Digestion of Inositol Phosphates by Dairy Cows: Method Development and Application

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ABSTRACT

Successful implementation of dietary P management strategies demand improved understanding of P digestion dynamics in ruminants and this is not possible without a reliable and accurate phytate (Pp) quantification method.

The objective of the first study was to develop a robust, accurate, and sensitive method to extract and quantify phytate in feeds, ruminant digesta and feces. Clean-up procedures were developed for acid and alkaline extracts of feed, ruminant digesta and feces and clarified extracts were analyzed for Pp using high performance ion chromatography (HPIC). The quantified Pp in acid and alkaline extracts was comparable for feed but alkaline extraction yielded greater estimates of Pp content for digesta and feces than did acid extraction. Extract clean-up procedures successfully removed sample matrix interferences making alkaline extraction compatible with HPIC. The developed method was applied to investigate the disappearance of Pp from the large intestine of dairy heifers. Eight ruminally- and ileally-cannulated crossbred dairy heifers were used and each heifer was infused ileally with 0, 5, 15, or 25 g/d Pp and total fecal collection was conducted. On average 15% of total Pp entering the large intestine was degraded

but the amount of infused Pp did not influence the degradability of Pp. Net absorption of P from the large intestine was observed.

A feeding trial was conducted to investigate the effect of dietary Pp supply on ruminal and post-ruminal Pp digestion. Six ruminally- and ileally-cannulated crossbred lactating cows were used and dietary treatments were low (0.10% Pp), medium (0.18% Pp), and high (0.29% Pp) Pp, and a high inorganic P (Pi; 0.11% Pp; same total P content as high Pp). Ruminal Pp digestibility increased linearly with dietary Pp. As in the infusion study, net disappearance of Pp from the large intestine was only 16% of total Pp entering the large intestine and not influenced by dietary Pp. Fecal P excretion increased linearly with increasing dietary Pp but was not affected by form of dietary P. In lactating cows Pp digestibility was not affected by dietary Pp and fecal P excretion was regulated by total dietary P rather than by form of dietary P.

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Chapter 1 INTRODUCTION

Attention to dietary phosphorus (P) supply has increased greatly in recent years due to growing global concern about excreted fecal P leading to environmental pollution. Different nutritional strategies have been developed and implemented to minimize the P excretion in dairy cows without impairing production (Knowlton et al., 2004; Vandehaar and St-Pierre, 2006). In dairy cows, fecal P excretion is highly correlated with dietary P intake. Dietary P concentration (g/kg DM) for lactating cows in the US is often above the concentration recommended by NRC, 2001 (Dou et al., 2003). Therefore, reducing dietary P is a very efficient way to reduce fecal P excretion in dairy cows.

As is true for almost all living organisms P plays an important role in maintaining production and performance of high producing dairy cows. Phosphorus plays key role in energy metabolism and is required for bone accretion (Wu et al., 2001). In ruminants, the importance of P in microbial growth and activity in the rumen was reported by several researchers (Bryant et al., 1959; Durand and Komisarczuk, 1988). In addition to these physiological functions in ruminants, P is constantly secreted in the milk and recycled into the rumen. Therefore, care needs to be taken while formulating a low P diet to make sure sufficient P is supplied both for the host and for rumen microbes.

One challenge limiting application of dietary P manipulation is uncertainty in P availability or digestibility in feedstuffs (Bravo et al., 2000; Mjoun et al., 2008). Phytate is the major form of P in grains and many by-product feed ingredients (Ravindran et al., 1994; Eeckhout and Paepe, 1994). Ruminants can utilize phytate P (Pp) because ruminal microorganisms synthesize the enzyme phytase which hydrolyzes Pp into simpler organic forms of P or inorganic P. Because it is the major form of P in the concentrate portion of dairy rations,

any variation in the hydrolysis of Pp in the rumen may cause variation in P availability. The conventional thought was that ruminants can hydrolyze Pp completely in the rumen because Pp was not detected in the feces of lactating cows fed Pp (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992). In early studies, Pp quantification methods were insensitive to low-phytate samples and thus the results might have biased the conclusion.

Moreover, high concentrate diets and high DM intake in high-producing dairy cows cause faster digesta passage which in turn may result in incomplete degradation of Pp in the rumen. Other factors which have been shown to cause variation in the hydrolysis of Pp in the rumen are the type of grain, processing of feedstuffs, and supplementation with exogenous enzymes (Park et al., 1999; Bravo et al., 2003; Kincaid et al., 2005). Incomplete hydrolysis of Pp in the rumen can be compensated if rumen-escape Pp can be degraded post-ruminally and released phosphate can be utilized by the animal. The hydrolysis of Pp and lower inositol phosphates (intermediates of partial Pp hydrolysis) was reported in the small intestine of non-ruminants (Hu et al., 1996). In rodents and non-ruminants, large intestinal Pp hydrolysis was reported by several researchers and attributed to the microbial phytase activity in the large intestine (Wise and Gilburt, 1982; Matsui et al., 1999). The similarity of large intestinal microbial population with ruminal microbes indicates the possibility of microbial Pp hydrolysis in the large intestine of ruminants (Maki and Picard, 1965; Hoover, 1978). The only study investigating post-ruminal Pp hydrolysis was with sheep and Pp hydrolysis was observed in the large intestine but not in the small intestine (Park et al., 2002). But, there is no data available regarding the Pp degradation in the small and large intestine of large ruminants.

Therefore, the objectives of this research project were to 1) develop and validate extraction and quantification methods to measure Pp content in feed, intestinal digesta, and fecal

samples from ruminants, 2) assess Pp degradation in the large intestine of heifers infused ileally with different amounts of Pp, and 3) assess the effect of dietary Pp on P digestibility in lactating dairy cows.

Chapter 2 REVIEW OF LITERATURE

Environmental implications of excess phosphorus excretion

Phosphorus (P) is an essential macromineral in the metabolism of dairy cows. As P cannot be synthesized by animals, sufficient dietary P supplementation is required for optimum animal performance. Attention to dietary P supply has increased greatly in recent years due to growing global concern about excreted fecal P leading to environmental pollution. Manure P can lead to soil P buildup, and runoff from agricultural land or livestock farms is a source of P contamination of surface water (Jordan et al., 2002; Wu et al., 2003). As a consequence P is more available for algal growth leading to the eutrophication which kills aquatic life (Sharpley and Tunney, 2000). The environmental pollution due to P runoff is a growing global concern leading to efforts to develop and implement nutritional strategies to minimize P excretion without impairing production (Vandehaar and St-Pierre., 2006). Therefore, it is very important to improve our understanding of the fate of dietary P in the digestive tract to increase P utilization in dairy cows for the purpose of reducing environmental impact.

In dairy cows, fecal P excretion is highly and positively correlated with P intake (Morse et al., 1992b; Wu et al., 2000; Knowlton and Herbein, 2002; Valk et al., 2002; Wu, 2005). The increased fecal P excreted by dairy cows fed high P diets is mainly water-soluble; this fraction is more susceptible to runoff losses (Dou et al., 2002; Kleinman et al., 2002). Dietary P concentration (g/kg DM) for lactating cows in the US is well above the concentration recommended (Wu et al., 2000; Dou et al., 2003). But, more than half of the dietary P cannot be used by dairy cows and the major proportion of P is excreted via feces (Morse et al., 1992b). Reducing dietary P from 0.47 to 0.31% (DM basis) did not affect production and reproductive performance of lactating Holstein cows (Wu et al., 2001). Similarly, Odongo et al. (2007) did not

observe any negative effect of reducing dietary P from 0.42 to 0.35% (DM basis) on the production of lactating Holstein cows but fecal P excretion decreased. A mechanistic modeling approach was used by Kebreab et al. (2008) to simulate fecal P excretion by Ontario dairy cows to evaluate the influence of nutrition strategies adopted to reduce fecal P excretion on the environment and the economy. Reduction in dietary P from 0.42% to 0.35% reduced total P contribution from dairy farms by 1.3 kt/yr while saving CAN $20 on each cow annually.

Phosphorus containing compounds in feeds, digesta, and feces

Phosphorus is present in both inorganic and organic forms in feeds and other samples of interest in dairy nutrition i.e. digesta, feces (Toor et al., 2005). Inorganic forms include orthophosphate (PO_4^{3-}), pyrophosphate ($P_2O_7^{4-}$), and polyphosphate (contains more than 2 P atoms). Phytate (*myo*-inositol hexakisphosphate) is the major organic P containing compound in cereal grains and is reviewed below. Other organic P containing compounds in feed or digesta samples may include P in nucleic acids of plant and microbial origin, phospholipids, and lower inositol phosphates (inositol penta- to tri- phosphate).

In grain feeds, a significant proportion of total P is present as phytate (Eeckhout and Paepe., 1994; Ravindran et al., 1994; Steiner et al., 2007). Phytate consists of an inositol ring with six covalently linked phosphate groups and is mostly present as a salt conjugated with divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} (Maenz et al., 1999, Tang et al., 2006). Phytate also complexes easily with protein or starch (Fontaine et al., 1946; Bourdillon, 1951; Kies et al., 2006). The immediate intermediate of partially hydrolyzed phytate (a.k.a. inositol pentaphosphate) was also reported to have protein binding capacity (Yu et al., 2012). Because of this reactivity with divalent cations, starch or protein, phytate makes these other nutrients less soluble, leading to the reduced absorption from animals' gut.

According to Nelson et al., (1976) natural phytate is present only in feed of seed origin, not in the ingredients derived from stem and leaves. Phytate plays an important role in P and mineral homeostasis during seed development. In seeds, minerals are stored mainly as mineral deposits which are composed of phytate. Minerals are stored in phytate-complexes in three different regions of germinating seed: Mn-phytate crystals in the endoplasmic reticulum, Zn-phytate crystals in vacuoles, and phytate complexed with other minerals (Ca, Mg, K) in protein reserve vacuoles (Otegui et al., 2002). In plant seeds, the type of plant and the proportion of other nutrients regulate the distribution of phytate (Lin et al., 2005). Therefore, it is difficult to find a single method that extracts or quantifies phytate satisfactorily from different types of feed.

Quantification of the phytate content of complex samples

Better understanding of the fate of P in the digestive tract demands the estimation of different fractions of P in feed, digesta, and feces. The first step for the quantification of Pp is the extraction of that form of P from the sample. Unlike the extraction of Pp from feed, Pp extraction from digesta and feces is difficult as this form of P is always bound with cations or other compounds. Incomplete extraction obviously leads to the erroneous estimation and the presence of interfering compounds can cause damage to the analytical columns by clogging.

Challenges to measurement of Pp influences interpretation of early work on Pp excretion by ruminants. Three research groups (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992a) reported little or no excretion of Pp (results discussed later in this review), but none measured or reported extraction and detection efficiency. The method of Pp estimation used by Nelson et al. (1976) consisted of several steps including extraction of dried sample with 0.5 N HCl followed by neutralization with NaOH and re-acidification with HCl. After that, ferric chloride was used to precipitate phytate, and the precipitate was subjected to repeated heating, cooling,

centrifuging, and washing with acid and distilled water. The other two commonly cited early studies (Clark et al., 1986; Morse et al., 1992a) used a simpler method of Pp quantification starting with the same concept of extracting with dilute acid (1.2% HCl) followed by precipitation of Pp using ferric chloride. Morse et al. (1992a) used the molybdenum blue method to determine the concentration of P after Pp extraction. This method consists of digesting and oxidizing the extracted sample with a suitable oxidizing agent (70-72% perchloric acid and concentrated nitric acid), formation of a phosphoric-molybdic acid complex, and reduction of this complex with a reducing solution (elon) to give the molybdenum blue color. Clark et al. (1986) digested Pp with sulfuric acid and then determined released P with Technicon Auto Analyzer II using the principle of blue color formation due to phosphomolybdate complex reduction by ascorbic acid.

Extraction of P-containing compounds

A series of methodologies have been developed for the quantification of Pp and majority of them involved acid or base extraction (Skoglund et al., 1997; Kemme et al., 1999; Carlsson, et al., 2001; Harland, et al., 2004; Leytem et al., 2008). Different researchers tried different concentrations of acid or base as extractants with variable extraction times but still there is no established method for the extraction of Pp. There are several accepted methods of analyzing feeds for Pp but none has been validated in feces or digesta samples in all sample types.

Researchers have tried different concentrations of acid or base as extractants with variable extraction times. McDowell et al. (2005) used sequential extraction to analyze P in both fresh and air-dried feces of dairy cattle, sheep, and deer. In fresh feces, when P in each fraction was calculated as percent of total extracted P, water, sodium bicarbonate, and NaOH were more effective in extraction of inorganic P than was HCl, while in dry feces HCl was the most

powerful extractant for inorganic P. The reason suggested for this difference in extracted P concentrations between fresh and dry feces was that drying may cause redistribution of P among fractions. Organic P in each fraction was not different between fresh and dry feces except for NaOH extraction.

McDowell et al. (2008) reported that the efficiency of the extractants vary with the dry matter content of the sample. They found that less P was extracted by water and dilute HCl (0.012 M) from dry fecal samples as compared to wet samples, whereas P extraction efficiency of NaOH-EDTA (0.25 M-50 mM) was higher for dry feces than for wet fecal samples.

Chapuis-Lardy et al. (2004) found a nonlinear decrease in water-soluble inorganic P in dairy feces due to the drying-grinding process. As a solution to this problem, Dou et al. (2007) used dilute HCl as extractant and found that the differences between wet-based and dry-based extractable inorganic P diminished as the acid concentration increased. The least difference between wet and dry sample was with 0.4% HCl. As an intermediate step of sequential extraction, total P extraction efficiency of NaOH was lower for dry feces than for wet feces from cattle (McDowell and Stewart, 2005).

Alkaline extraction has been reported to be the most efficient in extracting P from feed and manure or feces samples (Toor et al., 2005; McDowell et al., 2008). Interference of divalent cations such as Ca^{2+} with P extraction using NaOH has been reported (McDowell et al., 2008) but addition of EDTA as a chelating agent alleviates this problem (Turner, 2004).

Analytical methods for phytate quantification

During the last few decades, several quantitative analytical techniques have been used to quantify the content of Pp (myoinositol hexaphosphate), other lower inositols, and other organic forms of P in extracted samples. Before chromatographic methods were developed, the

quantitative analysis of Pp involved ***precipitation of the insoluble iron-phytate complex*** due to the reaction of phytate with ferric ion (Makower, 1970). One of the disadvantages of this method was lack of applicability in low Pp samples. Wheeler and Ferrel (1971) found that Pp estimation method using the iron-phytate precipitation principle was applicable at Pp concentrations between 0.10 and 0.65 mg per mL extracted and digested sample with a reasonable degree of accuracy.

Ion exchange chromatography replaced quantification methods based on the principle of iron-phytate complex precipitation. Harland and Oberleas (1977) extracted Pp directly with 1.2% HCl and then eluted inorganic P and Pp with 0.05 *M* and 0.7 *M* sodium chloride solution, respectively, through anion-exchange resin (200-400 mesh AGI-X8 chloride form anion exchange resin). The eluted Pp was then digested with concentrated sulfuric acid and concentrated nitric acid, and the released P measured colorimetrically. Latta and Eskin (1980) used this same anion-exchange principle for the separation of Pp from inorganic P but replaced the digestion step with the colorimetric assessment of Pp concentration using Wade's reagent. This was found to give inaccurate results in low Pp samples. Ellis and Morris (1983) modified the anion-exchange column chromatography method by adding EDTA to the extract and adjusting the pH to 6 with NaOH to reduce the interference of plant proteins and metal ions naturally present in complex with phytate. Interference of metal ions was reduced by the chelating effect of EDTA while protein interference was suppressed by the pH 6 (above the isoelectric point of most of the amino acids present in protein).

The advantage of this modified ion-exchange procedure over iron-phytate precipitation methods is that Pp concentration is not a limiting factor with this modified ion-exchange procedure. However, over-estimation of Pp may be an issue with ion-exchange methods.

Phillippy and Johnston (1985) found that estimated phytic acid contents of food samples were lower for ion chromatographic methods than for ion exchange methods and suggested that this difference was due to the inability of ion exchange methods to separate phytic acid breakdown products from phytic acid. Later, Lehrfeld and Morris (1992) also reported overestimation of phytic acid content of processed foods by anion-exchange method due to the inability of this method to distinguish between phytic acid and lower inositol phosphates.

Now, advanced, simple, and rapid analytical methods have been developed for the quantification of Pp in a variety of samples. ***Ion pair chromatography*** and ***high performance chromatography methods*** are the most common followed by ***^{31}P nuclear magnetic resonance spectroscopy*** (^{31}P-NMR). These novel analytical techniques have been applied to determine inositol hexaphosphate and other lower inositol phosphates.

Sandberg and Ahderine (1986) first developed the ion pair chromatographic procedure and used high-performance liquid chromatography to separate and determine inositol hexaphosphate and other lower inositol phosphates. Isomer specific high-performance ion chromatographic approach was first established by Phillippy and Bland (1988).

As an improved method to separate and quantify inositol di- to hexaphosphates and their isomers, Skoglund et al. (1997) used the combination of HCl extraction and high-performance ion chromatography (HPIC). Inositol phosphates were eluted using a gradient elution program and detected by UV detector after post column reaction. The samples in this study included food, and ileal and fecal samples from human. Anion-exchange high-performance ion chromatography was used by Talamond et al. (2000) to quantify phytic acid in food and was similar to the method described by Skoglund et al. (1997) except that sample pretreatment was simplified by removing solid phase extraction and that lower inositol phosphates cannot be separated by this method.

When Carlsson et al. (2001) used the method described by Talamomd et al. (2000), they observed interference of lower inositol phosphates with phytate (IP6) peaks and used a gradient elution program containing 1 M HCl and water to elute inositol bis- to hexa- phosphates. To simplify and improve the throughput of the method, they replaced solid phase extraction and evaporation steps with centrifugal ultrafiltration as sample pretreatment. But the use of 1 M HCl as eluent may cause damage to the expensive analytical column.

