.06 ± 0.013 % and 3.05 ± 0.014 % for corn based diets. Thus, grain type did not affect milk yield or composition.

Yang et al. (1997) observed higher milk yield in primiparous cattle fed corn based diets than barley based diets and no differences in milk composition. However, they found no differences in milk yield or in composition in multiparous cows fed the same diets. DePeters and Taylor (1985) reported no difference in milk yield or composition when they fed corn or barley based diets of similar chemical composition to lactating Holstein cows. Casper et al. (1990) also found similar milk production in cows fed corn or barley diets supplemented with either soybean meal or urea. However, whereas the protein percent in milk did not differ in cows fed different diets, milk fat percentage was lower in cows fed barley diets. Similar to the current study, Khorasani et al. (2001) found no difference in milk yield or milk composition in multiparous cows fed either barley or corn based diets. The protein content in milk is difficult to alter by manipulating the diet

(Thomas, 1983) and a lack of effect of the grain source on milk protein content has been observed previously (DePeters and Taylor, 1985). Although milk fat percentage is affected by dietary fiber all the treatment diets in the current study had similar chemical compositions.

Milk production efficiency, whether expressed as milk yield per kg of DMI or milk yield per kg of digestible DM was unaffected by the dietary treatments (Table 4). A similar result was reported by Yang et al. (1997) who compared barley, hull-less barley, and corn based diets of multiparous lactating dairy cattle and found no difference in efficiency of milk production. However, in primiparous cows they found a higher ratio of milk yield to digestible DM when fed hull-less barley as the grain source compared to corn or barley.

Concentrations of P and Ca in milk

The % P in milk was 0.09% and was not affected by dietary treatment (Table 4). The % P in milk primarily is a function of milk protein percent and is not affected by dietary P level (Forar et al., 1982; NRC, 2001). Similarly, the percent Ca in milk was 0.13% and there was no effect of dietary treatments.

Concentrations of Pi and Ca in serum

The addition of exogenous phytase to the diets increased ($P < 0.05$) serum Pi in cows fed both the diets containing barley (5.8 vs 6.6 mg/dL) and corn (5.5 vs 6.0 mg/dL) (Figure

1). The serum Pi values were with the normal physiological range 4.0 - 8.0 mg/dL (Harris et al., 1993). There was no difference in serum Pi between cows fed corn or barley as the grain source. Because blood Pi is affected by dietary P intake (Read et al., 1986), the addition of phytase to the diets apparently increased the amount of absorbable P in the small intestine of the cows. The similarity of serum Pi concentrations in cows fed the barley or corn based diets indicates P digestibility was not affected by grain source.

In contrast to P, the concentrations of Ca in serum were not affected in cows either by the exogenous phytase or the grain source. The serum Ca levels for the barley, barley + phytase, corn and corn + phytase diets were 9.5, 10.0, 9.6, and 10.1 mg/dL, respectively. The serum Ca levels were within the normal physiological range for cattle, 9.0 to 10.0 mg/dL (NRC, 2001).

Apparent digestibility of P

The addition of exogenous dietary phytase increased ($P < 0.05$) phytate P digestibility in lactating cows fed TMR containing either corn or barley (Figure 2 and Table 5). Total P digestibility tended to be greater ($P < 0.1$) in diets with exogenous phytase (Figure 3). Total excretion of fecal P tended to be decreased ($P < 0.1$) in cows fed the diets with added phytase (Table 5). There was no significant difference in P digestibility or phytate P digestibility between the barley and corn based diets (Table 5). The total P digestibility ranged from 42.9 to 53% in barley, 40 to 66% in barley + phytase, 42 to 56% in corn, and 40 to 66% in corn + phytase diets. The digestibility values were consistent with reported

results of 38–44 % (Guyton et al. 2003), 50% (NRC, 2001), 60% (Gueguen et al. 1989), and 70% (Kirchgessner, 1993). The percentage digestibilities calculated from AIA were higher compared to digestibilities from lignin, however, the trends were seen similar in digestibilities from AIA and ADL for total and phytate P. Dry matter digestibility (DMD) were similar among diets and using AIA led to higher predictions of DMD compared to using ADL (Table 5).

