Disappearance of Phosphorus in Phytate from Concentrates In Vitro and from Rations Fed to Lactating Dairy Cows[1]

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ABSTRACT

Objectives were to determine concentrations of P in phytate in selected concentrates, disappearance of P in phytate from these concentrates in vitro, and extent of hydrolysis of phytate in vivo. Total P and P in phytate were determined for eight concentrates; 32 to 81% of total P was in phytate. Six concentrates were incubated in vitro to determine the extent of phytate disappearance from solids and its appearance and disappearance from solution. Greater than 90% of P in phytate disappeared from solids between 6 and 8 h of incubation in vitro (wheat middlings, rice bran, hominy, soybean meal, and dried distillers grains) or between 12 and 24 h (cottonseed meal). Phosphorus in phytate in solution was hydrolyzed by 12 h except for cottonseed meal (by 24 h). Hydrolysis of the inositol ring to release P in vivo was greater than 99%, based on total fecal collection from 11 cows and the use of Cr as an indigestible marker in excreta, and between 94 to 98% for the same samples when acid detergent lignin was used as an indigestible marker to calculate phytate disappearance. These results further indicate that P in phytate should be considered available to lactating dairy cows when rations to meet their P requirements are being formulated.
(Key words: phytate phosphorus, concentrates, in vitro, hydrolysis)

Abbreviation key: ADL = acid detergent lignin.

INTRODUCTION

High producing lactating dairy cows require large intakes of energy, protein, vitamins, minerals, and water to maintain lactation. Typically, their intake of DM from concentrates, such as cereal grains, grain by-products, and grain supplements, exceeds that from forages. Recommended concentration of total P in ration DM for high producing cows is about .41% (15), of which 60 to 80% is from the concentrate portion. Forages are low in content of P in phytate (2, 3, 18), whereas, for concentrates, 50 to 75% of total P is in phytate. For this source of P to be available for absorption and utilization, the inositol ring of phytate must be hydrolyzed by the enzyme phytase (13, 19, 20, 21). Extent and location of this hydrolysis in the digestive tract affect availability of P in phytate for productive uses by lactating dairy cows.

The P in phytate fed to monogastrics may be only partially available for absorption because of low intestinal phytase (16, 20, 21). Including dietary phytase can enhance availability of P in phytate-rich diets, such as when wheat, wheat or rye brans, barley, or triticale was fed to pigs (20, 21). In ruminants, hydrolysis of dietary phytate occurs by action of phytase produced by rumen microorganisms (22) or is of dietary origin. However, availability of P in phytate may be affected adversely by other dietary constituents (7, 16). In sheep, availability of P was reduced when they were fed Ca phytate and also dicalcium phosphate and bone meal or monocalcium phosphate (22). Cows fed highly reactive limestone excreted up to one-half of the dietary P in phytate (12). This presumably occurred because the phytate precipitated as Ca phytate and was less well hydrolyzed.

Newly weaned calves fed concentrate diets containing .5% P, of which 58% was in phytate, had only traces of phytate in their feces because >99% was hydrolyzed (17). Results were similar for steers fed twice daily 1 kg of

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concentrate that contained .47% P, of which 49% was in phytate (17). In an additional study (17), steers deprived of feed for 18 h had no phytate in solids collected from rumen, abomasum, or small or large intestines. Although those authors (17) concluded that hydrolysis had been complete, phytate in the fluids of these compartments was not determined.

Most studies to evaluate the availability of P in phytate have used cattle fed small quantities of grain and DM (<12 kg/d of DM). High producing dairy cows fed phytate-rich diets (38.3 and 42.6 g/d) during early lactation showed almost complete hydrolysis of P in phytate (2). Neither source nor quantity of Ca (.6 and .9% Ca in dietary DM) reduced hydrolysis. For high producing dairy cows, supplementation with excess amounts of P should be avoided because it is costly, wasteful, and a possible environmental pollutant, and it may affect production adversely (1, 10). Therefore, it is important to know the availability to the cow of this source of P in various concentrates in order to formulate rations that are sufficient but not in excess of P requirements (14, 15).

Objectives of the current experiment were to determine concentrations of P in phytate in concentrates commonly used in rations fed to lactating dairy cows, to measure rate and extent of hydrolysis of phytate during in vitro incubation, and to measure in vivo the overall hydrolysis of phytate to release P.

MATERIALS AND METHODS

In Vitro

Concentrates evaluated were cottonseed meal, dried distillers grains, hominy, two sources of rice bran, soybean meal, and four sources of wheat middlings. All samples of concentrates were obtained from a commercial feed mill in southeast Florida (United Feed Cooperative, Okeechobee, FL). Cottonseed meal was sieved through a 2-mm screen to remove any remaining lint; other samples were small enough to pass through the 2-mm sieve.