A comparatively weaker eluent (0.25 M HNO_3) was used in an isocratic elution program when similar ion chromatographic approach was taken to quantify Pp in food and satisfactory results were obtained with 96-105% spike recovery (Phillippy et al., 2003). This method is not capable of separating and quantifying lower inositol phosphates including Pp. Kwanyuen and Burton (2005) developed an ion chromatographic method to quantify Pp in soy products using a 30 min gradient elution program which includes 0.01 *M* 1-methylpiperazine, and combination of 0.5 *M* $NaNO_3$ and 0.01 *M* 1-methylpiperazine as eluents. The pH of the eluents was 4.0 and retention time for Pp was little more than 20 min. Similar chromatographic approach was used by Leytem et al. (2008) to characterize P fractions in broiler ileal digesta, manure, and litter samples. In this method different analytical and guard column were used with relatively shorter elution program (20 min). Phytate (IP6) and lower inositol phosphates (IP3-IP5) were eluted separately with the retention time of 11.3, 10.91, 8.7, and 6.9 for IP6, IP5, IP4, and IP3, respectively. In the same study, ^{31}P NMR was also used to characterize different fractions of P and there was a very high correlation between Pp concentration quantified by ion chromatography and ^{31}P NMR. It was not possible to separate lower inositol phosphates by ^{31}P NMR because of poor resolution of phosphate monoester spectral region. The problem of separating inositol phosphates in NMR spectra was also reported by Turner et al. (2004) in the

effort to analyze Pp in cattle manure using ^{31}P NMR and the authors mentioned that the problem can be solved by separating NMR peaks either using deconvolution software or hypobromite oxidation of sample extracts before running NMR. Both these approaches would require either expertise or more complicated sample preparation.

Most of the Pp extraction and quantification methods have been developed and evaluated using human food, soil, monogastric digesta or manure samples rather than digesta or fecal samples from ruminants. Phytate P extraction and quantification method development and evaluation studies using ruminant digesta and feces are very limited. In ruminant digesta and fecal samples, inositol phosphate-P is likely complexed with other organic materials and minerals differently than it is in feed and monogastric samples. Thus it is challenging to extract inositol phosphates-P from ruminant samples efficiently. Moreover, these samples respond differently to different extractants (acid, base, strength of extractants) in terms of Pp extraction. Also, Pp quantification method is sensitive to pH of extracted samples. Therefore, it is important to find an extractant which is appropriate for efficient extraction as well as convenient for the quantification method.

Factors affecting ruminal P digestion in ruminants

Phytate hydrolysis activity of ruminal microorganisms

The microorganisms which can produce phytase include bacteria, yeasts, and fungi (Powar and Jagannathan, 1982; Nayini and Markakis, 1984; Ullah, 1988). Sequeilha et al. (1993) evaluated Pp hydrolysis ability of a yeast strain (*Schwanniomyces castellii*) and found that 90-95% of Pp in soft wheat bran and glandless cottonseed flour was hydrolyzed by the yeast.

Several strains of rumen anaerobic bacteria and other ruminal microorganisms have phytase activity. Yanke et al. (1998) found that the ruminal bacteria mainly responsible for

phytase production include *Selenomonas ruminantium, Megasphaera elsdenii, Prevotella ruminicola,* and *Mitsuokella multiacidus*. Reid et al. (1947) reported Pp hydrolysis in the sheep rumen and Raun et al. (1956) observed Pp hydrolysis capability in rumen microorganisms using an artificial rumen technique. Therefore, in contrast to monogastric animals with their limited capacity to digest Pp, ruminants can hydrolyze Pp into free inorganic P.

Efficiency of microbial phytase depends on certain physical and chemical factors such as temperature, pH, and presence of cations or anions. *Selenomonas ruminantium* was reported as the most efficient ruminal anaerobe in terms of phytase activity but optimum phytase activity can be achieved within certain range of temperature (50-55°C) and pH (4-5.5; Yanke et al., 1999). Phytase activity from *Selenomonas ruminantium* was inhibited in the presence of Fe^{2+}, Fe^{3+}, Zn^{2+}, and Cu^{2+} but was not affected by the concentration of phosphate. Similar conditions were found to be optimum for the most efficient phytase activity from *Mitsuokella jalaludinii* (Lan et al., 2011). Endogenous ruminal phytase activity varies also with the type of diet and P source. Yanke et al. (1998) reported that ruminal phytase activity was higher in steers fed grain diets than fed hay diets. Godoy and Meschy (2001) estimated the effect of Pp supply on the phytase activity of rumen microorganisms using a semi-continuous culture system fermentor (RUSITEC) and concluded that phytase activity of rumen bacteria was higher with the high Pp supply.

While limited data are available, some studies suggest limited ruminal Pp degradation. Tillman and Brethour (1958) observed 63% and 70% digestibility of P using calcium phytate and monobasic calcium orthophosphate, respectively, in sheep. In a similar kind of study using sheep, Lofgreen (1960) found that true digestibility of P was 33% in calcium phytate and 50% in dibasic calcium orthophosphate. The difference in the P digestibility between the inorganic calcium phosphate and the organic calcium phytate may be due to more complex nature of

phytate with cations, as compared to phosphates which can be easily made available as free phosphate by dissolution or by phosphatases or other enzymes. Also, the bioavailability of Pp reported in these studies may be overly optimistic, as they utilized calcium phytate instead of natural Pp.

In 1976, Nelson et al. studied the digestibility of natural Pp in calves using corn and sorghum as two main grains of two diets and found that less than 1% of dietary Pp was present in feces. Clark et al. (1986) reported that Pp was almost completely hydrolyzed into inositol and inorganic phosphate in dairy cows. Morse et al. (1992a) found that the hydrolysis of inositol ring to release P in high producing dairy cows was greater than 99%. In interpreting these findings, it is important to consider the methods used in these studies to quantify the Pp. As discussed above, fecal material is complex containing undigested feed residues, partially digested microbial residues, and endogenous cellular material. Within this matrix, Pp is complexed with minerals and other nutrients. Therefore incomplete recovery of fecal Pp with these methods may be one of the reasons why very low or no Pp was detected in feces in these earlier studies.

High-producing dairy cows consume more dry matter, and their diets contain an increased concentrate to roughage ratio as compared to the animals used in the preceding studies (Phipps et al., 2000). Higher dietary Pp intake due to increased concentrate feeding together with higher passage rate of digesta due to more dry matter intake may result in incomplete degradation of Pp in the rumen. Other factors which cause variation in the hydrolysis of Pp in the rumen or P digestion are the type of grain, processing of feedstuffs, and supplementation with exogenous enzymes. For these reasons, it is important to revisit the question of bioavailability of high Pp feeds in lactating cows.

Effect of the type of grain on P digestion

In Holstein dry cows, ruminal P digestion was more than 85% for cereals and cereal by-products when measured using nylon bag technique but was least for corn distillers grain (Bravo et al., 2000). Bravo et al. (2003) reported that apparent P digestion varied with the type of the grain in sheep. They found that apparent P absorption was higher in sheep fed soybean meal than in sheep fed other grains (rapeseed meal, sunflower meal, groundnut meal, and linseed meal). Kincaid et al. (2005) reported that total tract P digestibility was higher with corn than with the combinations of corn with different varieties of barley in lactating cows (35 vs. 11-29%). Similarly, Wang et al. (2008) found a wide variation in effective rumen degradability of P of corn (maize), sorghum, and rice using in situ nylon bag technique in wether goats. Mjoun et al., (2008) also used the nylon bag incubation technique in lactating cows to compare the ruminal P disappearance of corn, corn by-products, and different soybean feedstuffs. They found that effective disappearance of P was greatest in corn by-products (93.5%) followed by corn (83.3%), soybean meals (87-88%), and soyhulls (69.1%).

Effect of heat treatment of grains on P digestion

Heat treatment is a common feedstuff processing technique to increase the availability of protein in the small intestine by reducing protein degradation in rumen. In grains, phytic acid is present as phytate-protein complexes (Fontaine et al., 1946; Bourdillon, 1951). Due to this complex-forming nature of phytate, heat treatment that affects protein degradation kinetics may also reduce the degradation of Pp in the rumen.

Heat treatment of rapeseed meal at two different temperatures (133°C and 143°C) caused 37% and 55% Pp escape from the rumen in sheep in comparison to 22% Pp escape from untreated rapeseed meal (Park et al., 2000). Blaabjerg et al. (2007) found that in vitro

degradation of Pp was decreased in wheat/soybean meal by heat treatment. Konishi et al. (1999) reported that 31-58% of Pp in heat-treated (143°C) rapeseed meal was not degraded in the rumen of sheep as compared to 18-41% in untreated rapeseed meal. In both of these experiments, ruminal outflow rate was 0.02-0.08 per hour. They also reported that effective degradability of Pp was more intensely affected by heat treatment in rapeseed meal than in soybean meal. The suggested reason for this difference in responses to heat treatment was the difference in the distribution of phytate in these two oilseed meals. Phytate in rapeseed is localized in globoid crystals, while in soybeans, phytate is uniformly distributed in the protein matrix.

Effect of formaldehyde treatment of grains on P digestion

Park et al. (1999) found that formaldehyde treatment of soybean meal or rapeseed meal decreased ruminal degradation of dietary Pp in sheep. Bravo et al. (2002) reported decreased rumen P solubility of rapeseed meal and soybean meal after formaldehyde treatment in lactating cows and in goats. In 2003, Bravo et al. found that formaldehyde treatment increased apparent P digestibility of rapeseed and sunflower meals in mature sheep while apparent P digestibility of soybean meal was unaffected. The authors attributed this discrepancy to possible Pp degradation by colon microbiota and stimulation of P absorption from lower digestive tract due to homeostatic regulation. In an *in situ* study with Holstein cows, ruminal degradation rate of total and Pp was lower for formaldehyde treated rice bran than for untreated rice bran (Martín-Tereso et al., 2009).

Effect of exogenous phytase supplementation on P digestion

It is well documented that microbial phytase supplementation in the diet improves the bioavailability of Pp in poultry and pigs (Nelson et al., 1971; Cromwell et al., 1993; Olukosi et al., 2007a; Olukosi et al., 2007b;). While the endogenous phytase activity of the ruminal

microorganisms leaves less room for improvement, some recent research (Bravo et al., 2002; Kincaid et al., 2005; Knowlton et al., 2007) suggests an opportunity to increase bioavailability of feed P with exogenous phytase in ruminants.

Kincaid et al. (2005) found that supplementation with exogenous phytase improved the hydrolysis of Pp leading to increased total P digestibility in lactating cows. They also reported that physical properties of diet and ruminal passage rates may prevent total hydrolysis of Pp in the rumen of lactating cows and thus, exogenous dietary phytase might improve P digestibility in dairy cows in some dietary situations.

Bravo et al. (2002) found that addition of fungal phytase increased rumen P solubility in lactating goats fed untreated soybean meal, and in both control and formaldehyde-treated rapeseed meal. No improvement was observed, however, in rumen P solubility in lactating cows with fungal phytase supplemented to high forage (70%) diets. The probable reason for the reduced effectiveness of exogenous phytase in high forage diets was that lower rumen pH (5.77±0.19) due to high concentrate diet may cause maximal efficiency of exogenous phytase but high rumen pH (6.47±0.16) resulting from high forage diet may reduce the exogenous phytase activity.

Another hypothesis for explaining this improved rumen P solubility in phytase-supplemented high concentrate diets may be the reduced rumen retention time in cows fed the high concentrate diets. Reduced rumen retention time gives the endogenous microbial phytase less time to hydrolyze Pp and thus Pp degradability decreases. Therefore, Pp escaping microbial phytase hydrolysis can be degraded by exogenous phytase resulting increased rumen P solubility. In contrast, the higher rumen retention time in cows fed high forage diet leaves little room for further improvement in Pp hydrolysis.

Knowlton et al. (2007) found that digestibility of P was unaffected by the supplementation of exogenous phytase and cellulase in diet containing 37% forage. The contrasting result in this study as compared to the previous studies may be due to the variation in the basal diet. Moreover, the dietary Pp was relatively low in this study. More research is needed on the effect of exogenous phytase with different forage to concentrate ratio and grain types on the digestibility of P.

In a recent study with male finishing lambs, total tract P digestion increased linearly with increasing exogenous phytase supplementation (0, 750, 1500, and 2250 phytase units/kg as fed) to sorghum based diet (Buendía et al., 2010). Phytate degradation in rapeseed cake increased with the addition of phytase into the *in vitro* system containing rumen fluid from heifers but there was no effect of phytase on Pp degradation in wheat (Brask-Pedersen et al., 2011). This difference in the effect of phytase on Pp degradation was attributed to the presence of larger amount of natural phytase in wheat than in rapeseed cake. Therefore, the authors concluded that any positive effect of phytase supplementation to diet would depend on the type of feed ingredients present in the diet.

Degradation of phytate and lower inositol phosphates in the lower digestive tract

Phytate and lower inositol phosphates produced from incomplete degradation of Pp in the rumen can further be degraded in lower digestive tract. Bitar and Reinhold (1972) demonstrated some Pp hydrolysis activity in extracts of the mucosa of the small intestine of the rat, chicken, calf, and man. Sandberg et al. (1987) found that 58% of dietary Pp was hydrolyzed into lower inositol phosphates in the stomach and small intestine of humans consuming unprocessed wheat bran as a source of Pp. Hu et al. (1996) incubated pig intestinal mucosal samples with purified inositol hexaphosphate (IP6) and found hydrolytic enzymatic activity towards IP6. They

measured Pp using ferric chloride precipitation principle which did not allow them to differentiate between Pp and lower inositol phosphates and thus Pp may be overestimated. They also reported highest hydrolytic enzymatic activity for jejunum followed by duodenum and ileum. They added zinc and magnesium during incubation and maintained the pH of incubation solution at 7.9.

Phytate degradation in the large intestine

These reports of possible degradation in the small intestine are the exception; most evidence of lower tract degradation is from the large intestine. Wise and Gilburt (1982) measured phytic acid hydrolysis in germfree and conventional rats and found negligible phytic acid hydrolysis in germfree rats while 22-56% phytic acid was hydrolyzed in conventional rats. They concluded that intestinal bacteria were responsible for phytic acid hydrolysis. Park et al. (2002) studied post-ruminal Pp degradation in sheep and found that of the 24% dietary Pp that escaped ruminal degradation, none was degraded in the abomasum or small intestine and 60% of inositol hexaphosphate flowing from the small intestine was partly degraded in the large intestine. The data on the degradation of Pp in large intestine of ruminants are scarce and more study is needed to confirm large intestinal Pp degradation.

Degradation of lower inositol P forms

Some methods of quantifying Pp mistakenly include partial degradates (lower inositol phosphates) in the Pp fraction. Attention to this possible source of error is needed because these compounds may not behave the same as inositol heaxaphosphate in the digestive tract. Hu et al. (1996) reported small intestinal hydrolytic enzymatic activity toward the lower inositol phosphates [inositol triphosphate (IP3), inositol tetraphosphate (IP4), inositol pentaphosphate (IP5)] in vitro with the highest hydrolytic activity towards IP3 followed by IP4 and IP5. Optimal

conditions (pH and the concentration of added zinc and magnesium) for the hydrolysis were different for different inositols. The pH for all hydrolyses was much higher (6.9-7.4) than normal intestinal pH. Park et al. (2002) found that the flow of inositol triphosphate (IP3) tended to decrease in the small intestine in sheep while the passages of inositol tetra- (IP4) and penta- (IP5) phosphates were not markedly different between the abomasum and the small intestine. They also reported that the passages of all lower inositol phosphates (IP3, IP4, and IP5) in the upper large intestine were less than in the small intestine, and concluded that IP3 may be degraded in both segments of the intestine but that IP4, IP5, and Pp are only degraded in the large intestine.

Predicting bioavailability of feed P

Phosphorus availability can be defined as the proportion of dietary P which can be absorbed and utilized by the animal. Therefore the term ‘availability’ can be interchangeably used with ‘true absorption coefficient’. But care must be taken in determining P availability because P absorption is regulated not only by feed characteristics but also by animal physiology and dietary situations.

Conceptually the approach of estimating P availability resembles the approaches of predicting digestibility of other nutrients such as fiber, starch or protein in the diet. The early approaches of estimating true digestibility of fiber included simple model using rate of digestion and passage (Waldo et al., 1972). This model would be ideal when feed intake and rate passage is constant, and then true digestibility can be represented as a primary function of rate of digestion. Thus, under controlled condition with minimum contribution to variability from the animal, true digestibility of the nutrient would depend on the intrinsic characteristics of the nutrient.

Considering the impracticality of conducting *in vivo* experiments to evaluate the effect of all main factors and their interactions on true digestibility of fiber and other nutrients, a dynamic modeling approach was taken to investigate the influence of dietary and animal factors on fiber digestion. A dynamic model was developed to simulate NDF digestibility and passage kinetics, particle size reduction, and digestion were included in the model (Mertens and Ely, 1979). Three submodels, one each for passage, particle size reduction, and digestion, were developed separately and then integrated into a complete model of fiber digestion. Neutral detergent fiber was fractionated into fast-degrading, slow-degrading, and undegradable pools. Different rate constants in the model were for passage, digestion, and transformation between different nutrient pools.

Similar concepts may be applied in the modeling approach of investigating P digestion and metabolism in ruminants. But major caution is required in estimating P availability as the utilization of P by ruminants is different from the utilization of other nutrients in terms of physiology. For example, if energy is available beyond the requirement, animal would utilize the excess energy and increased energy density in the diet would inhibit DM intake. This is not the case for P intake and absorption. High P diets do affect DM intake and P in excess of requirement, though available, is excreted. Moreover, recycling of salivary P in ruminants makes it difficult to determine the fraction of fecal P from the diet.

Hill et al. (2008) constructed a model of P digestion and metabolism in lactating cows and predicted P pool sizes (g) and fluxes (g/d) in the digestive tract from the data of Schwab et al. (2006) and Knowlton et al. (2001). Because insufficient data were available to say otherwise, the model assumed no degradation of Pp or other organic P forms in the large intestine, and assumes no absorption of phosphate from that segment. The authors used the model to derive

digestibility coefficients for various fractions of P (organic, inorganic), and reported higher predicted values of small digestion of organic P than of inorganic P in the small intestine. They concluded that improved knowledge of dynamics (site and extent) of organic P digestion and inorganic P absorption will allow more accurate prediction of absorbable P supply for different feeds.