Thus, added dietary phytase improved the digestibility of phytate P in lactating cows, presumably by enhancing ruminal hydrolysis of phytate. In cows with no added phytase enzymes, the phytate P digestibility ranged from 69 to 89%. The phytate P digestibility in cows fed phytase ranged from 75 to 98%. The results of the present study indicate that phytase supplementation improves phytate P and total P digestibility in cattle fed either corn or barley as the grain source. The results are in contrast to a previous study where more than 95% of phytate P hydrolysis occurred in the dairy cows without any addition of phytase (Clark et al., 1986). However, in the present study apparent total digestibility was determined and did not separate digestibility in the rumen and in the lower GIT. Shanklin (2001) observed no improvement of P absorption by addition of phytase to lambs. However, the current results are supported by the findings of Hurley et al. (2002) who reported higher P digestibility in feedlot cattle fed 500 units of phytase/kg DM. Similarly, Sansinena (1999) found ruminal escape of phytic acid was 28 to 47% with greater escape occurring increased dietary Ca (0.28 to 1.75% Ca, DM basis). One possible explanation for the improvement in P digestibility with added phytase in the current study is that the high-producing lactating cows had feed intakes of 4% of BW.

Thus, the accompanying rapid rumen turnover rate may limit phytate hydrolysis prior to the absorption sites in the SI.

The concentration of Ca in the diet can affect P digestibility (Bedford, 2000) and phytate hydrolysis (Sansinena, 1999). Although Clark et al. (1986) observed higher apparent digestibility of P when the dietary Ca was 0.9% than 0.6%, others have found reduced P absorption with higher concentrations of dietary Ca (Field et al., 1983; Mathur, 1953). Barth and Hansard (1962) reported phytate P utilization was 100% when the Ca: P ratio was 2:1, but fell to 67% when Ca: P ratio increased to 8:1. In the current study, Ca concentration ranged from 1.11 to 1.18% of the TMR (DM basis), and the Ca: P ratio in the TMR ranged from 2.4 to 2.6:1.

Fecal P, Ca and phytate P

Fecal P excretion (g/d) tended ($P < 0.1$) to be less in cows fed diets containing the added phytase (Table 5). The coefficient of variance (CV) in fecal P output among the cows was 17%. Fecal excretion of phytate P (g/d) was significantly lower ($P < 0.05$) with phytase treatment. The CV of phytate P in feces of cows was 30%. Fecal Ca was unaffected by added dietary phytase and the grain source fed to the cows. The fecal Ca concentration in feces on DM basis for barley, barley + phytase, corn, and corn + phytase diets were 2.5, 2.4, 2.6, and 2.3 %, respectively.

CONCLUSION

Addition of exogenous phytase to diets of lactating cows improved P digestibility. Apparently, phytate P hydrolysis is not 100% in the rumen of lactating cattle and the addition of dietary phytase enhanced the amount of P available for absorption in the SIT. There was no effect of grain source on phytate P digestibility, milk yield or composition. These results indicate that exogenous phytase can improve P absorption in lactating cows and potentially reduce P excretion. These findings have practical implications in P management on dairies, particularly when dietary P intakes are at or below the current recommendations.