Rumen fluid was obtained from a lactating dairy cow fitted with a rumen cannula after the morning milking and before the morning feeding. The cow was fed a diet of corn silage and concentrate mix (55:45, wt/wt, DM basis). The concentrate portion included ground corn, soybean meal, and dried distillers grains or whole cottonseed. Rumen fluid was obtained on five separate occasions (batches) and incubated with two concentrates per batch (runs). At collection, rumen fluid was placed in a preheated thermal jug and transported immediately to the laboratory, where it was mixed (1:4, vol/vol) with McDougall's buffer (23). After mixing, CO_2 was bubbled through the solution to adjust pH to 6.97 ± .01. Then 150 ml of solution were dispensed into 250-ml Erlenmeyer flasks containing 3 g (dry basis) of a concentrate. Solution and feed were mixed by gentle swirling, air in flasks was displaced with CO_2, flasks were stoppered with one-way valves, and flasks were placed in a 39°C incubator. Twenty-four flasks per concentrate were incubated with rumen fluid plus McDougall's buffer for each batch, and 3 each were removed after 1, 2, 4, 6, 8, 10, 12, or 24 h of incubation. Only 18 flasks were incubated during the first two runs; no samples were removed at 1 and 24 h. At each time interval, when flasks were removed from the incubator, remaining flasks were swirled gently. A set of samples (in triplicate) also was incubated for 24 h in McDougall's buffer alone to determine loss of P in phytate attributed to activity of phytase in the concentrate. At the time flasks were removed, they were unstoppered, pH was measured, and 12*N* HCl was added dropwise to kill bacteria and to inactivate phytase (pH <2). The pH was measured during the incubations, which contained the final three of the five collections of rumen fluid (six concentrates). Flask contents were transferred to 50-ml nalgene tubes and centrifuged (1020 × *g*, 15 min). All tubes corresponding to one flask were filtered through a common coarse filter paper (number 41, Whatman, Clifton, NJ). Filtered solids were rinsed three times with deionized distilled water and finally with acetone. After final rinsing, the filter paper containing the solids was rolled and placed in a 50-ml nalgene tube and then dried overnight at 50°C. Filtrate was stored in nalgene bottles. For the 2-, 6-, and 24-h incubations in three runs and also for the 1- and 12-h incubations for the other two runs, duplicate 10-ml samples of filtrate were used for determination of P in phytate, and duplicate samples of incubation solution were used to quantify P in phytate in

TABLE 1. Least squares ANOVA of variation in concentrations of P in phytate in concentrates after varying incubation times in batches of rumen fluid plus McDougall's buffer.

Source	df	SS Type III	F	P<
Batches (B)	4	21,339	1.00	.4855
Feed in B[1]	5	26,661	196.33	.0001
Hour (H)	7	61,854	26.22	.0001
B × H	25	26,051	3.09	.0015
Feed in B × H[2]	32	10,786	12.41	.0001
Residual	303	8229		

[1]Error term for batch.

[2]Error term for hour and batch × hour.

the absence of feed (blank). Quantity of P in phytate in filtrate was corrected for quantity in blank tubes.

Phytate in dried solids was extracted by the method of Earley (5), except that 1.2% HCl containing 10% Na_2HSO_4 was added (20 ml/g of dried sample) before tubes were capped and placed on the shaker for 6 h at room temperature. Although the method called for 2 h of shaking (5), no difference in extraction of phytate resulted when samples were shaken for 2, 4, or 6 h. Ten milliliters of extract were placed in each of two nalgene tubes, and phytate was precipitated with 5 ml of .4% $FeCl_3$ in .6% HCl containing 5% Na_2SO_4 (5). Tubes were heated in a 100°C oven for 60 min and then cooled on ice. A white precipitate formed that contained di- to hexaphosphates (4). Tubes were centrifuged (1020 × *g*, 15 min), supernatants decanted, and precipitates rinsed with .6% HCl containing 5% Na_2SO_4. Centrifugation and rinsing were repeated three times, and then precipitate was transferred into a 30-ml Pyrex beaker (Corning Glass Works, Corning, NY) using deionized distilled water. Beakers were placed in a 100°C oven until samples were dry, and then samples were ashed at 550°C for 4 h. Concentration of P was determined by the method of Harris and Popat (9) as described by Fick et al. (6). Sodium phytate from corn was used as the standard for the phytate procedure (Sigma Chemical Co., St. Louis, MO). Calculated concentration of P in phytate in standard was 229.6 mg/kg. The P in phytate measured was 228.4 ± 3.6 mg/kg (mean recovery was 99.5%).