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Chapter 3 QUANTIFYING PHYTATE IN DAIRY DIGESTA AND FECES: ALKALINE EXTRACTION AND HIGH PERFORMANCE ION CHROMATOGRAPHY

Abstract

Development of an analytical method with appropriate combination of extraction and quantification approaches for undigested phytate in ruminant feces and digesta will advance knowledge of phytate degradation in ruminants and reduce phosphorus excretion. Established quantification methods give satisfactory results for feedstuffs and nonruminant manure but recovery of phytate is incomplete for ruminant feces and digesta because of their complex sample matrix and low ratio of phytate to inorganic P. The objective was to develop a robust, accurate, sensitive, and inexpensive method to extract and quantify phytate in feeds, ruminant feces and digesta. Diets varying in phytate content were fed to dairy heifers, dry cows, and lactating cows to generate digesta and fecal samples of varying composition to challenge extraction and quantification methods. Samples were extracted with 0.5 M HCl or 0.25 M NaOH+0.05 M EDTA. Acid extracts were mixed with 20% NaCl, alkaline extracts were acidified to final pH < 2, and then both extracts were clarified with C_{18} cartridges and 0.2 µm filters. High performance ion chromatography (**HPIC**) was used to quantify phytate. In feed samples, the measured phytate was comparable in alkaline and acid extracts (2,965 vs. 3,085 µg/g DM). In digesta and fecal samples, alkaline extraction yielded greater estimates of phytate content than did acid extraction (40.7 vs. 33.6 and 202.9 vs. 144.4 µg/g DM for digesta and fecal samples, respectively). Analysis of alkaline extracts by HPIC is usually not possible because of sample matrix interferences; acidification and C_{18}-cartridge elution of alkaline extracts prevented this interference. Pure phytate added to dry samples before extraction was almost completely recovered (88 to 105%) indicating high extraction efficiency, no adverse effect of extract clean-

up procedures, and accurate quantification of phytate. The proposed method is rapid, inexpensive, robust, and combines the extraction power of NaOH-EDTA with the precision and sensitivity of HPIC quantification, allowing accurate quantification of phytate in feeds, ruminant digesta and fecal samples.

Key words: high performance ion chromatography, dairy feces, phytate

Introduction

The quantification of phytate in feed, digesta, and feces from dairy cows is important in nutritional and environmental research. Environmental pollution due to P runoff from livestock farms is a growing global concern leading to efforts to develop and implement nutritional strategies to minimize the P excretion without impairing production (Knowlton et al., 2004; Vandehaar and St-Pierre, 2006). Improved understanding of the fate of dietary P in the digestive tract may support improved P utilization by livestock to reduce their environmental impact.

One challenge limiting application of dietary P management is variation in P availability or digestibility in feedstuffs (Bravo et al., 2000; Mjoun et al., 2008; Cherry et al., 2010). Phytate is the major form of P in grains and many by-product feed ingredients (Ravindran et al., 1994; Eeckhout and Paepe, 1994). Ruminants can utilize phytate-P because ruminal microorganisms express the enzyme phytase which hydrolyzes phytate into simpler organic forms of P or inorganic P. Because phytate is the major form of P in the concentrate portion of dairy rations, incomplete hydrolysis of phytate in the rumen may limit P availability. While a body of research from previous decades documents complete hydrolysis of phytate in the rumen of lactating cows (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992), high-producing dairy cows consume more dry matter, and their diets contain less forage, as compared to the animals used in early studies (Phipps et al., 2000). The resulting higher dietary phytate intake and higher passage rate

of digesta due to high DMI may result in incomplete degradation of phytate P in the rumen. Other factors which have been shown to cause variation in the hydrolysis of phytate in the rumen are the type of grain, processing of feedstuffs, and supplementation with exogenous enzymes (Park et al., 1999; Bravo et al., 2003; Kincaid et al., 2005).

In interpreting earlier work on phytate digestion by ruminants, limitations in methods used to quantify phytate P in feces must also be considered. Classical methods are based on the precipitation of phytate as an insoluble iron-phytate complex but these methods are too insensitive for low phytate samples (Wheeler and Ferrel, 1971). Also, the complexity of feces with its undigested feed residues, partially digested microbial residues, and endogenous cellular material challenges traditional methods of phytate quantification. Within this matrix, phytate is complexed with minerals and other nutrients. If recovery of fecal phytate is incomplete due to methodological limitations, fecal phytate may be underestimated in these earlier studies.

The initial step for the quantification of phytate P is the extraction of that form of P from the sample. Quantitative extraction of phytate from digesta and feces is more difficult than from feed because this phytate is always bound with cations, protein, or other nutrients to a greater extent than it is in feed and in monogastric manure samples, leading to the erroneous estimation and(or) damage to the analytical instruments. Factors affecting recovery of P-containing compounds in different extraction schemes include type of extractant, sample matrix, and sample handling. Of two commonly used extractants, HCl gives satisfactory recovery of total and phytate P in feed and monogastric digesta and feces (Skoglund et al., 1997; Leytem et al., 2008) but recovery of P is poor in the complex matrix of ruminant feces (McDowell et al., 2008). Alkaline extraction, most commonly with 0.25 M NaOH-0.05 M EDTA, gives almost complete recovery of total and various forms of P in feed and fecal samples (Turner, 2004; Toor et al.,

2005; McDowell et al., 2008) but is incompatible with HPIC quantification method because the alkaline extract causes column damage and interference from the matrix of the extracted sample. Thus, phytate in alkaline extracts is quantified using **^{31}P NMR** (Nuclear Magnetic Resonance), but this method has shortcomings including long analysis time, relatively high cost, and insensitivity to low phytate samples such as ruminant feces. High performance of ion chromatography is compatible with acid extraction but recovery of total and phytate P from ruminant feces is not complete with acid extraction.

Development of robust, inexpensive, and reproducible techniques for accurate quantification of undigested phytate in complex animal manure and digesta samples is essential to advance knowledge of the effects of diet on phytate and P excretion in ruminants (Turner et al., 2002). Consequently, the objectives were to identify and evaluate an extraction method that adequately recovers phytate from feed, ruminant digesta, and fecal samples and is fully compatible with HPIC quantification.

Materials and Methods

Sample Collection

For this method development project, dairy heifers (n=2) and cows (n= 2 dry and 2 lactating) were fed two or three different diets in sequential periods. Diets were designed to generate coordinated feed, digesta, and fecal samples of widely varying composition to challenge extraction and quantification methods. The experiment was carried out according to procedures approved by the Virginia Tech Animal Care and Use Committee.

Animals were fed experimental diets (Table 3-1) in 10 d periods; 7 d of diet adaptation and 3 d for sample collection. On d 8 to 10, fecal grab samples were collected twice daily, pooled by diet, and frozen (-20°C). Lactating and dry cows were ruminally cannulated, allowing

simultaneous collection of omasal samples using a vacuum pump (Huhtanen et al., 1997). Total mixed rations, concentrate mixes, and individual grain and forage samples were collected once per period and stored frozen (-20°C). Diet sequence was fixed and diets were specific to a class of animals (heifers, dry cows, lactating cows) because the focus was on generating a diverse sample set rather than evaluating the effect of animal, period, or diet.

Sample Preparation and Conventional Analysis

All samples were oven-dried at 55°C to constant weight and ground with a Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA). Dried samples were analyzed in duplicate for NDF, ADF, and ADL (Van Soest et al., 1991), starch (Hall, 2009), total Kjeldahl N (TKN), Ca, P, Mg, Al, Fe, and K (AOAC, 1984). Fiber analysis was sequential and NDF was quantified using amylase and sodium sulfite. Samples were ground further through a 0.2 mm sieve (Z Grinder) and stored in air-tight containers for further analysis. A subset of fecal samples was thawed at room temperature and extracted wet (described below).

Extraction, Extract Clean-up, and Phytate Analysis

Chemical Extraction. Samples were subjected to one of four extraction protocols: 0.5 M HCl, 1 M HCl, 0.25 M NaOH-0.05 M EDTA, and sequential extraction (0.5 M HCl extraction followed by 0.25 M NaOH-0.05 M EDTA). For the extraction of dry samples, 1 g of dried ground sample was shaken with 20 mL of extractant on a horizontal shaker continuously for 4 h at room temperature. Then, samples were centrifuged at 30,000 × *g* for 15 min at 4°C and supernatants decanted into acid-washed vials. Samples were extracted in triplicate, and for each replicate extract, duplicate 5 mL aliquots of supernatant were analyzed for total P (AOAC, 1984). The 0.5 M HCl and 0.25 M NaOH-0.05 M EDTA extracted P more efficiently than did

the other two extraction methods, and these two were used for further experimentation focused on phytate quantification by high performance ion chromatography (**HPIC**).

Clean-up of Acid Extracts. Acidified extracts were categorized by sample matrix (high protein and starch feed samples vs. high fiber fecal and digesta samples) to determine appropriate clean-up procedure. To remove proteins and starch, grain and TMR sample extracts were mixed 1:1 with a 20% w/v NaCl solution and stored overnight at 4°C. Salt-treated extracts were centrifuged at 30,000 × *g* for 20 min at 4°C and supernatant was decanted to acid-washed vials. After centrifugation of salt-treated extracts, clear supernatants were diluted with deionized distilled water at 1:5 to bring the phytate concentration within the calibration range of the HPIC, and passed through a 0.2 µm ion chromatography (IC) membrane (filter) into Dionex sample vials for HPIC analysis.

Forage, omasal, and fecal sample extracts, neither salt-treated nor diluted, were centrifuged at 30,000 × *g* for 20 min at 4°C and supernatant was decanted to acid-washed vials. After centrifugation, clear supernatants were passed through a methanol-conditioned C_{18} cartridge (Waters, Milford, MA) and then through a 0.2 µm IC membrane (filter) into Dionex sample vials discarding the first 2 mL of supernatant. The C_{18} cartridge has a hydrophobic silica-based binding phase capable of adsorbing analytes even with poor hydrophobicity from solutions.

Clean-up of Alkaline Extracts. Alkaline extracts were again categorized by sample matrix (high protein and starch feed samples vs. high fiber fecal and digesta samples) to determine appropriate clean-up procedure. Alkaline extracts of grain and TMR samples were acidified with HCl-HF acid solution (6 M HCl and 1.2 M HF) by adding 500 µL of HCl-HF acid solution to 5 mL sample extract. Acidified extracts were stored overnight at 4°C and then

centrifuged at 30,000 × *g* for 20 min at 4°C. After centrifugation of acidified extracts, clear supernatants were collected and diluted with deionized distilled water at 1:5 to bring the phytate concentration within the calibration range of the HPIC. The diluted extracts were then passed through a methanol-conditioned C_{18} cartridge before passing through 0.2 μm IC membrane (filter) into Dionex sample vials for IC analysis. The first 2 mL of supernatant was discarded.

Forage, omasal, and fecal sample extracts were acidified with the HCl-HF acid solution as above and then centrifuged at 30,000 × *g* for 20 min at 4°C; supernatant was decanted to acid-washed vials. Supernatants of each sample were passed through a methanol-conditioned C_{18} cartridge (Waters, Milford, MA) and then through a 0.2 μm IC membrane (filter) into Dionex sample vials discarding the first 2 mL of supernatant.

Spike Recovery. To evaluate extraction efficiency and accuracy of IC analysis, spike recovery tests were performed on selected low-phytate samples. Dried ground forage and omasal samples (1 g DM in triplicate) were spiked using 1 mL of phytate solution (100 μg phytate-P/mL) while fecal samples (higher in phytate than forage and omasal samples) were spiked using 1 mL of higher concentration phytate solution (300 μg phytate-P/mL). Spiked samples were subjected to the same extraction, extract clean-up, and analysis protocols described above. Samples were allowed to sit for 1 h after spike addition to improve the assimilation of spiking solution into the sample matrix.

High Performance Ion Chromatography Analysis. Chromatographic quantification of phytate in pre-treated samples was achieved by HPIC (Dionex ICS 3000 with an HPLC pump, a Dionex 4 × 50 mm IonPac AG7 guard column and a 4 × 250 mm IonPac AS7 analytical column; Dionex, Sunnyvale, CA) using post-column reaction detection. Treated sample extracts were injected and phytate (inositol hexaphosphate) was eluted at 7.2 min with isocratic elution (0.25

M HNO_3) at a flow rate of 1 mL per min. Eluted phytate was mixed with 0.1% $Fe(NO_3)_3$ in 2% $HClO_4$ solution in a post column reactor. The phytate products were measured at 290 nm on UV-VIS detector. Calibration standards (3, 15, and 30 μg P/mL) were prepared from phytic acid, sodium salt hydrate (Sigma).

Statistical Analysis

Total P extraction efficiency data were analyzed using PROC GLIMMIX of SAS (SAS Institute, 2002). The model included extractant, sample type, and their interaction as fixed effects. The model evaluating the effect of feces drying on total P extraction efficiency of different extractants included extractant, physical property, and their interaction as fixed effects. Significance was declared at $P < 0.05$.

Results and Discussion

Sample Properties

As intended, selected samples varied widely in their protein, starch, and fiber content (Table 3-2; Table 3-3). Feed CP content varied from 7.8% to 54.8% with standard deviation (SD) of 18.6%. The variability in starch content of feed samples was ±19.9% (SD). The fiber and lignin content of feed samples also varied between classes with high SD. Within class, the variability in sample properties was less in omasal and fecal samples in comparison to the feed samples. Sample CP and starch composition may influence efficiency of different extractants for total P or phytate because phytate forms stable complexes with protein and starch (Fontaine et al., 1946; Bourdillon, 1951). Furthermore, in plant feed sources the distribution of phytate varies with the type of plant and with the proportion of other nutrients (Lin et al., 2005). Therefore, different extractants may be needed for different sample types and also for samples within a type that vary in composition.

Content of mineral elements varied widely among the sample types (feed, omasal contents, and feces) as well as within the sample type (Table 3-2; Table 3-3). Among the major elements, K concentration was the highest followed by Ca and Mg in feed and omasal samples; Ca was predominant in fecal samples. Aluminum and Fe were higher in omasal and fecal samples than in feed samples. He et al. (2009) suggested that the appropriate extractants for P depends on sample characteristics including the association of P with Ca, Al, or Fe because adverse effects of these on the solubility of P or on P extraction efficiency have been reported for a variety of extractants (McDowell and Stewart, 2005; McDowell et al., 2008; He et al., 2009). McDowell and Stewart (2005) reported that Al and Fe were the major cations having an adverse effect on P solubility in dairy cattle feces, but in another study McDowell et al. (2008) reported Ca as the major cation affecting total P extraction in feces of dairy cattle. Ajiboye et al. (2007) reported Ca as the most prevalent cation regulating P solubility in biosolids and hog, dairy, beef, and poultry manure.

Total P concentration in dried samples varied widely within sample type and across sample class (Table 3-4). The variability in total P concentration of fecal and omasal samples can be attributed to combined effects of variation in feed ingredients, individual animal variation in digestion, and P utilization efficiency related to the production stage of animals. In feed samples, total P concentration ranged from 1.6 to 14.8 (SD = 3.6) mg/g DM. Commonly used feed ingredients contain total P ranging from 2.3 to 11.6 mg/g DM (National Research Council, 2001). Total P concentration in feces ranged from 5.6 to 12.0 mg/g DM (Table 3-4), similar to values reported in the literature (4.6 to 14 mg/g DM; Dou et al., 2003; Ajiboye et al., 2007; McDowell et al., 2008). Total P concentration in omasal samples was higher than in feed and feces (12.9 to 21.6 mg/g DM) because of the incorporation of inorganic P into the flow of P from

the rumen through the release of recycled salivary P into the rumen (Valk et al., 2002; Puggaard et al., 2011). Also, absorption of organic compounds such as volatile fatty acids (VFAs) from the rumen concentrating P in the omasal contents.

Efficiency of Extraction of Total P

The efficiency of total P extraction from dried samples varied with extractant, sample type, and the interaction of extractant and sample type (Table 3-5). Alkaline extraction (NaOH-EDTA) was highly effective in extracting P from all sample types, and the extraction efficiency was generally lower with 0.5 M HCl than with other extractants (Table 5). The P in omasal contents (largely inorganic) was efficiently extracted by all extractants. The most commonly used acid and alkaline extractants are HCl and NaOH-EDTA, respectively, and water and $NaHCO_3$ are often added for sequential extraction procedures (Dou et al., 2000). Little direct comparison of different extractants used individually to extract total P in feed, digesta, and fecal samples from dairy cows has been reported. In a recent study, McDowell et al. (2008) used acid and alkaline extractants (0.012 M HCl and 0.25 M NaOH-0.05 M EDTA) to extract P from feces of dairy cows and observed higher total P recovery in alkaline extracts than in HCl extracts.

Effectiveness of Acid Extraction in Different Types of Samples. Acid extraction was highly effective for omasal samples (Table 3-5) because of the dominance of salivary (inorganic) P in that sample type. In contrast, HCl extracted only 75-79% of the P in feces, similar to the 61 to 86% of total P extracted from dairy fecal samples with dilute HCl (0.012 M) by McDowell et al. (2008). With repeated extractions of dairy feces with 1 M HCl recovery of P was maximized at 90% (Dou et al., 2000). In the current study the two concentrations of HCl (0.5 and 1 M) were equally effective in omasal samples (and equally ineffective in feces, Table 3-5). The HCl extractant was more effective in feed samples than in fecal samples, perhaps because P is mostly

present as salts of K, Mg, and Ca in feed samples but is present also as P complexed with Al and Fe in feces (McDowell et al. 2008). Hydrochloric acid extractable P was thought associated with Ca while NaOH-EDTA extractable P was considered to be associated with Al and Fe in feces (McDowell and Stewart, 2005). The greater recovery of total P from feces was in alkaline extraction than in acid extraction and therefore support the hypothesis that more P was associated with Al, and Fe than with Ca in feces. Within feed samples, the HCl extraction was more effective in forage samples (average 90.2%) than in grains and TMRs (average 80.6%). In most studies, 1 M HCl is used as a step of sequential extraction of total P, and the 0.5 M concentration is used for phytate quantification studies using HPIC.

Effectiveness of Alkaline Extraction in Different Samples. The 0.25 M NaOH-0.05 M EDTA extractant extracted nearly all P from feces (94.1% of total P; Table 5) as has been reported by others (Toor et al., 2005; McDowell et al., 2008). The efficiency of extraction of P from feeds with the alkaline extractant was also essentially complete (95.2% for forage; 98.1% for grains and TMR) and higher than reported by McDowell et al. (2008; 60-87% recovery of total P in extracts of grain, forage, and TMR).

Polyvalent cations in the sample affect P extraction efficiency and should be considered when choosing the extractant. The Ca content of feeds may challenge alkaline extraction (Chapuis-Lardy et al., 2004; McDowell et al., 2008) because NaOH is less efficient in extracting Ca than is HCl and Ca-P complexes influence solubility of P (Turner and Leytem, 2004; Ajiboye et al., 2007). Likewise, the Al and Fe content of feces affects recovery of P with alkaline extraction. The use of EDTA to chelate the polyvalent cations can help resolve these problems. In cattle manure, improved recovery of total P was reported with the use of NaOH-EDTA instead of NaOH alone (80% vs. 32%; Turner, 2004). Very low recovery of total P in 0.25 M NaOH-

0.05 M EDTA extract of cattle feces was reported by McDowell and Stewart (2005); this poor recovery was attributed to high concentrations of Al and Fe in their samples (8,500 and 4,300 mg/kg DM respectively), so high that these minerals could not be fully chelated by EDTA. Samples in the current study were comparatively low in Al and Fe (888 and 1,035 mg/kg DM mg/kg DM respectively; Table 3-2, Table 3-3).