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Table 1. Ingredient composition of TMR fed to lactating cows containing barley or corn, with or without added phytase

	Dietary treatments			
	Barley	Barley + 427 FTU phytase/kg	Corn	Corn + 427 FTU phytase/kg
	% dry matter basis			
Alfalfa haylage	25.6	25.6	25.6	25.6
Alfalfa hay	22.3	22.3	22.3	22.3
Barley, steam rolled	25.8	25.7		
Corn, steam rolled			25.8	25.7
Whole cottonseed	9.9	9.9	9.9	9.9
Wheat millrun	6.6	6.6	6.6	6.6
Peas, culled	5.3	5.3	5.3	5.3
Soybean meal	1.6	1.6	1.6	1.6
Sodium carbonate	0.71	0.71	0.71	0.71
Limestone	0.53	0.53	0.53	0.53
Trace mineral premix[1]	0.53	0.53	0.53	0.53
Magnesium oxide	0.14	0.14	0.14	0.14
Molasses	0.89	0.89	0.89	0.89
Vitamin A premix[2]	0.018	0.018	0.018	0.018
Vitamin D premix[3]	0.018	0.018	0.018	0.018
Vitamin E premix[4]	0.0036	0.0036	0.0036	0.0036
4-plex[5]	0.035	0.035	0.035	0.035
Pellet binder	0.018	0.018	0.018	0.018
Phytase[6]		0.09		0.09

[1]Consists of 97% NaCl, 0.18 Mn, 0.35% Zn, 0.2% Fe, 0.037% Mg, 0.035% Cu, 0.01% I, 0.006% Co, and 0.009% Se.
[2]Consists 30,000 IU/g
[3]Consists 8810 IU/g
[4]Consists 500 IU/g
[5]Contains 2.58% Zn as Zn Met, 1.43% Mn as Mn Met, 0.9% Cu as Cu Lys, and 0.18% Co as Co glucoheptonate. Zinpro Corp., Eden Prairie, MN.
[6]Phytase (*Aspergillus niger*) 600 PAK manufactured by ADM Animal Health & Nutrition Division, Des Moines, IA 50313. Contains 600 FTU/g. One FTU is the amount of enzyme that liberates 1 μmole of phosphate/min from 0.0051 mol/L of Na phosphate at 37°C and pH 5.5.

Table 2. Chemical composition of TMR for lactating cows

	Dietary treatments			
	Barley	Barley + phytase	Corn	Corn + phytase
	% dry matter			
CP, %	18.0	18.1	18.2	18.2
NDF, %	34.9	34.7	34.8	35.1
ADF, %	19.4	19.1	19.6	18.9
Ca, %	1.16	1.14	1.11	1.18
P, %	0.45	0.47	0.46	0.45
Phytate P, %	0.235	0.237	0.236	0.238
ADL, %	3.2	3.1	3.3	3.6
AIA, %	1.42	1.52	1.40	1.36

Table 3. Phytate P determination: Solutions for the P standard curve

Tube#	1	2	3	4	5	6
1 mM P (µL)	0	25	50	100	200	400
Zero digest (µL)	100	100	100	100	100	100
Deionized water (ml)	3.9	3.88	3.85	3.8	3.7	3.5
Chen's reagent (ml)	4.0	4.0	4.0	4.0	4.0	4.0

Chen's reagent: 1 volume of 6N H_2SO_4
1 volume 2.5% ammonium molybdate
1 volume of 10% ascorbic acid
2 volumes of deionized water

Table 4. Effect of grain type and added phytase on body weight, feed intake and milk yield per kg feed intake (on DM basis)

	Dietary treatments				SE	Statistical value, P <		
	Barley	Barley + phytase	Corn	Corn + phytase		Grain	Phytase	G x P
Body weight (kg)	704.4	699.5	702.7	705.9	79.0	0.69	0.62	0.15
Dry matter intake (kg)	28.3	28.8	29.0	28.2	0.88	0.45	0.35	0.24
Milk yield kg/d	44.5	43.5	43.5	43.0	4.5	0.29	0.25	0.57
4% FCM, kg/d	40.1	40.2	39.6	39.0	4.7	0.39	0.40	0.64
Milk protein, %	3.1	3.1	3.0	3.1	0.014	0.91	0.13	0.73
Milk fat, %	3.4	3.5	3.4	3.4	0.09	0.92	0.88	0.70
Milk Ca, %	0.131	0.131	0.131	0.132	0.011	0.84	0.80	0.92
Milk P, %	0.09	0.09	0.09	0.09	0.008	0.89	0.89	0.88
PE[1]	1.6	1.5	1.5	1.5	0.11	0.26	0.50	0.69