Sequential Extraction. For both feed and fecal samples, lower P extraction efficiency was observed with sequential extraction than with alkaline extraction (0.25 M NaOH-0.05 M EDTA) alone (Table 3-5). Sequential extraction (acid followed by alkaline, with or without water and $NaHCO_3$) is the most common approach to fractionating P forms in soil, animal manure, and other organic amendments when the research purpose is to address the issue of environmental pollution from P runoff (Sharpley and Moyer, 2000; Hansen et al., 2004; McDowell and Stewart, 2005). In this study, we used HCl extraction followed by NaOH-EDTA extraction as the sequential extraction scheme and omitted the H_2O and $NaHCO_3$ steps because our interest was to select the best two extractants for complete recovery of total P rather than identifying different fractions of P.

Effect of Drying Fecal Samples on Extraction Efficiency

Drying did not affect the efficiency of P extraction from feces (Table 3-6). The variability in extraction efficiency within extractant was higher in wet feces compared with dry feces, possibly due to the errors inherent in mixing and weighing wet fecal samples. Also, the interaction of the extractant and sample matrix may differ between dry and wet samples.

In most studies, observed changes with drying are usually transformation between different P forms rather than changes in total extractable P. For instance, McDowell et al. (2008) did not find any significant effect of drying on total P recovery from dairy feces but reported

increased molybdate-reactive P (mostly inorganic phosphate) in NaOH-EDTA extracts of dry feces relative to wet feces; the opposite was observed in water extracts. EDTA nullifies any effect of drying on total P extractability because it acts as a chelating agent on humic compounds. In contrast, McDowell and Stewart (2005) observed that when used as a step of sequential extraction, NaOH recovered less total P from dry cattle feces than from fresh feces probably because of increased hydrophobicity as a result of rearrangement of humic compounds. Chapuis-Lardy et al. (2004) observed that oven drying at 65°C reduced water extractable inorganic P. Due to the inconsistent pattern of extraction efficiency in dry and wet feces for different extractants and the difficulty in storing wet feces, most studies use dry feces for convenience and reduced variation. Our results support that preference.

Phytate Analysis by High Performance Ion Chromatography (HPIC)

Based on the total P recovery data two extractants, 0.5 M HCl and 0.25 M NaOH-0.05 M EDTA, were selected for phytate quantification. The NaOH-EDTA extraction was chosen because it yielded almost complete recovery of total P and the 0.5 M HCl extraction was used to reflect easily soluble P, likely available for absorption by the animal . Given the lack of improvement in total P recovery with sequential extraction and the increased opportunity for analytical error, sequential extraction was not explored further.

Phytate concentration in HCl and NaOH-EDTA extracts of feed ranged from <5 to 10-11,000 µg/g DM (Table 3-7) with values similar to previously reported for comparable human foods and livestock feeds (Eeckhout and Paepe, 1994; Talamond et al., 2000; McDowell et al., 2008). Phytate was also detectable in HCl and NaOH-EDTA extracts of omasal and fecal samples but at lower concentrations (<5 to 80-100 µg/g DM and <50 to <400 µg/g DM respectively; Table 3-8).

Spike Recovery. In the spike recovery tests, the recovery of added phytate from spiked samples was almost complete with 0.25 M NaOH-0.05 M EDTA extraction (88-106% of spiked phytate; Table 3-9). Recovery of spiked phytate was not complete in many samples with HCl extraction. Poor recovery of spiked phytate with HCl extraction was observed primarily with low-phytate samples, likely due to a complex interaction among phytate, extractant, and sample matrix. Complete recovery (95-105% of spiked phytate) of phytate spiked to food samples was reported with HCl extraction and HPIC analysis (Skoglund et al., 1997; Brooks and Lampi, 2001; Chen, 2004), although in these studies inositol hexaphosphate (**IP6**) spikes were added after extraction, so evaluated just analytical efficiency rather than extraction efficiency. The current study utilized pre-extraction spikes (spikes added to dried ground samples before extraction). The full recovery of phytate spiked to samples pre-extraction suggests both the absence of any adverse effect of the alkaline extraction on phytate and also the applicability of 0.25 M NaOH-0.05 M EDTA extraction for phytate quantification using HPIC.

Utility of HPIC vs. NMR. High performance ion chromatography and high performance liquid chromatography (**HPLC**) have previously been used to quantify phytate in monogastric feces and digesta (Leytem et al., 2008), though NMR analysis is sometimes used. Leytem et al. (2008) found HPLC (acid extraction) to yield comparable results to NMR (alkaline extraction) when quantifying IP6 in broiler ileal digesta. For ruminant feces, ^{31}P NMR is typically used to analyze NaOH-EDTA extracts for various P-containing compounds (Toor et al., 2005; McDowell and Stewart, 2005; McDowell et al., 2008). McDowell et al. (2008) used 0.012 M HCl as extractant in feed and feces from dairy cows prior to ^{31}P NMR analysis but mandatory pH adjustment to an alkaline pH before NMR analysis caused precipitation of certain P fractions with polyvalent cations leading to underestimation of some P forms.

Although ^{31}P NMR provides a full spectrum of different P forms in one run with just a single extraction, this technique is relatively insensitive and more expensive than HPLC or HPIC. Another disadvantage to NMR is that identification of inositol phosphates is difficult due to poor resolution in orthophosphate monoester region of NMR spectra; this compromises the separation of inositol phosphate esters from other orthophosphate monoesters leading to overestimation of IP6. This problem can be overcome by using deconvolution software but this requires considerable expertise. Due to its greater sensitivity, relative simplicity, and lower cost, HPIC is preferred to ^{31}P NMR for quantification of different P forms in a wide range of samples.

Special Problems with Soybean Meal. While NaOH-EDTA was at least as effective as 0.5 M HCl in extracting phytate from most samples, SBM appears to pose a special problem (Table 3-7). When quantified using HPIC, the phytate concentration in NaOH-EDTA extracts of SBM samples was lower than in HCl extracts. This may be attributed to the acidification of 0.25 M NaOH-0.05 M EDTA extracts before chromatographic analysis rather than to inefficiencies of the alkaline extraction *per se*. Acidification of alkaline extracts is necessary to make extracted samples compatible with chromatographic analysis but in SBM it may cause the formation of insoluble complexes between phytate and polyvalent cations and(or) soy proteins. We speculate that the NaOH-EDTA extractant releases protein not only from phytate-protein complexes in the sample but also from complexes of protein with other non-phytate compounds. Also the alkaline extract would theoretically be able to interrupt metal-ligand coordination within proteins and break their H bonds and physical molecular forces, possibly even covalent bonds. To the eye, the supernatant of the alkaline extracts were obviously not true solutions with simple salts but instead contained colloids. When the alkaline extracts of SBM were acidified in preparation for HPIC, the protonated (H^+) sites on proteins in the extract likely formed insoluble complexes with

the (negatively charged) phytate in the extract. Precipitate was visually obvious in the alkaline extracts following acidification and we hypothesize that the precipitate was comprised of insoluble protein-phytate complexes, removing phytate from the solution.

This is a problem specific to SBM because, in soy, more phytate is associated with protein than with Ca and Mg (Brooks and Morr, 1982). Tzeng et al. (1990) reported that proteins and metal ions compete with each other to form complexes with phytic acid in acidic media. Hartman (1979) reported that in pH range of 2 to 4.5, phytate removal from SBM extract was inefficient due to strong phytate-protein complexes; phytate can be displaced from this complex only in the presence of excess divalent cations.

Thus the apparently low recovery of phytate from SBM with alkaline extraction in our study was likely due to problems imposed with the acidification step of the post-extraction treatment. Therefore, although it gave incomplete recovery of total P (Table 3-4, Table 3-5), we conclude that HCl extraction is still preferred for SBM samples unless alkaline extracts can be made compatible with HPIC in some way other than acidifying the extract. More research is needed with other high protein feeds to evaluate this hypothesis.

Conclusions

A method is described for analysis of phytate in ruminant digesta and fecal samples consisting of extraction of samples, extract clean-up, and HPIC analysis. Alkaline extraction was able to extract P almost completely from all sample types; acid extraction was adequate for feeds and omasal samples but not for feces. The described extract clean-up procedures successfully removed the interference from sample matrix allowing accurate measurement of low concentrations of phytate in fecal and digesta samples from dairy cattle with HPIC. Soy samples may pose special problems when alkaline extracted because of protein-phytate interactions in the

extract. The described method combines the extraction power of NaOH-EDTA with the precision and sensitivity of HPIC quantification to measure phytate in feeds, ruminant digesta, and feces. This method is simple, rapid, accurate, and robust and may help advance knowledge of dynamics of digestion of phytate and P in ruminants.

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Table 3-1. Ingredient composition of experimental diets

	Mixed ration 1	Mixed ration 2	Mixed ration 3	Mixed ration 4	Mixed ration 5	Mixed ration 6[1]	Hay
Fed to	Lactating cows	Lactating cows	Lactating cows	Heifers	Heifers	Dry cows	Dry cows
Ingredient				*% of diet DM*			
Grass hay	-	9.03	-	71.3	71.3	64.0	100
Alfalfa haylage	-	20.1	-	-	-	-	-
Alfalfa silage	9.24	-	11.6	-	-	-	-
Corn silage	35.9	27.3	48.2	8.83	8.67	21.6	-
Barley straw	-	-	1.89	-	-	-	-
Grain mix 1[2]	54.8	-	-	-	-	-	-
Grain mix 2[3]	-	43.6	-	-	-	-	-
Grain mix 3[4]	-	-	38.4	-	-	-	-
Grain mix 4[5]	-	-	-	19.9	-	-	-
Grain mix 5[6]	-	-	-	-	20.0	-	-
Grain mix 6[7]	-	-	-	-	-	14.4	-

[1]Dry cows were pen fed and reported proportions of dietary ingredients are approximate.

[2]Grain mix 1 contained 60.3% corn, 28.0% soybean meal, 3.66% soybean hulls, 4.33% tallow, 1.67% limestone, 1.00% sodium bicarbonate, 0.33% salt, 0.33% urea, 0.17% potassium magnesium sulfate, 0.13% monoammonium phosphate, 0.04% Se, 0.03% mineral premix, 0.03% vitamin ADE, 0.01% vitamin E.

[3]Grain mix 2 contained 64.4% corn, 33.1% cottonseed meal, 1.81% limestone, 0.50% trace mineral mix, 0.16% vitamin ADE.

[4]Grain mix 3 contained 13.27% cottonseed meal, 13.27% soybean meal, 11.75% corn distillers whiskey (dehydrated), 7.5% corn, 15.33% hominy, 15.16% wheat middlings, 11.63% citrus pulp (dehydrated), 2.07% megalac plus, 1.60% cane molasses (dehydrated), 0.63% tallow, 2.11% limestone, 2.33% pro-lak, 0.42% urea, 0.63% salt, 0.42% magnesium oxide, 0.42% potassium magnesium sulfate, 1.26% sodium bicarbonate, 0.07% Se (600), 0.06% trace mineral premix, 0.04% vitamin ADE, 0.04% vitamin A.

[5]Grain mix 4 contained 50.25% corn, 49.3% soybean meal, 0.35% trace mineral mix, 0.10% vitamin ADE.

[6]Grain mix 5 contained 61.64% corn, 37.91% cottonseed meal, 0.35% trace mineral mix, 0.10% vitamin ADE.

[7]Grain mix 6 contained 39.9% corn, 29.9% soybean meal, 12.5% soybean hulls, 11.5% wheat middlings, 3.03% limestone, 1.00% salt, 0.50% magnesium oxide, 0.50% magnesium sulfate, 0.28% Se (0.06%), 0.13% trace mineral premix, 0.13% vitamin ADE, 0.27% vitamin E.

Table 3-2. Nutrient content of feed samples

Sample ID	CP	Starch	NDF	ADF	ADL	Ca	K	Mg	Al	Fe
				% of DM					*mg/kg DM*	
Grains										
Soybean meal sample 1	52.1	7.6	21.6	6.34	3.01	0.35	2.59	0.31	545	230
Soybean meal sample 2	54.8	7.1	39.3	6.98	0.59	0.47	2.59	0.36	37	120
Soybean meal sample 3	50.7	6.9	29.8	8.73	1.58	0.43	2.51	0.37	752	564
Corn	9.5	69.5	10.6	2.71	0.12	0.01	0.39	0.12	14	32
Cottonseed meal	50.2	4.3	46.7	22.2	13.7	0.28	1.84	0.81	58	237
Grain mix 3	22.3	23.8	26.8	10.7	5.57	1.57	1.16	0.62	128	352
Grain mix 6	19.1	36.1	31.4	15.5	2.09	1.49	1.17	0.70	255	495
Forages										
Alfalfa hay	17.4	1.1	54.9	40.9	14.0	1.07	2.30	0.27	592	763
Grass hay	10.8	1.7	70.5	41.4	7.74	0.40	2.64	0.19	363	366
Corn silage sample 1	7.8	33.6	42.4	22.1	1.13	0.26	1.32	0.22	29	163
Corn silage sample 2	8.2	38.9	36.4	19.1	1.95	0.19	1.14	0.15	51	142

Mixed rations										
Mixed ration 1	15.3	29.3	31.7	16.2	3.72	0.80	1.4	0.29	237	312
Mixed ration 2	19.7	29.7	32.1	17.8	2.93	0.73	1.36	0.33	225	324
Mixed ration 3	15.6	24.6	38.7	20.2	4.15	1.36	1.37	0.59	1,112	1,047

Table 3-3. Nutrient content of digesta and feces samples

Sample type	Diet	CP	Starch	NDF	ADF	ADL	Ca	K	Mg	Al	Fe
					% of DM					*mg/kg DM*	
Omasal contents											
Dry cow	Mixed ration 6	17.6	0.9	36.9	19.8	8.70	0.52	3.90	0.27	1,515	935
Dry cow	Grass hay only	14.2	0.9	44.2	25.2	12.0	0.65	8.90	0.28	521	359
Lactating cow	Mixed ration 1	31.9	1.9	49.3	28.3	13.4	1.02	3.24	0.37	441	538
Lactating cow	Mixed ration 2	29.3	1.4	55.8	30.3	18.3	0.80	2.37	0.41	316	467
Lactating cow	Mixed ration 3	22.6	1.0	36.7	19.7	9.40	1.02	3.11	0.42	233	362
Feces											
Heifer	Mixed ration 4	19.3	5.8	41.6	22.6	6.09	1.42	1.43	0.80	714	808
Heifer	Mixed ration 5	15.2	16.4	51.5	32.2	6.84	0.89	1.30	0.56	468	701
Dry cow	Mixed ration 6	15.8	1.1	59.6	33.3	9.89	1.69	1.23	0.67	1,902	1,608
Dry cow	Grass hay only	11.2	0.7	59.3	35.7	18.1	1.06	0.85	0.46	952	776
Lactating cow	Mixed ration 1	16.7	1.2	50.6	27.9	8.47	2.28	0.80	0.66	946	1,340
Lactating cow	Mixed ration 2	17.5	9.7	54.7	31.4	7.60	2.14	0.68	0.71	743	1,110

Lactating cow	Mixed ration 3	16.5	1.4	53.5	30.7	7.78	2.33	0.71	0.84	492	903

Table 3-4. Total P content and P extraction efficiency of feed, digesta, and feces samples with HCl, NaOH-EDTA, and sequential extraction

	Total P	0.5 M HCl	1 M HCl	NaOH-EDTA	Sequential
	μg/g DM	*Extraction efficiency, % of total P*			
Grains					
Soybean meal sample 1	7,157	66.8	77.9	97.6	82.8
Soybean meal sample 2	8,467	71.3	76.3	95.6	84.0
Soybean meal sample 3	7,900	65.4	78.8	95.1	83.8
Corn	3,065	89.4	91.0	90.8	94.5
Cottonseed meal	14,794	82.0	88.8	96.4	89.5
Grain mix 3	7,289	89.0	95.5	104.2	98.2
Grain mix 6	5,233	76.5	81.1	93.0	86.4
Forages					
Alfalfa hay	2,915	92.0	89.4	92.6	85.3
Grass hay	2,395	99.6	103.9	103.1	89.1
Corn silage sample 1	1,632	91.8	93.3	89.5	87.4

Corn silage sample 2	2,327	77.4	84.9	95.7	75.8
Mixed rations					
Mixed ration 1	4,296	94.4	98.9	107.7	99.7
Mixed ration 2	4,988	90.8	92.7	101.7	92.9
Omasal contents					
Dry cow fed mixed ration 6	19,662	95.4	97.7	100.8	100.6
Dry cow fed grass hay only	21,582	95.8	94.9	96.7	98.2
Lactating cow fed mixed ration 1	12,864	93.0	91.9	97.2	97.3
Lactating cow fed mixed ration 2	16,309	95.9	95.9	100.0	96.6
Lactating cow fed mixed ration 3	18,394	92.4	94.3	96.5	95.8
Feces					
Heifer fed mixed ration 4	12,022	84.2	88.1	97.0	89.6
Heifer fed mixed ration 5	9,034	78.6	74.7	93.3	79.0
Dry cow fed mixed ration 6	7,019	69.5	76.4	97.8	80.1
Dry cow fed grass hay only	6,143	62.9	67.3	84.9	67.4
Lactating cow fed mixed ration 1	5,623	73.7	72.6	92.7	75.3

Lactating cow fed mixed ration 2	7,438	75.4	84.6	100.0	91.6
Lactating cow fed mixed ration 3	8,955	82.0	87.4	92.9	85.5

Table 3-5. Phosphorus extraction efficiency from four classes[1] of feeds with HCl, NaOH-EDTA, and sequential extraction

	Extraction efficiency, % of total P[2]			
	0.5 M HCl	1 M HCl	NaOH-EDTA	Sequential
Grains and mixed rations	80.6 ± 0.92[gf]	86.8 ± 0.92[de]	98.1 ±0.93[a]	93.3 ± 0.95[ab]
Forages	90.2 ± 1.38[bcd]	93.2 ± 1.41[abc]	95.2 ± 1.38[ab]	87.1 ± 1.41[cde]
Omasal contents	94.5 ± 1.23[ab]	95.0 ± 1.23[ab]	98.2 ± 1.23[a]	98.6 ± 1.23[a]
Dry feces	75.2 ± 1.05[h]	78.7 ± 1.04[gh]	94.1 ± 1.04[ab]	84.1 ± 1.04[ef]

[1]Classes are groupings of the feeds described in tables 1 and 2. Values reported are LS means and SEM.

[2]Values with different superscripts are significantly different ($P < 0.05$).

Table 3-6. Effect of drying on extraction efficiency of total P in feces

	Extraction efficiency, % total P[1]			
	0.5 M HCl	1 M HCl	NaOH-EDTA	Sequential
Dry feces	75.2 ± 2.04[d]	78.7 ± 2.01[cd]	94.1 ± 2.01[a]	84.1 ± 2.01[bc]
Wet feces	72.4 ± 2.04[d]	80.3 ± 2.03[cd]	90.1 ± 2.01[ab]	91.1 ± 2.7[ab]

[1]Values with different superscripts are significantly different ($P < 0.05$).

Table 3-7. 0.5 M HCl and 0.25 M NaOH-0.05 M EDTA extractable phytate P in feed samples

Class	Feed	HCl Phytate P, μg/g DM	NaOH-EDTA Phytate P, μg/g DM
Grain	Soybean meal 1	4,729	3,831
Grain	Soybean meal 2	5,282	4,237
Grain	Soybean meal 3	3,630	4,047
Grain	Corn	2,567	2,577
Grain	Cottonseed meal	10,871	10,520
Grain	Dry cow pellet	3,384	3,662
Grain	Lactating cow pellet	4,457	4,311
Forage	Alfalfa silage	6	15
Forage	Grass hay	22	39
Forage	Corn silage sample 1	3	10
Forage	Corn silage sample 2	2	9
Mixed ration 2	For lactating cow, containing cottonseed meal, corn silage, and grass hay	2,377	2,855
Mixed ration 3	For lactating cow, containing cottonseed meal, SBM, alfalfa and corn silage	1,230	2,434

Table 3-8. 0.5 M HCl and 0.25 M NaOH-0.05 M EDTA extractable phytate P in digesta and feces samples

Sample type	Diet	HCl Phytate P, µg/g DM	NaOH-EDTA Phytate P, µg/g DM
Omasal contents			
Dry cow	Mixed ration 6	80	103
Dry cow	Grass hay only	11	5
Lactating cow	Mixed ration 1	ND[1]	ND[1]
Lactating cow	Mixed ration 2	3	7
Lactating cow	Mixed ration 3	31	48
Feces			
Heifer	Mixed ration 4	176	231
Heifer	Mixed ration 5	270	369
Dry cow	Mixed ration 6	65	138
Dry cow	Grass hay only	39	68
Lactating cow	Mixed ration 1	57	95
Lactating cow	Mixed ration 2	167	196
Lactating cow	Mixed ration 3	237	324

[1]ND: not detected

Table 3-9. Effect of extraction and extract clean-up on the recovery of phytate spiked to samples

	HCl	NaOH-EDTA
	Phytate recovery, % of quantity spiked	
Alfalfa silage	29.7	88.0
Omasal contents		
Mixed ration 2 fed lactating cow	42.8	87.5
Mixed ration 3 fed lactating cow	97.2	90.4
Feces		
Mixed ration 5 fed heifer	81.5	105.5
Mixed ration 3 fed lactating cow	85.0	100.2

Chapter 4 DISAPPEARANCE OF INFUSED PHYTATE FROM THE LARGE INTESTINE OF DAIRY HEIFERS

Abstract

The objective of this study was to investigate the disappearance of phytate from the large intestine of dairy heifers. Uncertainty about the availability of phosphorus (P) in different feeds may limit implementation of dietary strategies to reduce fecal P excretion by dairy cows. Increased understanding of the dynamics of phytate degradation and disappearance of P in the large intestine may improve prediction of intestinal P digestion and absorption. Eight ruminally- and ileally-cannulated crossbred dairy heifers were used in two 4 x 4 Latin squares with 9 d periods including 3 d of washout. All heifers were fed a high forage diet containing 0.14% P throughout the study. Ytterbium (Yb)-labeled corn silage and Co-EDTA were dosed to the rumen four times daily as particulate and liquid phase markers, respectively, to measure ileal digesta flow. On d 4 to 7 of each period, each heifer was infused ileally with 0, 5, 15, or 25 g/d phytate (phytic acid) in solution and total fecal collection was conducted. When infusion ceased (d 8 and 9) ileal digesta was sampled to measure P flow to the ileum from the basal diet. Feed, digesta, and feces samples were dried, ground, and analyzed for phytate, inorganic P, and total P using high performance ion chromatography, inductively coupled plasma atomic emission spectroscopy (ICP-AES), and the molybdovanadate yellow method, respectively. Phytate degradation in the large intestine was observed but was not complete, and the amount of infused phytate did not influence the degradability of phytate. Fecal excretion of total P increased with increasing total P infused. There was net absorption of P from the large intestine and greater disappearance of P from dietary P flowing to the ileum than from the infused pure phytate (44 vs.

25%). This data will support mechanistic modeling efforts to improve prediction of P digestion allowing more accurate estimation of P bioavailability in feeds.

Key words: phytate digestion, phosphorus absorption, large intestine, dairy heifer

Introduction

Phosphorus (P) is required for almost all living organisms. In high producing dairy cows, P plays an important role in maintaining production and performance. Absorbed P is constantly secreted in milk irrespective of dietary P intake (Cohen, 1980; Knowlton et al., 2001). More than 80% of the total body P is present in bone. Bone mineral resorption plays a key role in regulating blood P, and replenishment of bone P is critical to maintain animal performance and production (Cohen, 1980; Peterson et al., 2005; Wu et al., 2001). Also P is part of the structural basis of cell membranes and nucleic acids.

The salient physiological characteristic of salivary P recycling in ruminants demands continuous and sufficient dietary P supply (Horst, 1986; Valk et al., 2002). In ruminants, a relatively constant proportion of blood P is recycled through saliva and not all the P absorbed from the digestive tract is available for animal use. Therefore, inadequate dietary P supply may compromise other physiological functions. Other physiological functions of P include rumen microbial growth, energy metabolism, and transport of fatty acids. In ruminants offered P-deficient diets, feed intake was depressed and ruminal digestion impaired (Bryant et al., 1959; Durand and Komisarczuk, 1988; Komisarczuk et al., 1987). Reduced ATP production and transfer in the rat myocardium during P depletion reflects the importance of the role of P in energy production and transfer, and similar relationships were observed in ruminants fed P-deficient diets (Brautbar et al., 1982; Louw, 1979; Riddell et al., 1933).

The use of inorganic P supplements has been reduced for last few decades because of increasing cost due to depletion of natural sources of phosphate (Leng, 2008). Also, P losses from farms, a problem exacerbated by excess dietary P, contribute to eutrophication of streams, lakes, and other water bodies. Dietary P content in dairy rations in the US typically exceeds the amount recommended by the NRC (Dou et al., 2003) making P supplementation unnecessary. But, care needs to be taken in formulation of low P diets to be sure sufficient P is supplied both for the rumen microbes and the cow.

Current NRC (2001) recommendations for dietary P supply are based on the requirement of absorbed P for milk production, maintenance, growth, and reproduction. Specific absorption coefficients were assigned for each source of inorganic P, but fixed values were assigned for forages (0.64) and concentrates (0.70) and these are based on relatively sparse research. The single availability coefficient for forages and for grains ignores the observation that ruminal P solubility and(or) P availability is greatly influenced by type of feed or feed processing (Bravo et al., 2003; Kincaid et al., 2005). Using these fixed absorption coefficients may limit accurate calculation of dietary P supply.

Ruminants are more efficient than non-ruminants in extracting P from phytate, the major form of P in grains and cereals. While the expression of the enzyme phytase by ruminal microorganisms allows ruminants to hydrolyze phytate (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992), phytate degradation by ruminants may be incomplete in certain dietary situations (Park et al., 1999; Park et al., 2000; Bravo et al., 2002). Grain type, processing of feed ingredients, and supplementation with exogenous phytase have also been shown to influence ruminal phytase activity (Yanke et al., 1998) or phytate hydrolysis (Park et al., 2000; Bravo et al., 2003; Kincaid et al., 2005). In lactating cows, high phytate intake with feeding of high

concentrate diets and fast rate of digesta passage due to high DMI may result in flow of phytate P to the lower digestive tract.

The large intestinal degradation of phytate has been widely investigated in monogastrics (Williams and Taylor, 1985; Matsui et al., 1999; Marounek et al., 2003) but data describing the amount and fate of phytate flowing to the large intestine in ruminants are scarce. Large intestinal phytate degradation is attributed primarily to microbial phytase activity. Though the bacterial population in the large intestine is not exactly same as the rumen, some species are in common (Maki and Picard, 1965; Yanke et al., 1998) and the total viable count of bacteria per g of wet weight in cecal and colon digesta collectively was the same as in ruminal digesta (Edwards et al., 2005). Therefore, phytate digestion in the large intestine seems likely. In sheep fed a 50:50 forage:concentrate diet, daily passage of inositol phosphates through the upper large intestine was less than through the small intestine, likely due to microbial phytase activity in sheep large intestine (Park et al., 2002).

The release of inorganic P due to phytate hydrolysis is of nutritional significance only if P is absorbed from the large intestine. Though the extent is highly variable, disappearance of P from the ruminant large intestine has been reported (Breves and Schroder, 1991; Scharrer, 1985). We hypothesized that phytate entering the large intestine of ruminants can be hydrolyzed, and the released inorganic P absorbed. The objective of this study was to quantify phytate P degradation in the large intestine of dairy heifers and to estimate the disappearance of P from the large intestine. The data from this experiment may support more accurate prediction of feed P availability for ruminants.

Materials and Methods

Animals and Experimental Design

Eight crossbred [Swedish Red or Brown Swiss × (Holstein × Jersey)] heifers averaging 420 kg BW were fitted surgically with a half T-type ileal cannula made of tygon (5 to 6 cm anterior to ileo-cecal junction) and a ruminal cannula (Bar Diamond, Inc., Parma, ID) 60 d and 21 d prior to the experiment, respectively. All surgeries were conducted aseptically using local anesthesia. Ileal cannulas were checked daily to ensure fixed position and cleaned twice a week.

Heifers were in late gestation period and were housed in a freestall barn with constant access to water and fed once daily using Calan doors. A high forage diet (91% forage; Table 4-1) was formulated to contain very low total P (0.14% of dietary DM) to minimize the amount of organic P reaching the ileum allowing unconfounded measurement of disappearance of the infused phytate. Diets were formulated to meet NRC (2001) recommendations for heifers weighing 420 kg for all nutrients except P. Feed was offered ad libitum during a 21 d diet acclimation period. To maintain constant DM proportions of forage and concentrate in diet, the DM content of corn silage was measured weekly and the amount of forage adjusted accordingly. After 21 d of diet acclimation, heifers were moved to individual tie stalls in a metabolism unit for infusion and sampling.

Heifers were randomly assigned to one of four treatment sequences in a duplicated Latin square design with four, 9 d periods. Treatments were infusion of 0, 5, 15, or 25 g/d phytate (0, 1.41, 4.22, and 7.04 g/d P, respectively) in solution of total 960 mL/d (40 mL/h) using an infusion pump (Ardus Medical, Inc., Cincinnati, OH). Solutions of varying phytate-P concentrations were prepared using 50% (w/w) phytic acid solution (Sigma-Aldrich Co., St.

Louis, MO). The pH of infusion solutions were adjusted to neutral or mildly alkaline (7 to 8.1) before infusion.

A 7-d adjustment period to the metabolism stall was allowed before the start of the first period and then each period included 3 d of washout, 4 d of infusion, and 2 d of ileal sampling. During the washout periods heifers were fed at 0600 and 1800 h. During infusions, heifers were fed four times daily at 0600, 1200, 1800, and 2400 h. On day 3 of each period, heifers were fitted with urinary catheters to prevent mixing of feces and urine. Heifers were observed for signs of illness and rectal temperatures were recorded daily.

Ytterbium (Yb)-labeled corn silage and Co-EDTA were dosed daily as particulate and liquid phase markers, respectively, to measure ileal digesta flow. Dried corn silage retained on bottom sieve of Penn State Particle separator was labeled using $YbCl_3$ solution by the method described by Harvatine et al. (2002). Lithium Co-EDTA was prepared by the method of Uden et al. (1980). Markers were mixed with rumen digesta four times daily throughout the study at a rate of 0.11 g Yb or Co/heifer per d. All protocols and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Sample Collection and Preparation

Total mixed ration and feed refusals were sampled daily for the last 6 d of each period. Total fecal collection was conducted during the 4 d infusion (d 1 to 4 of each period). Every 24 h, water was added (50% of total feces weight) to increase feces homogeneity, and feces from each heifer was blended and sampled. When phytate infusion ceased (d 8 and 9) ileal digesta was collected through the ileal cannula four times a day (0600, 1200, 1800, and 2400 h) to quantify P flow to the ileum from the basal diet. All samples were stored at -20°C immediately following collection.

Frozen samples were later thawed, TMR samples composited by period, refusals composited by heifer x period, and ileal samples composited by heifer x day. Samples were oven dried (Wisconsin Oven, Memmert, Schwabach, Germany) at 55°C until constant dry weight was achieved. Dried samples were ground through Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA) and then through a Z grinder (0.2-mm screen; ZM 100 USA, Retsch, Haan, Germany). Ground samples were stored in air tight containers.

Sample Analysis

Dried ground feed samples were analyzed for N, total P (AOAC, 1984), and NDF and ADF sequentially (Van Soest et al., 1991). Dried ground fecal samples were analyzed for N and total P (AOAC, 1984), and ileal digesta samples were analyzed for total P (AOAC, 1984). Ileal digesta samples were analyzed for Yb and Co using Inductively Coupled Plasma Atomic Emission Spectroscopy (CirOS VISION model, SPECTRO Analytical Instruments Inc., Mahwah, NJ).

Ileal and fecal samples were analyzed for inositol hexaphosphate (IP6) and lower inositol phosphates (IP5, IP4, IP3) following the method of Ray et al. (2012) except that a different high performance ion chromatography (**HPIC**) quantification approach was used. In brief, dried ground (0.2 mm) samples were extracted using 0.25 *M* NaOH-0.05 *M* EDTA and then alkaline extracts were acidified with HCl-HF acid solution (500 µl of 6 *M* HCl and 1.2 *M* HF added to 5 mL sample extract). Acidified extracts were stored overnight at 4°C and centrifuged at 30,000 × *g* for 20 min at 4°C. Clear supernatants were collected and passed through methanol-conditioned C_{18} column (Sep-Pakplus, Waters, MA) and then through a 0.2 µm IC membrane (PTFE filter; IC Millex-LG, Fisher, PA) into Dionex sample vials (Dionex, Sunnyvale, CA) for HPIC analysis.

Chromatographic quantification of phytate (inositol hexaphosphate) in clear sample extracts was achieved by HPIC (Dionex ICS 3000 with a Dionex 4 × 50 IonPac AG7 guard column and a 4 × 250 mm IonPac AS7 analytical column; Dionex, Sunnyvale, CA) using pH 4 elution and post-column reaction detection (Rounds and Nielsen, 1993; Kwanyuen and Burton, 2005; Leytem et al., 2008). Treated sample extracts (50 µl) were injected into the column with an autoinjector. Phytate was eluted at 11.8 min with gradient elution (0.01 M Methylpiperazine and 0.01 M $NaNO_3$-0.01 M Methylpiperazine) at a flow rate of 1 mL per min. Eluted phytate was mixed with Wade's reagent (0.015% $FeCl_3$ + 0.15% sulfosalicylic acid solution) in a post column reactor and detected using UV-VIS detector by monitoring absorbance at 500 nm. Three IP6 standards (6, 30, and 60 µg P/mL) were prepared using sodium salt of phytic acid (Phytic acid, sodium salt hydrate powder, Sigma, St. Louis, MO). Within the range of calibration standards, the relationship between response and concentration was linear.

The combination of alkaline extraction and HPIC quantification (Ray et al., 2012) makes possible quantification of phytate and the lower IP (IP3, IP4, and IP5) in ruminant digesta and fecal samples in a single HPIC run using the same sample extract after a common post-extraction clean-up process. The elution time for IP5, IP4, and IP3 were 10.5, 8.9, and 6.8 min, respectively. Calculation of concentration of these lower IPs used two secondary standards (mixtures of inositol phosphates) prepared by hydrolyzing 50 mg sodium phytate in 5 mL of 6 M HCl at 140°C for 1 and 2 h, respectively. Pure standards of IP3, IP4, and IP5 were used to standardize the concentration to peak-area ratio in the two secondary standards for lower inositol phosphates and IP6. This ratio was used to calculate the concentrations of lower IP in unknown samples. The following equation was used to calculate lower IP concentration in samples:

$$Cs = \frac{Css}{Ass} \times As$$

where:

Cs = concentration of lower IP in sample extract

Css = concentration of lower IP in secondary standard

Ass = area of lower IP peak in secondary standard

As = area of lower IP peak in sample extract

Statistical Analysis

All intake, excretion, and disappearance data were analyzed using PROC GLIMMIX in SAS (SAS Institute, 2002). The model included square, infusion dose, and period as fixed effects. For excretion and disappearance data, basal ileal flow was used as covariate. Data for one cow were excluded from second period as the cow was suffering from ruminal stasis. Polynomial contrasts were used to test the effect of infusing increasing quantities of phytate (5, 15, and 25 g/d) on large intestinal phytate disappearance and fecal Pi excretion. Statistical significance was declared at $P < 0.05$. Treatment means were separated using a multiple comparison test following the Tukey-Kramer method.

Results and Discussion

Diet Composition, Intake, and Digestibility

As intended, quantified total P concentration in the diet averaged 0.14% (DM basis) across all periods (Table 4-1). Similarly, observed dietary phytate P concentration was very low (3.8% of dietary total P).

Heifers infused with saline or assigned phytate solution consumed an average of 7.7 kg DM/d, and DMI, fecal DM, and apparent DM digestibility was similar for all treatment groups

(Table 4-2). Average DM digestibility was 68.8% which is similar to the DM digestibility reported by others in growing heifers fed high forage diet (Moody et al., 2007). Total P intake (average 10.8 g/d) was similar for all infusion groups but fecal P increased with increased infusion of phytate P (Table 4-2).