[1]PE = Milk production efficiency was calculated as kg of milk per kg feed intake (DM basis)

G x P is the response due to grain and phytase interaction

Table 5. Effect of grain source and added phytase on P intake, P secretion in milk, digestibility of total dietary P and phytate P in dairy cows

	Dietary treatments				SE	Statistical value, P <		
	Barley	Barley + phytase	Corn	Corn + phytase		Grain	Phytase	G x P
Total P intake, g/d	128	134.1	134	127.2	10.2	0.23	0.24	0.32
Phytate P intake, g/d	66.5	68.2	68.4	67.1	6.4	0.21	0.26	0.30
P in milk, g/d	48.6	52.3	50.1	52.5	2.5	0.57	0.32	0.72
Estimates using ADL as internal marker								
P in feces, g/d	65.8	64.0	66.8	60.4	4.5	0.45	0.09	0.32
P absorbed, g/d[1]	62.2	70.0	67.1	66.8	3.1	0.13	0.11	0.27
P digestibility, %	48.6	52.3	50.1	52.5	3.4	0.56	0.07	0.67
Phytate P in feces, g/d	14.3	9.8	13.5	9.9	0.8	0.43	0.03	0.28
Phytate P digestibility, %	78.4	85.4	80.3	85.1	4.9	0.56	0.02	0.43
DM digestibility, %	68.5	67.5	68.0	66.8	3.3	0.38	0.22	0.38
Estimates using AIA as internal marker								
P in feces, g/d	59.7	56.1	62.0	57.0	5.8	0.44	0.08	0.73
P absorbed, g/d[1]	68.3	78.0	72.0	70.2	4.5	0.52	0.43	0.44
P digestibility, %	53.4	58.2	53.8	55.2	3.3	0.23	0.08	0.17
Phytate P in feces, g/d	7.8	6.0	8.5	6.6	0.9	0.57	0.05	0.45
Phytate P digestibility, %	88.1	91.0	87.3	90.1	4.9	0.43	0.03	0.95
DM digestibility, %	71.0	69.9	72.7	72.6	3.8	0.33	0.35	0.45

[1]Calculated from the P intake - P in feces, not taken P in urine into consideration