Intake, Ileal Flow, and Disappearance of Phytate from the Large Intestine

Phytate P intake did not differ with treatment (Table 4-3). Dietary phytate P reaching the ileum was similar for all treatment groups (Table 4-3). On average, 68% of dietary phytate P was degraded in the rumen and small intestine. Several researchers have reported that ruminants hydrolyze the majority of feed phytate (78-100%) with the rumen the major site of phytate degradation (Clark et al., 1986; Morse et al., 1992; Kincaid et al., 2005). The digestion of phytate P in the rumen (and upper intestine) was comparatively low in this study (68%). Park et al. (1999) reported 84% ruminal effective digestibility of phytate P when ruminal passage rate was 0.02/h. Ruminal degradability of phytate P decreased to 69 and 57% when ruminal passage rate increased to 0.05 and 0.08/h, respectively. The high forage diet in this study would not seem to suggest a passage rate fast enough to reduce ruminal phytate P digestibility (Park et al. 1999).

The relatively low ruminal phytate degradation in this study may instead be due to the effect of the type of diet (high forage) and dietary phytate content on ruminal phytase activity. Lower ruminal phytase activity was reported in steers fed all hay diets than fed high-grain diets (Yanke et al., 1998), and lower phytase activity was observed in vitro (semi-continuous culture fermenter) with low phytate than high phytate diets (Godoy and Meschy, 2001). High dietary phytate is often the result of feeding a high concentrate diet and phytate P hydrolysis was greater in dairy cows fed an 80% grain (0.38% phytate P) diet as compared to cows fed a 52% grain (0.24% phytate P) diet (99% vs. 78-85% phytate P hydrolysis; Morse et al., 1992; Kincaid et al.,

2005). The low rumen pH resulting from high grain diets creates an environment favoring efficient phytase activity (Lan et al., 2011; Yanke et al., 1999), and the endogenous phytase activity in many cereal grains contribute to phytate hydrolysis in high grain diets (Viveros et al., 2000; Selle et al., 2003). The relatively low phytate hydrolysis in the rumen and upper intestine in this study supports these general observations that diet affects ruminal phytate hydrolysis, and also contradicts the conventional wisdom that phytate is completely hydrolysable in the rumen.

There was a linear increase ($P = 0.02$) in the amount of phytate which disappeared from the large intestine when infused phytate level increased from 5 to 25 g/d, and daily fecal phytate P output increased with increased phytate P infusion to the ileum. However phytate P disappearance (% of total supplied) from the large intestine was similar for all levels of phytate infusion (Table 4-3). Within the 5-25 g/d range, the amount of phytate reaching the large intestine did not influence phytate degradation in the large intestine. This range of infusion was chosen as representative of the range associated with diets of lactating cows. For instance, a lactating Holstein cow of 680 kg BW, 55 kg/d milk yield, 30 kg/d DMI at 90 DIM would require 0.38% (DM basis) dietary P (NRC 2001). If 70% of the dietary P is phytate P, dietary phytate intake would be 80 g/d. Applying the foregut phytate digestibility observed in this study (68% of consumed phytate P), 25 g/d phytate P would reach the large intestine. These assumptions are extreme in terms of dietary phytate offered (extremely high) and foregut phytate hydrolysis (probably lower than would be observed in a high phytate diet), and still the amount of phytate reaching the large intestine is within the upper limit of infused phytate into the large intestine in this study.

While ruminal phytate hydrolysis does appear to be influenced by type of diet and feed processing method, a fixed value for large intestinal phytate hydrolysis can probably be used in

estimating P availability from phytate P. In this study, average disappearance of phytate P from the large intestine was 15% of the total phytate entering the large intestine (Table 4-3). This is similar to the 19% of phytate inflow digested between the upper and lower large intestine of sheep fed a 50% forage diet (Park et al., 2002). In that study, more than 10% of total dietary inositol phosphate was not digested. In the present study, considering 68% phytate hydrolysis in the rumen and then 15% hydrolysis of phytate entering the large intestine, total tract phytate degradation would be 73% rendering 27% undigested and excreted in the feces. Combined, the results from this study and from the study by Park et al. (2002) indicate that large intestinal phytate degradation contributes to the overall digestibility of phytate but ruminal phytate hydrolysis is the key regulatory factor.

Large intestinal phytate degradation in sheep is considered to be due to the microbial phytase activity in the large intestine in ruminants (Park et al., 2002) and also in rodents and other nonruminants (Wise and Gilburt, 1982; Matsui et al., 1999). The microbial population in the large intestine is similar to that in the rumen (Maki and Picard, 1965; Hoover, 1978). Moreover, various species of *Bacillus* have been detected in the cecum and colon of cows and steers, and *Bacillus* strains have phytase capability (Joseph and Raj, 2007). The phytase activity in the large intestine must be less efficient than in the rumen, as less than 20% of influent phytate was digested in the large intestine in this study and in the sheep study by Park et al. (2002). Likely this inefficiency is due to the neutral or mild alkaline pH of the large intestine compared to the acidic pH reported ideal for the most efficient phytase activity (Yanke et al., 1999; Lan et al., 2011) and also to the very short digesta retention time in the large intestine of cows (Hecker and Grovum, 1975).

Disappearance of lower IP from the Large Intestine

The absence of lower IP in ileal digesta indicates either that lower IP was completely degraded in rumen and small intestine or their concentration in ileal samples was too low to be detected by HPIC. The former seems likely. In an *in vitro* study with pig small intestinal mucosa, phytase activity was higher towards IP3 and IP4 than IP5 and IP6 (Hu et al., 1996). In sheep fed corn and rapeseed meal-based diet (50% forage and 50% concentrate), daily passage of lower IP to the upper large intestine was less than 50% of that entering the small intestine indicating extensive digestion of the lower IP in that segment of the digestive tract. In the current study, the amount IP5 and IP4 infused daily to the ileum was different between treatment groups and fecal output of each increased with increased infusion amount (Table 4-4). Thus, the apparent disappearance of IP5 and IP4 from the large intestine did not differ with amount infused when expressed as percent of total supplied. The apparent disappearance of IP5 and IP4 from the large intestine averaged 34% and 64% of total supplied, respectively. No IP3 was detected in the feces due either to complete hydrolysis in large intestine or fecal concentration below the detection limit.

This is the first experiment where large intestinal lower IP disappearance has been investigated and observed in large ruminants. In ruminants the disappearance of lower IP from a very small large intestinal pool may not be of nutritional significance but the quantification method may be useful to investigate lower IP disappearance from the hind gut of non-ruminants.

Fate of Pi in the Large Intestine

The observed phytate disappearance from the large intestine was much lower than ruminal phytate disappearance (15 vs. 68%) but however modest, phytate degradation would

increase the pool of available P (Pi) in the large intestine. Inorganic P release from phytate degradation in the large intestine has nutritional importance only if released Pi is absorbed.

As expected, basal flow of Pi to the ileum was not different between treatment groups (Table 4-5), representing~43% of total P flow to the ileum daily. Inorganic P in infusion solutions increased with increased phytate content because infusion solutions were prepared from a phytic acid solution which contained Pi as an impurity (~18% of infused P). Despite the impurity, the amount of basal (dietary) Pi flowing to the ileum was higher than flow of infused Pi for each treatment group.

Fecal Pi increased in a linear manner ($P = 0.05$) with increasing phytate infusion level (5 to 25 g/d) and was greater than Pi entering the large intestine (Pi in ileal flow + Pi in infusion solution; Table 4-5). The difference between the amount of Pi in feces and the sum of Pi in basal flow and infusion solution is an estimate of net Pi release from hydrolysis of IP6 and lower IP.

There are reports of large intestinal P absorption in ruminants and our data also suggest this. When ileal total P flow and infused total P were used as continuous variables, the slope coefficient for total ileal P flow (dietary only) to feces was 0.56 ± 0.26, while the slope coefficient for infused P was 0.75 ± 0.13. Thus 44% of ileal P flow and 25% of infused P disappeared from the large intestine. Similarly phosphate absorption was observed from proximal colon descendens in lambs when ligated intestinal segments were filled with 1.2 mM phosphate solution ($HPO_4^{2-} + H_2PO_4^-$) for 30 min inside the abdominal cavity of anesthetized animals (Scharrer, 1985). Breves and Schroder (1991) observed that in sheep fed 1.0 to 4.1 g/d P, net disappearance of P from hindgut was 2 to 30% of total P entering hindgut.

The greater relative disappearance of P from dietary P flowing to the ileum than from the pure phytate infusion (44% vs. 25%) was likely due to higher proportion of Pi in total P flowing

to the ileum than in total P infused to the ileum. In sheep, the ratio of inorganic to total P decreased to less than 0.2 in lower small intestine indicating that most of the P was absorbed as soluble inorganic P (Poppi and Ternouth, 1979). In a study conducted on cannulated sheep, the flow rate (mmol/h) of total and soluble P decreased by 8% and 95%, respectively, from ileum to feces (Ben-Ghedalia et al., 1975). Therefore, the forms of P and their solubility in the digestive tract are two of the major factors regulating P absorption in the digestive tract. Ben-Ghedalia et al. (1975) observed that the solubility of P in sheep digesta decreased by 43% from the duodenum to the terminal ileum. The ratio of insoluble non-nucleic acid P to total P increased in lower small intestine and proximal colon of sheep indicating the formation of higher degree P-complex at high pH in these segments of digestive tract (Poppi and Ternouth, 1979).

The primary mechanism of phosphate absorption differs in ruminants and non ruminants. In non-ruminants, the small intestinal epithelium contains Na^+-dependent phosphate transporter (Murer et al., 1994). In ruminants, however, the uptake of phosphate by intestinal epithelium occurs through an electroneutral pH-dependent H^+/Pi cotransporter, and acidic extracellular pH is required to maintain phosphate uptake (Shirazi-Beechey et al., 1991). The neutral pH in the large intestine, therefore, may limit active P absorption. Therefore, passive diffusion may be the predominant mechanism of P absorption in the large intestine at high luminal concentrations of P. More research is needed to elucidate the mechanism of P absorption in the large intestine of ruminants. But this data will support mechanistic modeling efforts to improve prediction of P digestion allowing more accurate estimation of P bioavailability in feeds.

Conclusions

Infused phytate was degraded in the large intestine of dairy heifers and Pp digestibility was not dependent on the amount of phytate entering the large intestine. Degradation of phytate

and lower IPs increased the pool of large intestinal Pi; released Pi disappeared partially from the large intestine. This data will support mathematical modeling approach of predicting P digestion allowing more accurate estimation of P bioavailability in feeds. Further study is needed with phytate from dietary sources to evaluate its flow to and degradability in the large intestine of ruminants.

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Table 4-1. Composition of basal diet fed to eight crossbred heifers

	% of dietary DM
Ingredient	
Barley straw	38.3
Corn silage	14.7
Beet pulp, dried	38.6
Cane molasses, dehydrated	4.98
Slow release non protein nitrogen[1]	2.72
Trace mineral mix[2]	0.11
Selenium 90[3]	0.42
Vitamin ADE mix[4]	0.18
Nutrient	
CP	12.6
NDF	54.4
ADF	34.7
P	0.14
Phytate P[5]	0.005

[1]Optigen (Alltech, Springfield, KY)

[2]Contained 37% Na, 60% Cl, 0.03% K, 0.3% Mg, 14% S

[3]Contained 90 mg Se per kg of DM

[4]Contained 26,400 KIU vitamin A; 8,800 KIU vitamin D; and 44,000 IU vitamin E per kg of DM

[5]Phytate P was 3.8% of dietary total P.

Table 4-2. Dry matter intake, digestibility, total P intake, and fecal excretion in eight crossbred heifers infused withdifferent amounts of phytate P

	Dosage of infused phytate P[1]					
	0	X	3X	5X	SEM	P <
DMI, kg/d	7.48	7.60	7.36	8.28	0.54	0.65
Fecal DM, kg/d	2.12	2.37	2.33	2.65	0.24	0.52
DM digestibility, %	70.1	67.8	69.1	68.4	1.54	0.67
P intake, g/d	10.5	10.7	10.5	11.6	0.76	0.72
Fecal P, g/d	8.60[a]	11.6[ab]	13.7[bc]	16.3[c]	1.12	0.0001

[1]Daily infusion of phytate P to ileum (g/d): 0 = 0, X = 5, 3X = 15, 5X = 25

[a-c]Values with different superscripts differ significantly ($P < 0.05$) within a row.

Table 4-3. Effect of ileal phytate infusion on disappearance of phytate from the large intestine of eight crossbred heifers

	Dosage of infused phytate P[1]					
	0	X	3X	5X	SEM	P <
Phytate intake, g/d	0.40	0.41	0.39	0.44	0.03	0.66
Phytate flow, g/d	0.13	0.11	0.16	0.11	0.02	0.23
Phytate infused, g/d	0.00[a]	1.02[b]	3.08[c]	5.03[d]	0.13	0.0001
Fecal phytate, g/d	0.12[a]	1.05[b]	2.63[c]	4.30[d]	0.28	0.0001
Phytate disappearance, g/d	0.05[a]	0.15[ab]	0.62[b]	0.62[b]	0.14	0.01
Phytate disappearance, % total supplied	14.9	17.3	15.8	13.7	8.80	1.0

[1]Daily infusion of phytate P to ileum (g/d): 0 = 0, X = 5, 3X = 15, 5X = 25

[a-c]Values with different superscripts differ significantly ($P < 0.05$) within a row.

Table 4-4. Effect of different levels of lower IP infusion on large intestinal lower IP disappearance

	Dosage of infused phytate P[1]				
	X	3X	5X	SEM	P <
IP5[2] infused, g/d	0.76[a]	2.44[b]	3.79[c]	0.08	0.0001
Fecal IP5, g/d	0.5[a]	1.62[b]	2.29[c]	0.19	0.0001
Net IP5 disappearance, % of total supplied[4]	29.7	36.1	36.8	5.97	0.59
IP4[3] infused, g/d	0.28[a]	0.91[b]	1.73[c]	0.04	0.0001
Fecal IP4, g/d	0.13[a]	0.35[b]	0.44[c]	0.05	0.0001
Net IP4 disappearance, % of total supplied[4]	58.6	65.1	68.8	5.88	0.41

[1]Daily infusion of phytate P to ileum (g/d): X = 5, 3X = 15, 5X = 25

[2] Inositol penta phosphate

[3] Inositol tetra phosphate

[4]Net disappearance is the difference between infused and fecal flow. Unknown is the precise fate of each IP. Disappearance of an IP may indicate partial degradation (to IP4, IP3, etc) or complete degradation.

[a-c]Values with different superscripts differ significantly ($P < 0.05$) within a row.

Table 4-5. Effect of ileal phytate infusion on disappearance of inorganic P from the large intestine of eight crossbred heifers

	Dosage of infused phytate P[1]					
	0	X	3X	5X	SEM	P <
P flow, g/d	7.90	8.23	7.94	8.21	1.12	0.99
P infused, g/d	0.00[a]	2.03[b]	6.02[c]	9.67[d]	0.22	0.0001
Fecal P, g/d	8.59[a]	11.6[ab]	13.7[bc]	16.3[c]	1.12	0.0001
Pi flow, g/d	2.81	3.70	4.20	3.19	0.75	0.54
Pi infused, g/d	0.00[a]	0.26[b]	0.79[c]	1.31[d]	0.03	0.0001
Fecal Pi, g/d	5.80[a]	7.22[ab]	7.69[ab]	9.30[b]	0.72	0.03

[1]Daily infusion of phytate P to ileum (g/d): 0 = 0, X = 5, 3X = 15, 5X = 25

[a-c]Values with different superscripts differ significantly ($P < 0.05$) within a row.

Chapter 5 EFFECT OF DIETARY PHYTATE ON PHOSPHORUS DIGESTIBILITY IN DAIRY COWS

Abstract

The objective was to evaluate the effect of dietary phytate P (Pp) supply on ruminal and post-ruminal Pp digestion and net disappearance of P from lower digestive tract of lactating cows. Six ruminally-and ileally-cannulated crossbred lactating cows were used in two incomplete Latin squares with four 21-d periods (17 d of diet adaptation, 4 d of total collection). Dietary treatments were low Pp, medium Pp, and high Pp, and a high inorganic P (Pi) diet with the same total P content as the highest phytate diet but with P mostly from inorganic sources. The diets contained 0.10, 0.18, 0.29, and 0.11% Pp and 0.43, 0.48, 0.54, and 0.53% total P on DM basis, with cottonseed meal used to increase Pp content. Ytterbium-labeled corn silage and Co-EDTA were used as particulate and liquid phase markers to measure omasal and ileal digesta flow. Omasal and ileal digesta were collected every 6 h on d 20 and 21 and rumen contents were collected on d 21. Samples were analyzed for total P (molybdovanadate yellow method), Pi (blue method), and Pp (high performance ion chromatography). Phytate and total P intake increased linearly with increasing dietary Pp. Ruminal Pp disappearance also increased linearly with dietary Pp but the magnitude of change was small. Small intestinal net disappearance of Pi was not affected by dietary Pp. Phytate P was hydrolyzed in the large intestine but its hydrolysis was not influenced by dietary Pp. Net disappearance of Pi form the large intestine did not vary with dietary Pp. Dry matter (DM) digestibility decreased linearly with increasing dietary Pp, as did apparent digestion of P, and fecal P increased linearly. Dry matter digestibility was higher for high Pi than for high Pp, likely due to the effect of cottonseed meal in the latter diet. Replacing a portion of Pp with Pi resulted in decreased P excretion but this effect was confounded with

increased fecal DM for the high Pp (high cottonseed meal) diet. In lactating cows Pp digestibility was not negatively influenced by dietary Pp and fecal P excretion was regulated by dietary total P rather than by form of dietary P.

Key words: phytate, digestibility, dairy cows

Introduction

Accurate prediction of phosphorus (P) availability could allow new dietary P manipulation strategies to reduce P excretion without affecting production and performance of dairy cows. The digestibility of P is higher in inorganic P supplements than in grains and by-product feed ingredients (Chicco et al., 1965; Witt and Owens, 1983), and digestibility of organic P in the latter types of feed may also vary. Phytate (Pp) contributes the majority of P in grains and most of the by-product feed ingredients (Eeckhout and Depaepe, 1994; Ravindran et al., 1994). Ruminants can utilize P from phytate because ruminal microorganisms are capable of synthesizing phytase enzyme which can release phosphate group from the phytate molecule (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992), but ruminal Pp hydrolysis is variable. It is influenced by the type of grain, processing of feed ingredients, and supplemental exogenous phytase enzyme (Park et al., 2000; Bravo et al., 2002; Kincaid et al., 2005). The variation in ruminal Pp hydrolysis may be due to the alteration in endogenous phytase activity (Yanke et al., 1998) or due to physical and(or) chemical alterations of the phytate molecule.

Modern dairy rations contain large amounts of high-phytate grains and by-product feed ingredients. Increased phytate intake together with high DMI may limit ruminal Pp hydrolysis by reducing the duration of Pp exposure to microbial phytase while increasing the amount of substrate (phytate). In addition, saturation of ruminal phytase activity may occur on high grain (high-phytate) diets. High grain diets also are associated with reduced secretion of saliva, possibly decreasing salivary P available for microbial use and for absorption in the small intestine (Scott and Buchan, 1985). Incomplete ruminal hydrolysis of Pp can be compensated only if large intestinal hydrolysis of remaining phytate occurs and if released Pi is absorbed from the large intestine. Little data is available on changes in ruminal and post-ruminal Pp hydrolysis

with dietary Pp concentration in ruminants. Therefore, the objective of this study was to investigate the effect of dietary phytate on ruminal and post ruminal P digestibility in dairy cows.

Materials and Methods

Animals and Experimental Design

Six crossbred [Swedish Red or Brown Swiss X (Holstein X Jersey)] first lactation cows averaging 463 kg BW and 90 (± 35) DIM were used in two incomplete 4 × 4 Latin squares. These animals were from the 8 animals used in the previous study. The cows were fitted surgically with a half T-type ileal cannula made of tygon (5 to 6 cm anterior to ileo-cecal junction) and a ruminal cannula (Bar Diamond, Inc., Parma, ID). Ileal cannulas were cleaned every other day and checked daily for correct position.

During diet adaptation, cows were housed in a freestall barn with constant access to water and fed once daily at 1200 h in a Calan door system (American Calan, Northwood, NH). Dietary treatments were low, medium, and high phytate, and a high inorganic P diet with the same total P content as the high phytate diet but with P mostly from inorganic sources. The diets contained 0.10, 0.18, 0.29, and 0.11% phytate P and 0.43, 0.48, 0.54, and 0.53% total P on DM basis (Table 5-1), with cottonseed meal (CSM) used to increase phytate content. Diets were formulated to meet or exceed NRC (2001) recommendations for all nutrients. Feed was offered ad libitum during diet adaptation.

After 15 d of diet adaptation, cows were moved to individual tie stalls in a metabolism barn for a 2-d barn acclimation period (d 16 and 17) followed by 4-d of total collection (d 18-21). While in the metabolism barn cows were fed ad libitum four times daily at 0600, 1200, 1800, and 2400 h to minimize diurnal variation in digesta flow. Cows were milked twice daily at 0600 and 1800 h. On d 17 of each period, cows were fitted with urinary catheters for total

collection of urine. Cows were observed for symptoms of infection, and rectal temperatures were recorded daily.

Ytterbium (Yb)-labeled corn silage (Harvatine et al., 2002) and Co-EDTA (Uden et al., 1980) were used as particulate and liquid phase markers, respectively, to measure omasal and ileal digesta flow. Beginning on d 16 of each period, markers were dosed into the rumen before each feeding at a rate of 0.11 g Yb or Co/cow/d. All protocols and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Sample Collection and Preparation

Total mixed ration (TMR) and feed refusals were collected daily from d 16 to 21of each period. Total fecal collection was conducted for last 4 d of each period. Feces from each cow was blended daily and a representative sample was collected. Total daily urine was collected into a 12 L jug surrounded by ice to prevent evaporation and a sub-sample was collected daily for each cow (Knowlton et al., 2010). All samples were stored at -20°C immediately after collection.

On d 20 and 21, omasal and ileal samples were collected every 6 h with the collection time shifted by 2 h on the second day to get sample representative of every 4 h over a 24 h period. Omasal contents were collected using an omasal sampler placed in the omasal orifice and attached to a vacuum pump (Huhtanen et al., 1997). Ileal digesta was collected through the ileal cannula. All samples were stored at -20°C immediately following collection.

Rumen evacuation was conducted on the last day of each period. Rumen contents were weighed and two representative sub-samples collected, then contents were returned to the rumen. One sub-sample was stored at -20°C immediately following collection and the other sub-sample was composited by period to isolate rumen microbes (Cecava et al., 1990). Briefly, 500 mL saline was added to 1,500 mL of composited rumen contents and the mixture was blended in a

Waring blender for 2 min. Blended rumen contents were strained through 4 layers of cheesecloth to yield a homogenized fluid with small particles. Strained fluid was centrifuged twice at 500 × *g* for 20 and 10 min. Pellets were discarded and the remaining supernatant was centrifuged at 8,000 × *g* for 20 and 10 min to capture the microbes in the pellet. Fresh isolated microbes were stored at -80°C before being freeze dried (Genesis 25EL, Stone Ridge, NY).

Frozen samples were later thawed and TMR, refusals, omasal, and ileal samples were composited by cow × period. Total mixed ration and refusals were oven dried (Wisconsin Oven, Memmert, Schwabach, Germany) at 55°C until constant dry weight was achieved. Rumen, ileal, and fecal samples were freeze-dried (Genesis 25EL, Stone Ridge, NY). Dried samples were ground through Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA) and then through a Z grinder (0.2-mm screen; ZM 100 USA, Retsch, Haan, Germany). Ground samples were stored in air tight containers.

Because of possible bias in collecting heterogeneous omasal samples, omasal contents were fractionated into fluid, small, and large particulate phases following the method described by Reynal and Broderick (2005). Briefly, omasal contents were strained through single layer cheesecloth to separate large particles from fluid and small particles. Then fluid containing small particles was centrifuged at 1,000 × *g* for 5 min to separate small particles from fluid. The three phases were freeze dried (Genesis 25EL, Stone Ridge, NY). Dried small and large particulate phases were ground through a Z grinder (0.2-mm screen; ZM 100 USA, Retsch, Haan, Germany). Omasal fluid phases were ground using a freezer mill (6850 Freezer Mill, Santa Clara, CA) because of the hygroscopic nature of the material.

Sample Analysis

Dried ground TMR, feed refusals, and feces were analyzed for N, total P (AOAC, 1984), and NDF and ADF sequentially (Van Soest et al., 1991). Feed samples were analyzed for Ca using Inductively Coupled Plasma Atomic Emission Spectroscopy (CirOS VISION model, SPECTRO Analytical Instruments Inc., Mahwah, NJ). Urine samples were analyzed for total P (AOAC, 1984). Omasal, ileal, and fecal samples were analyzed for Yb and Co using Inductively Coupled Plasma Atomic Emission Spectroscopy (CirOS VISION model, SPECTRO Analytical Instruments Inc., Mahwah, NJ). Milk samples were analyzed for fat, protein, lactose, solids non-fat, milk urea N, and somatic cell count (DHIA laboratory Blacksburg, VA).

Total mixed ration, refusals, rumen, the three phases of omasal contents, ileal, and fecal samples were extracted with 0.5 *M* HCl (extracted for 4 h at ambient temperature, then centrifuged at 30,000 × *g* for 20 min at 4°C) and analyzed for inorganic P using the molybdenum blue method as described by Murphy and Riley (1958).

Feed samples were analyzed for inositol hexaphosphate (IP6; phytate) using the method developed by Ray et al. (2012a). Rumen contents, the three phases of omasal contents, ileal and fecal samples were analyzed for IP6 and lower inositol phosphates (IP5, IP4, IP3, a.k.a. lower IPs) following the method described by Ray et al. (2012b). In brief, IP6 and lower IPs were extracted from dried ground samples using 0.25 *M* NaOH-0.05 *M* EDTA and then centrifuged at 30,000 × *g* for 20 min at 4°C. Alkaline extracts were acidified with HCl-HF acid solution (500 µl of 6 *M* HCl and 1.2 *M* HF added to 5 mL sample extract) and stored overnight at 4°C. After overnight storage, acidified extracts were centrifuged at 30,000 × *g* for 20 min at 4°C and clear supernatants were collected and passed through methanol-conditioned C_{18} column (Sep-Pak plus, Waters, MA) fitted with a 0.2 µm IC membrane (PTFE filter; IC Millex -LG, Fisher, PA).

Inositol hexaphosphate and lower IPs in clear sample extracts were quantified by HPIC (Dionex ICS 3000 with a Dionex 4 x 50 IonPac AG7 guard column and a 4 x 250 mm IonPac AS7 analytical column; Dionex, Sunnyvale, CA) using pH 4 elution and post-column reaction detection (Rounds and Nielsen, 1993; Kwanyuen and Burton, 2005; Leytem et al., 2008). Phytate (inositol hexaphosphate), IP5, IP4, and IP3 were eluted at 11.8, 10.5, 8.9, and 6.8 min, respectively, with gradient elution (0.01 M Methylpiperazine and 0.01 M $NaNO_3$-0.01 M Methylpiperazine) at a flow rate of 1 mL per min. Eluted phytate and lower IPs were detected using UV-VIS detector with absorbance monitored at 500 nm after the derivatization reaction with Wade's reagent (0.015% $FeCl_3$ + 0.15% sulfosalicylic acid solution) in a post column reactor.

Statistical Analysis

All intake, flow, and excretion data were analyzed using PROC GLIMMIX in SAS (SAS Institute, 2002). The model included square, diet, and period as fixed effects and cow as random effect. Pre-planned contrasts were used to test the effect of increasing dietary phytate on intake, disappearance, and fecal excretion, and to compare high phytate and high inorganic P diets; contrasts to evaluate increasing dietary phytate were polynomial. Statistical significance was declared at $P < 0.05$.

Results and Discussion

Nutrient Intake, Digestibility, and Milk Production and Composition

Dry matter intake was not affected by either dietary Pp concentration or by the form of dietary P (Table 5-2). Digestibility of DM decreased linearly with increasing dietary Pp and was also lower in the high Pp diet than in the high Pi diet. This effect may be due to inclusion of CSM in the medium and high Pp diets (12.5 and 24.7% of dietary DM). The decrease in DM

digestibility for CSM supplemented diets was associated with reduced NDF digestibility. Fiber digestibility tended to decrease linearly ($P = 0.06$) with increasing dietary Pp and fiber digestibility was less in cows fed the high Pp diet compared with cows fed the high Pi diet. Others have observed decreased ruminal digestion of OM (Zinn et al., 1997), NDF and N (Goetsch and Owens, 1985) with increased CSM content of diets for lactating cows, and this is usually associated with concomitant reductions in total tract digestion. Post-ruminal digestion is less often affected by CSM content but post-ruminal NDF digestion was decreased in one study (Goetsch and Owens, 1985). Apparent digestibility of N decreased linearly with increasing dietary Pp, likely because protein in CSM is relatively slowly degraded (Pires et al., 1997).

Milk yield and composition were not affected by dietary Pp or dietary P form except for milk fat content (Table 5-3). Milk fat yield showed a trend for a quadratic relationship ($P = 0.07$) with dietary Pp, with fat yield lower in cows fed medium Pp than in cows fed low or high Pp. This aligns with the numerically lower NDF intake and digestibility in cows fed the medium Pp diet compared with low and high Pp diets, but the ultimate cause of these effects is unclear.

Phosphorus Intake, Flow, and Excretion

Effect of dietary phytate. As planned, Pp intake increased linearly with increasing dietary Pp (Table 4) as did total P intake but Pi intake decreased linearly with increasing dietary Pp.

Rumen pool size of total P, Pi, and Pp was not affected by dietary Pp (Table 5-5). Ruminal disappearance (% of intake) of Pp increased linearly with increasing dietary Pp. This increase in Pp digestibility was probably due to increased microbial phytase activity in the rumen. Phytase activity increased with increasing Pp in microbes isolated from the rumen (Yanke et al., 1998) and in a semi-continuous culture system fermentor (Godoy and Meschy, 2001). While ruminal hydrolysis of dietary Pp was almost complete others have reported that

substantial amounts of phytate can escape the rumen (Park et al., 1999b; Kincaid et al., 2005). The results of this study and previous studies showed a wide range (57% to > 90%) of ruminal phytate hydrolysis which varied with several factors such as type of feed ingredient, type of diet, feed processing method (Konishi et al., 1999, Park et al., 1999; Ray et al., 2012b).

Omasal flow of total P was not affected by dietary Pp (Table 5-4). Though total P intake increased with increasing dietary Pp the large amount of salivary P entering the rumen nullified any difference in the rumen pool of total P. Similarly, omasal flow of Pi and Pp were not influenced by dietary Pp. The flow of total P, Pi, and Pp to the ileum increased linearly with increasing dietary Pp (Table 5-4). These increases were due to increases in ileal DM flow; the concentration of total P, Pp, and Pi in ileal digesta did not vary. In one study with lactating Holstein cows, the effect of dietary P on apparent digestibility of P was confounded with the effect of diet of fecal DM (Wu, 2005). On average, small intestinal net disappearance of Pi was 71% of omasal Pi flow and was not influenced by dietary Pp or total P (75, 70, and 68% for LPP, MPP, and HPP diets). Ileal flow of Pm was not influenced by dietary Pp and ranged from 2.35-2.70 g/d.

In this study, phytate P was degraded in the large intestine and net disappearance of Pi in the large intestine was observed (8.4, 12.3, and 9.6% of ileal Pi flow for LPP, MPP, and HPP diets). Large intestinal Pp degradation was not influenced by dietary Pp and on average, 16% of ileal Pp flowing into the large intestine was degraded (22.8, 9.8, 11.6, and 21.4% of ileal Pp flow for LPP, MPP, HPP, and HPI diets). Similar extent of Pp degradation (19% of Pp entering the large intestine) was observed in the large intestine of sheep (Park et al., 2002). Large intestinal Pp hydrolysis in the present study was likely due to microbial phytase activity in the large intestine as was reported by others (Wise and Gilburt, 1982b; Matsui et al., 1999). Similar

microbial population in the rumen and the large intestine, and the presence of phytase activity in some bacterial strains isolated from the large intestine of ruminants would reinforce this conclusion (Hoover, 1978; Joseph and Raj, 2007). Inorganic P flowing into the large intestine or released from Pp degradation would be nutritional significance if animals can absorb that in the large intestine. Net disappearance of Pi in the large intestine as observed in this study and reported by other researchers indicated possible nutritional significance of large intestinal P digestion in ruminants (Ben-Ghedalia et al., 1975; Scharrer, 1985).

Average urinary P excretion was 0.26 g/d and was not affected by dietary Pp. Microbial P in the feces ranged from 3.48-4.75 g/d and was not affected by dietary Pp (Table 5-4). Fecal excretion of total P, Pi, and Pp increased linearly with increasing dietary Pp (Table 5-4). A key question is whether fecal excretion of these was caused by increased fecal DM excretion for medium and high Pp diets, by the effect of the form of dietary P, or both. The concentration of total P, Pp, and Pi in feces increased linearly with increasing dietary Pp, and thus we conclude that dietary P directly influenced fecal P excretion, independent of the effect of CSM on fecal DM excretion. This still leaves open the question of whether the effect of dietary P was amount of dietary P or form, as these are confounded in the first three diets. Several authors have shown increased fecal P with addition of inorganic P to the diet (Wu et al., 2000; Knowlton and Herbein, 2002). Almost complete ruminal degradation of Pp in this study eliminated any possible effect of dietary Pp on post-ruminal P digestion and fecal P excretion. Similar to the observation by Dou et al. (2002), the increment in fecal P was associated with increased fecal Pi in this study. Therefore, it can be concluded that Pi was available in the digestive tract and the degradation of higher form of P was not affected by dietary Pp. Linear decrease in apparent digestibility of P

with increasing dietary Pp or P (47, 37, and 32% of P intake for LPP, MPP, and HPP diets) was in agreement with the results in previous studies (Wu et al., 2001; Knowlton and Herbein, 2002).

Effect of dietary P form. As intended, total P intake was not different between the high Pp and the high Pi diet (Table 5-4). Phytate P intake was higher and Pi intake was lower for high Pp diet than for high Pi diet.

Rumen pool size of total P was higher in high Pp group than in high Pi group but form of dietary P did not influence rumen pool size of Pp and Pi (Table 5-5). Ruminal disappearance (% of intake) of Pp was higher for high Pp diet than for high Pi diet. Larger total P pool in the rumen of cows fed high Pp diet was probably due to smaller ruminal DM pool in high Pi diet compared with high Pp diet because ruminal total P concentration was not affected by dietary P form. Moreover, initial pool of readily soluble fraction of total P i.e. Pi was higher in high Pi diet compared with high Pp diet and a portion of Pi was probably transported at faster rate across the rumen wall (Breves et al., 1988). Increased ruminal Pp digestibility for high Pp diet was likely due to the high phytase activity in the presence of high Pp. Another possible explanation could be the negative influence, if any, of high Pi on the production and activity of microbial phytase as observed in different species of *Aspergillus* and several strains and varieties within the same species (Shieh and Ware, 1968). But this explanation may not be applicable in case of microbial phyase synthesis in the rumen because phytase activity in the rumen is primarily of bacterial origin and bacterial phytase synthesis is cell-associated and independent of Pi concentration unlike extracellular phytase in *Aspergillus* spp. (Yanke et al., 1998). The question can then be raised about the physiological significance of expressing the enzyme phytase by rumen microbes given that there is always sufficient Pi available for the microbes to use for their metabolism.

Omasal flow of total P, Pp, and Pi was not affected by form of dietary P (Table 5-4). Similarly, total P, Pp, and Pi flow to the ileum was not different between high Pp and high Pi diet. But ileal flow of Pp tended to be higher (P = 0.08) in high Pp diet than in high Pi diet. Ileal flow of microbial P was not affected by dietary P form (2.46 g/d for HPP vs. 2.44 g/d for HPI; Table 5-4). Net disappearance of Pi form the small intestine was not influenced by form of dietary P and was 68 and 71% of omasal Pi flow for high Pp and high Pi diet, respectively. Large intestinal Pp disappearance was not different between high Pp and high Pi diet (11.6 vs 21.4% of ileal Pp flow). Unchanged large intestinal Pp disappearance with different amount of Pp entering the large intestine confirmed that large intestinal Pp degradability was constant. In this study, no effect of replacing a portion of dietary Pp with Pi on small intestinal disappearance of Pi was similar to the observation that small intestinal net disappearance of Pi did not vary with increasing dietary Pp or total P as observed in this study. Bravo et al. (2003) reported that when the range of dietary P was 0.25 to 0.50% (DM basis), P absorption coefficient appeared to be constant and independent of dietary P supply.