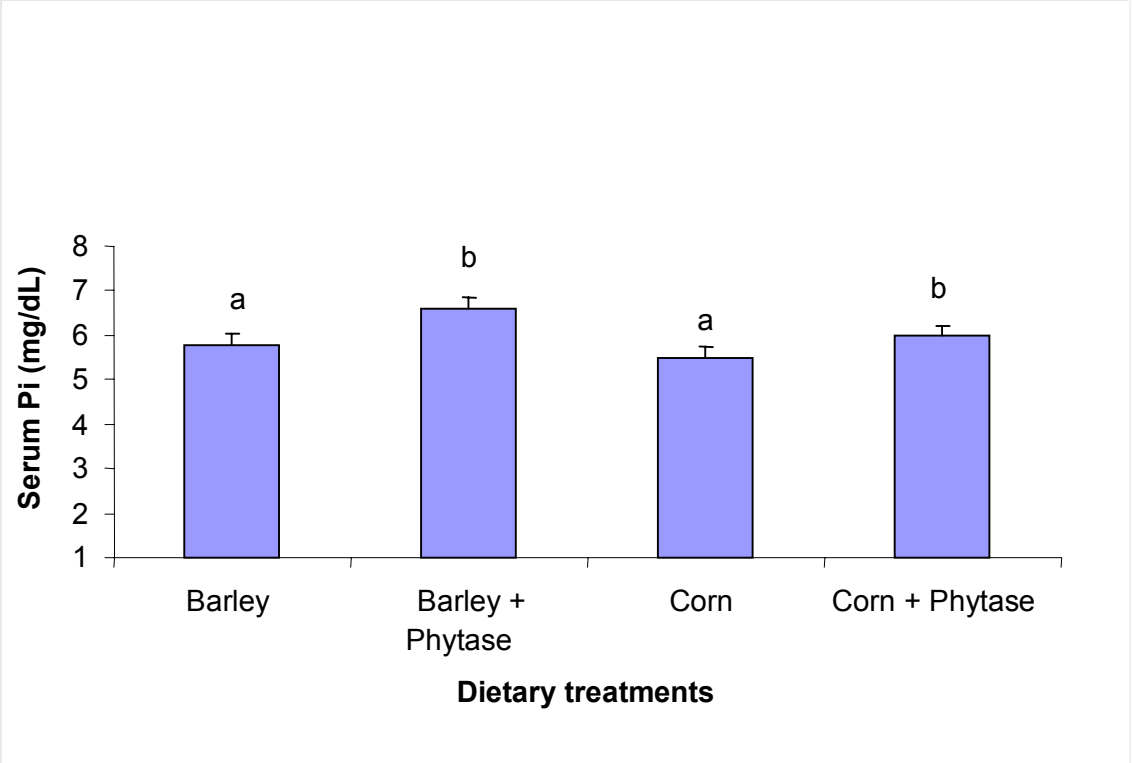

Figure 1. Effect of exogenous dietary phytase on serum Pi in lactating cows fed total mixed rations containing either barley or corn. Treatments with different letters differ ($P < 0.05$). MSE = 0.227