As was not the case for ileal flow, fecal excretion of total P, Pp, and Pi was less for high Pi diet than for high Pp diet (Table 5-4). Form of dietary P did not influence fecal excretion of microbial P. Total tract P digestibility was lower in high Pp diet than in high Pi diet (32 vs 45% of P intake). Bravo et al. (2003) mentioned that Pp appeared as one of the factors affecting P digestion when the authors used a modeling approach to evaluate the effect of different variables on P digestibility. The concentration of total and other forms of P in fecal DM did not vary with dietary P form and fecal DM was higher for high Pp diet than for high Pi diet. We can speculate that the effect of dietary P form on fecal P excretion was influenced by fecal DM excretion partly if not completely.

Conclusions

Dietary Pp concentration influenced ruminal and post-ruminal digestion of P but it appeared that most of the effects were associated with dietary total P concentration rather than dietary P form. The exceptions were ruminal and large intestinal Pp degradation, both influenced by dietary Pp content. Ruminal Pp hydrolysis was almost complete and was not negatively influenced by dietary Pp concentration. Phytate P digestion and net disappearance of Pi was observed in the large intestine but were affected neither by dietary Pp nor by dietary P form. Net disappearance of Pi in the small intestine was not affected by either dietary Pp or form of dietary P. Increasing dietary P intake resulted in increased fecal P excretion.

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Table 5-1. Ingredient and nutrient composition of diets

	Diet			
	LPP[1]	MPP[2]	HPP[3]	HPI[4]
Ingredients	% of dietary DM			
Corn silage	46.1	45.6	45.2	45.8
Grass hay	9.42	9.33	9.25	9.36
Corn, ground	9.32	9.23	9.14	9.26
Cottonseed meal	.	12.5	24.7	.
Soybean meal, 48%	14.1	7.00	.	14.0
Beet pulp, dried	9.63	7.16	4.73	9.57
Cane molasses, dehydrated	2.99	2.96	2.93	2.97
Pro-Lak	4.82	2.63	0.47	4.79
Urea	0.63	0.31	.	0.63
Limestone	0.92	1.25	1.54	0.91
Vitamin-mineral mix[5]	2.10	2.08	2.06	2.08
Monoammonium phosphate	.	.	.	0.65
Nutrient				
DM	56.2	54.0	54.5	54.1
CP	18.2	17.6	18.6	17.0
NDF	34.7	31.9	35.0	36.1
ADF	18.3	16.7	18.7	19.0
Ca	1.04	1.07	0.90	0.90

P	0.43	0.48	0.54	0.53
Phytate P	0.10	0.18	0.29	0.11

[1]Low phytate

[2]Medium phytate

[3]High phytate

[4]High inorganic P

[5]Contained vitamin A 26,400 KIU, 8,800 KIU vitamin D, and 44,000 IU vitamin E per kg of DM; 37% Na, 60% Cl, 0.03% K, 0.3% Mg, 14% S, 90 ppm Se

Table 5-2: Effect of diet on nutrient intake and digestibility

	Diet				SE	*P* <			
						Diet	Dietary phytate		HPP[3] vs. HPI[4]
	LPP[1]	MPP[2]	HPP[3]	HPI[4]			Linear	Quadratic	
DMI, kg/d	17.9	18.6	18.1	17.8	0.61	0.71	0.82	0.35	0.71
DM digestibility, %	69.6	65.8	64.5	68.9	0.96	0.002	0.0009	0.24	0.004
NDF intake, kg/d	5.75	5.48	6.24	6.23	0.33	0.27	0.26	0.19	0.98
NDF digestibility, %	50.7	42.9	44.2	54.6	3.7	0.02	0.06	0.28	0.008
N intake, g/d	569	544	602	528	30	0.27	0.41	0.24	0.07
N digestibility, %	71.7	64.6	63.2	67.4	1.9	0.03	0.005	0.25	0.11

[1]Low phytate

[2]Medium phytate

[3]High phytate

[4]High inorganic P

Table 5-3: Effect of diet on milk yield and composition

	Diet					P <			
							Dietary phytate		
	LPP[1]	MPP[2]	HPP[3]	HPI[4]	SE	Diet	Linear	Quadratic	HPP[3] vs. HPI[4]
Milk yield, kg/d	33.0	32.6	32.8	34.0	1.08	0.78	0.84	0.79	0.41
Milk fat, kg/d	1.52	1.34	1.54	1.57	0.08	0.21	0.81	0.07	0.81
Milk protein, kg/d	1.14	1.12	1.15	1.19	0.05	0.80	0.93	0.72	0.53
Milk lactose, kg/d	1.60	1.60	1.60	1.65	0.05	0.74	0.89	0.83	0.37
SNF, kg/d	3.03	2.99	3.02	3.13	0.10	0.75	0.97	0.76	0.42
MUN, mg/dL	18.7	16.9	16.5	15.9	0.83	0.11	0.06	0.46	0.60

[1]Low phytate

[2]Medium phytate

[3]High phytate

[4]High inorganic P

Table 5-4. Effect of diet on intake, omasal flow, ileal flow, and fecal excretion of P, Pi, Pp, and Pm

	Diet					$P <$			
							Dietary phytate		
	LPP[1]	MPP[2]	HPP[3]	HPI[4]	SE	Diet	Linear	Quadratic	HPP[3] vs. HPI[4]
Intake									
Total P, g/d	78.3	84.2	98.8	93.3	4.6	0.02	0.005	0.40	0.36
Inorganic P, g/d	51.4	47.3	40.7	63.9	2.7	0.0001	0.008	0.67	0.0001
Phytate P, g/d	18.2	32.4	56.2	19.6	2.4	0.0001	0.0001	0.10	0.0001
Omasal flow									
Total P, g/d	188	196	186	197	29.4	0.98	0.96	0.75	0.71
Inorganic P, g/d	178	188	183	185	29	0.99	0.86	0.74	0.96
Phytate P, g/d	2.70	2.75	3.02	3.06	0.67	0.96	0.72	0.88	0.96
Ileal flow									
Total P, g/d	46.0	64.7	74.9	56.0	8.7	0.03	0.006	0.56	0.11
Inorganic P, g/d	40.7	58.5	62.2	50.3	5.0	0.07	0.01	0.29	0.12
Phytate P, g/d	1.36	2.06	2.90	1.79	0.45	0.10	0.02	0.89	0.08

Microbial P, g/d	2.35	2.70	2.44	2.46	0.2	0.59	0.73	0.20	0.93
Fecal excretion									
Total P, g/d	41.5	58.2	64.6	50.5	3.3	0.0003	0.0001	0.34	0.003
Inorganic P, g/d	36.5	49.4	53.7	44.2	2.7	0.001	0.0002	0.18	0.02
Phytate P,g/d	0.98	1.86	2.41	1.38	0.36	0.02	0.004	0.69	0.04
Microbial P, g/d	3.48	4.75	4.03	3.53	0.45	0.15	0.34	0.07	0.41
Urinary P, g/d	0.20	0.26	0.31	0.61	0.20	0.51	0.86	0.86	0.26

[1]Low phytate

[2]Medium phytate

[3]High phytate

[4]High inorganic P

Table 5-5. Effect of diet on rumen pool size of P, Pi, and Pp and ruminal disappearance of Pp

	Diet					$P <$			
							Dietary phytate		
	LPP[1]	MPP[2]	HPP[3]	HPI[4]	SE	Diet	Linear	Quadratic	HPP[3] vs. HPI[4]
Rumen pool									
Total P, g	66.0	69.3	70.6	53.9	3.84	0.02	0.34	0.80	0.006
Inorganic P, g	57.6	60.5	61.4	51.1	4.8	0.40	0.53	0.84	0.13
Phytate P, g	0.70	0.65	0.57	0.44	0.11	0.42	0.35	0.92	0.42
Phytate P disappearance, % of intake	84.6	91.3	93.5	84.8	2.93	0.06	0.04	0.46	0.03

[1]Low phytate

[2]Medium phytate

[3]High phytate

[4]High inorganic P

Chapter 6 CONCLUSIONS

The method developed to quantify phytate in ruminant digesta and feces included alkaline extraction, extract clean-up, and HPIC quantification. Of four different extractants (0.5 M HCl, 1 M HCl, 0.25 M NaOH-0.05 M EDTA, 0.5 M HCl followed by 0.25 M NaOH-0.05 M EDTA) evaluated for their P extraction efficiency, and 0.25 M NaOH-0.05 M EDTA is the best universal extractant for feed, digesta, and feces. All the extractants were efficient in extracting P from omasal contents. Acid extractants gave satisfactory results in extracting P from feed but were least efficient in extracting P from feces. Sequential extraction showed lower P extraction efficiency for feces samples compared with alkaline extraction. Oven-drying of samples did not affect P extraction efficiency of any extractant.

The clean-up procedures developed for acid (0.5 M HCl) and alkaline extracts removed interference of sample matrix in chromatograph and prevented the damage to the analytical columns often associated with extracts of complex samples. The clean-up method in combination with HPIC analysis allowed improved accuracy in quantifying low phytate concentrations in ruminant digesta and feces. Moreover, the clean-up step of alkaline extraction made HPIC compatible with alkaline extraction which was the most efficient and robust extractant in extracting P. Thus, the combination of alkaline extraction and HPIC confirms that phytate quantification is ***not*** limited by extraction allowing more focus on improving the quantification approach. Almost complete recovery of phytate spiked to the sample before extraction validated the efficiency of the extraction, clean-up, and chromatographic quantification steps of this method. This simple, robust, and sensitive method of phytate quantification may help investigating phytate P digestion dynamics in ruminants.

Almost one third of dietary phytate escaped ruminal degradation in heifers fed high forage low P-low phytate diet. This contradicts the conventional thought that phytate can be completely degraded in the rumen. The relatively low ruminal phytate hydrolysis suggests that dietary factors influence ruminal phytate hydrolysis.

Phytate and its partially hydrolyzed products, i.e. inositol pentaphosphate (IP5), inositol tetraphosphate (IP4), and inositol triphosphate (IP3), were degraded in the large intestine of dairy heifers. Average phytate hydrolysis was 15% of the phytate entering the large intestine and was not influenced by increasing phytate supplied to the large intestine. Therefore, it can be concluded that a fixed value of large intestinal phytate digestibility can be used in estimating P availability in ruminants. Summed phytate degradation (ruminal+post-ruminal) indicated that more than 25% of phytate was not digested and excreted in the feces. Lower inositol phosphates (IPs; IP5, IP4, and IP3) were also degraded in the large intestine and their digestibility was higher than phytate. Inorganic P released from the degradation of phytate and lower IPs might be of nutritional significance as there was net disappearance of Pi from the large intestine. Considering relatively low degradation of phytate in, and net disappearance of Pi, from the large intestine, one might question the significance of large intestinal P digestion in ruminant nutrition. But large intestinal P digestion does have environmental implications as the form of P excreted in the feces would influence P runoff from the soil after manure application.

When feed phytate was added to the diet ruminal phytate degradation was almost complete and improved with increased dietary phytate indicating that dietary phytate does not negatively influence ruminal phytate digestion. In fact, ruminal phytate digestibility was higher for high phytate diet compared with high Pi diet indicating the positive influence of substrate phytate on the activity of enzyme phytase. Small intestinal net disappearance of Pi was not

affected by dietary phytate. Dietary phytate was degraded in the large intestine but was not affected by increasing dietary phytate intake and was not different for high phytate and high Pi diets indicating constant phytate digestibility in the large intestine. More fecal P was excreted in feces with higher phytate intake. The form of dietary P (high phytate vs. high Pi) did affect fecal excretion of total P, Pp, and Pi but this result was confounded with the increased fecal DM excretion associated with high cottonseed meal diets. Fecal P excretion was primarily regulated by dietary total P rather than by the form of dietary P.

APPENDIX

PREPARATION OF ILEAL CANNULA FOR CATTLE

May 14, 2012

The half T ileal cannula consisted of two pieces: body (barrel) and flanges. The materials used for preparing cannula were Tygon tubing (2.2 cm I.D. × 2.9 cm O.D. and 2.5 cm I.D. × 3.2 cm O.D.), Braided Vinyl tubing (5.1 cm I.D. × 6.4 cm O.D.), cyclohexanone (Fisher, Fair Lawn, NJ), and Dremel tool with cylindrical sand paper attachment (Dremel, Racine, WI).

Two pieces, 15.9 cm and 8.9 cm, were cut from Tygon tubing (2.5 cm I.D. × 3.2 cm O.D.) and a lengthwise slit of 1.3 cm width was cut from each of the pieces. The shorter piece of tubing (8.9 cm long) was placed centrally over the longer piece (15.9 cm long) aligning the lengthwise cuts; the convex surface of the longer piece was in contact with the concave surface of the shorter piece. These two pieces were fused together using cyclohexanone (99.8%) to prepare the flanges; double thickness provided needed strength and support for the cannula. To ensure a tight seal between the pieces, the glued tubing was placed and taped tightly around a 3" diameter wooden rod. At least 24 h was allowed for fusing of Tygon pieces.

A 2.9 cm diameter hole was cut in the middle of the double-layer segment of Tygon piece (the flanges) with a hollow metal punch. The hole was enlarged with the Dremel tool to maintain its circular shape and fit tightly to the Tygon tubing that forms the barrel of the cannula. To prevent injury to the intestine, sharp edges of the flanges were smoothed and rounded using a Dremel tool.

For the barrel of the cannula, a 14 cm long piece of Tygon tubing (2.2 cm I.D. × 2.9 cm O.D.) was cut. One end of the tubing was placed in the hole created in the middle of the flange segment and cyclohexanone was used to fuse the barrel to the flanges of the cannula. To make

the cannula stronger, another layer of Tygon tubing (length 4.4 cm; 2.5 cm I.D. × 3.2 cm O.D.) was fused with cyclohexanone to the barrel at the end where barrel meets the flanges. Prior to fusion the shorter piece of tubing was sanded to a sharp angle on one end and, on the opposite end, a concave curvature to fit the outside curve of the flanges.

An 8.9 cm long and 7 cm wide piece of braided vinyl tubing (5.1 cm I.D. × 6.4 cm O.D.) was prepared and served as a flat washer to prevent the barrel going into the body cavity. A hole of 3.2 cm diameter was made in the middle of the washer and the sharp edges were smoothed using the Dremel tool. The flat washer was slided over the exteriorized barrel of the cannula. A 2 cm long Tygon tubing (2.5 cm I.D. × 3.2 cm O.D.) was cut and served as a ring washer. The inner diameter of the ring washer was increased by applying heat and sanding the inner surface using Dremel tool. The ring washer was slided over the barrel of the cannula to secure the flat washer snugly against the body wall to reduce the movement of the cannula.

SURGICAL PROCEDURE FOR STANDING ILEAL CANNULATION OF CATTLE

May 14, 2012

Cows were fasted for 12 to 24 h prior to surgery. Procaine penicillin G (22,000 IU/kg, IM) and Flunixen meglumine (0.5 mg/kg, IV) were administered 2 h prior to surgery. Surgeries were performed with the animal standing under local anesthesia. Cows were restrained in a chute and head catch. The right paralumbar fossa was clipped from the dorsal midline to the flank fold, and from just caudal to the tuber coxae cranially to the 10th intercostal space. A rough scrub with soap and warm water was used to remove loose hair and dirt.

For the ileal cannulation, anesthesia of the right paralumbar fossa (and the intervertebral space where the cannulae exited) was accomplished by infiltrating the proposed incision sites with 2% lidocaine ("line block"). Following a surgical scrub of the paralumbar fossa with betadine and alcohol, a 60 x 80 cm impervious drape was placed over the dorsum of the animal and secured with towel clamps. A triangular fenestration, patterned by the paralumbar fossa, was created in the following manner. A 20 cm vertical skin incision, starting approximately 10 cm below the transverse processes was extended ventrally with a #10 Bard Parker blade. The incision was positioned 4 cm caudal to the last rib. Each muscle layer was identified and incised separately. The peritoneum was incised with the transverses abdominis muscle. Following fenestration, the ileum was located (generally passing horizontally through the center of the fenestration) and used to identify the cecum was served as a landmark. The ileum was pulled through the incision. A Penrose drain was placed around the small bowel approximately 5 cm proximal to the pylorus, tightened around the bowel, and clamped with a hemostat. A second drain was placed in a similar fashion about 16 to 20 cm proximal to the first, allowing enough space for placement of the cannula. These drains were to prevent continous contamination of the

surgical site once the intestinal incision was made.

A 4 cm longitudinal incision was made into the ileum midway between the Penrose drains using a #10 blade. The incision was extended if necessary with Metzenbaum scissors. During this time, copious lavage of the surgical site was provided with sterile saline solution. A half T cannula was inserted into the ileal incision and pushed toward one end. The intestinal mucosa was sutured using 2-0 synthetic absorbable suture (Vicryl) in a simple continuous pattern. The serosa was reapposed with 2-0 Vicryl in a continuous Lembert pattern. To ensure a good seal between the bowel and cannula a serosal purse string was placed around the base of the cannula using 2-0 Vicryl. Once secured, sterile gauze sponges was placed inside the cannula and a trocar was positioned on the end of the cannula and the Penrose drains were removed. The trochar was secured by a single circumferential ligature of #3 Vetafil.

The cannula exit site was identified by passage of the surgeon's left arm along the peritoneal surface of the abdominal cavity and application of outward digital force to the peritoneal surface. This site was located approximately 6-8 cm below the costochondral junction and cranial to the laparotomy. The external surface of the exit site was subjected to a sterile scrub and locally infiltrated with approximately 10 cc of 2% lidocaine. While applying internal digital force, the skin, muscles, and peritoneum were incised to form a small exit site. If necessaary, the opening was enlarged using blunt dissection with Mayo scissors.

The cannula was passed down along the abdominal wall with the trocar pointed ventrally. Once the cannula was positioned at the exit site, the trochar point was advanced through the incision. It was critical that the cannula fits tightly in the fistula to minimize postoperative problems. The point of the trochar was then removed and the T-handle was secured to the end to assist in pulling the cannula through the exit incision and snugly up against the body wall. Once

the cannula was positioned, the T-handle was removed. Tape (2.5 cm) was wrapped about 1 cm deep around the cannula close to the abdomen to prevent movement of the cannula internally until a peritoneal seal developed following surgery.

The laparotomy incision was closed in a routine three layer closure: peritoneum and transverses, internal, and external abdominal obliques, and skin. A simple continuous suture pattern using #3 chromic gut was used in the first two closure layers. Skin closure was accomplished with #3 Vetafil in a Ford interlocking pattern.