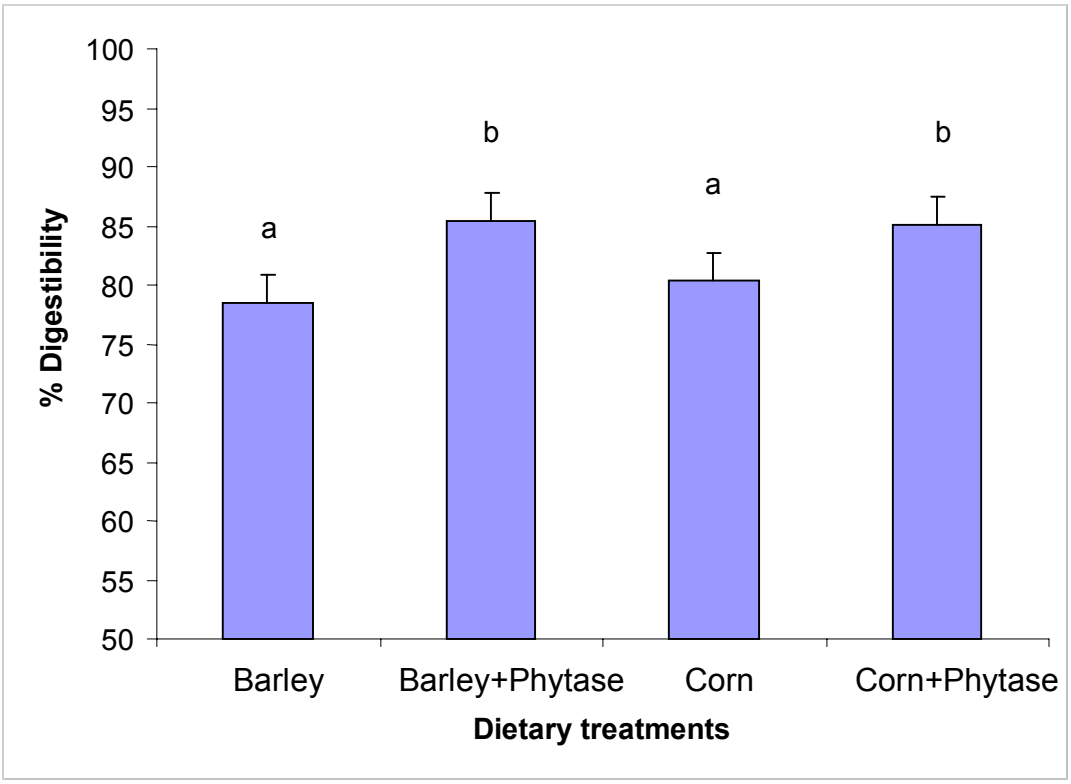

Figure 2. Effect of grain source and exogenous phytase addition on phytate P digestibility in lactating cows. Treatments with different superscripts differ ($P < 0.05$). MSE = 4.9

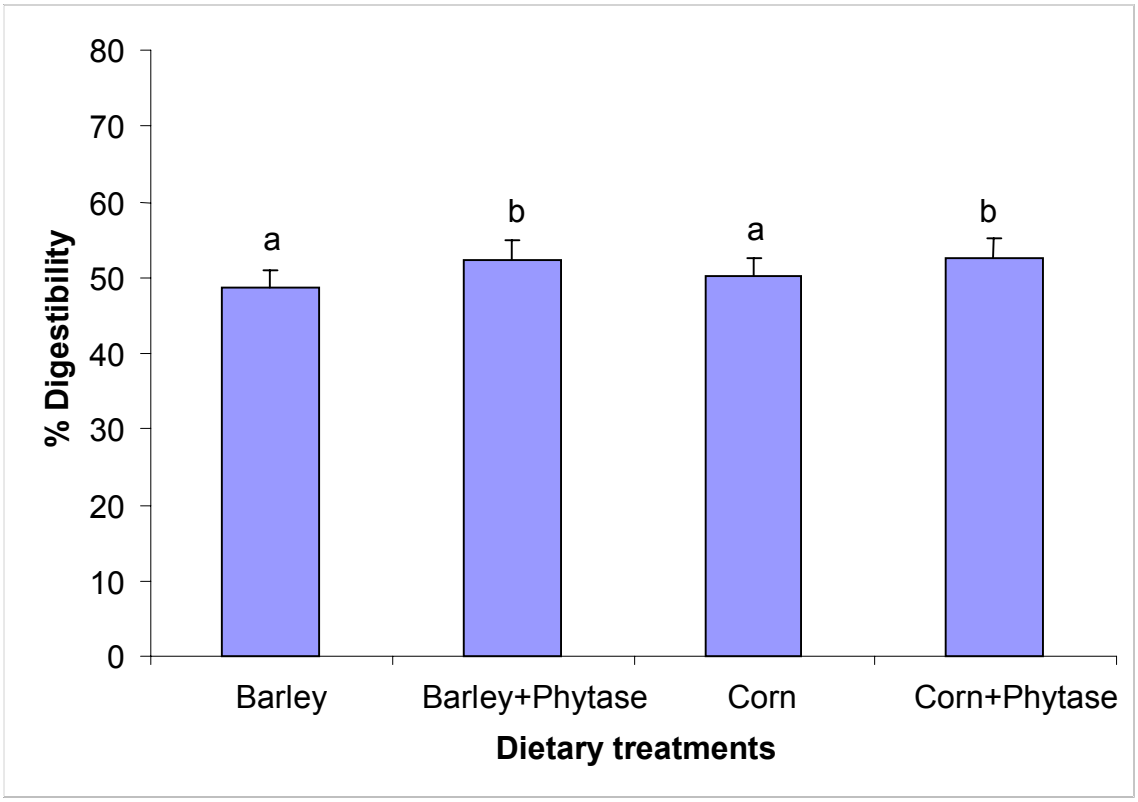

Figure 3. Effect of grain source and exogenous phytase addition on total P digestibility in lactating cows. Treatments with different superscripts differ ($P < 0.1$). MSE = 3.4