In Vivo

Digestibility of P in phytate was determined by total fecal collection of 11 Holstein cows (>60 DIM) during wk 9 of a digestion trial (14). Ingredient composition of basal diet fed as a TMR was corn silage, ground corn, cottonseed hulls, soybean meal, urea, trace-mineralized salt, magnesium oxide, and $CaCO_3$. Dicalcium phosphate was added to provide P intakes of 60 to 120 g of P daily, of which 34 g were in phytate. Fecal samples were analyzed for concentration of P in phytate, and digestibility of P in phytate was calculated as the difference between quantities in feed and feces. Digestibility also was determined using acid detergent lignin (ADL) and Cr (8, 11, 14, 24) as indigestible markers. Concentrations of ADL, Cr, and P in phytate in diets fed were 6.0, .075, and .17% of dietary DM. Additionally, 20 fecal samples collected from dairy cows on two south Florida dairies during a field study were analyzed for lignin and concentrations of P in phytate (8). The estimated forage:concentrate ratio fed on these dairies was 20:80, mean concentration of P in phytate in diets fed was .38% of dietary DM (P in dietary DM was .41 to .55%), and total intake of P was between 80 and 120 g/d. For the diets fed on these dairies, lignin comprised 6.1 or 5.0% of dietary DM. Fecal DM (3 g) was extracted using 40 ml of 1.2% HCl containing 10% Na_2SO_4, and the chemical analysis of phytate and P was as described.

Statistical Analyses

Amount of P in phytate was determined after in vitro incubation with rumen fluid plus McDougall's buffer (1:4, vol/vol) for up to 24 h. The first mathematical model used was a split-split-plot design with true replication (Table 1). Because no significant difference was found from five different batches of rumen

TABLE 2. Least squares ANOVA of concentrations of P in phytate in concentrates after varying times in an in vitro incubation.

Source	df	SS Type III	*F*	*P*<
Feed (F)	9	51,544	93.54	.0001
Hour (H)	7	60,409	140.95	.0001
F × H	57	37,769	10.82	.0001
Sample (F × H)[1]	128	7837	27.32	.0001
Rep[sample (F × H)][2]	175	392		

[1]Error term for those above.

[2]Error term for sample (F × H); Rep = replicate.

fluid used to prepare the incubation solution ($P > .49$), batch was deleted from the model. The reduced model permitted separation of components of the combined overall error term (Table 2). Mean square error was 2.24, and estimated sample variance was 31.6 with k value of 1.9. The k is the number of observations associated with one cell of that variance source for the balanced case (equal numbers); with unequal numbers, the k values are estimated by equation. Based on estimates of sample variance and mean square error, it was possible to estimate variance of the mean for various sampling schemes. As number of samples of a feed analyzed increased from 1 to 4, estimates of variance of the mean decreased from 33.8 to 18.0, 12.7, and 10.1. If each of these samples were replicated twice, estimates of variance of the mean would be 32.7, 16.9, 11.6, and 9.0. Based on availability of equipment, triplicates of each sample were incubated in vitro, and replicates were made prior to determination of phytate. For this procedure, variance of the mean was 11.6. Greatest reduction in variation would occur by increasing the number of samples per feed by hour combination (Table 2).

RESULTS AND DISCUSSION

In Vitro

Concentrations of P in phytate for concentrates sampled ranged from 1900 to 12,560 mg/kg of DM and total P from 2980 to 15,780 mg/kg of DM (Table 3); 32.0 to 80.7% of total P was in phytate. Dried distillers grains had the lowest concentration of P in phytate (32.0%); other sampled concentrates were greater than 55%, which is similar to reported concentrations (3, 18). Common (3) reported P in phytate, as a percentage of total P, for wheat (77.6%), oats (74.3%), corn meal (78.3%), bran (77.7%), and extracted soybean (53.9%). In contrast, percentages of total P in phytate were less than .01% for alfalfa hay; bermuda, brome, orchard, or fescue grasses (17); and corn silage (2).

Although effects of batch of rumen fluid on hydrolysis of phytate were not significant when the same concentrates were incubated in more than one batch, batches were considered to be different for analytical purposes to observe variation for repeated analysis of the same sample. Least squares means for disappearance of P in phytate from incubated solids were expressed as the percentage of total P initially in phytate (Table 4). Percentage of P disappearance in phytate at 1 h was greater than that at 2 or 4 h for the same concentrate. The reason for this finding is unknown. As incubation time increased from 2 to 24 h, percentage of P disappearance in phytate increased for most concentrates. Results for cottonseed meal were least consistent. Percentages of P disappearance in phytate for 1, 2, 4, 6, and 8 h were 35.2, 48.7, 17.8, 57.0, and 39.6%. Variation in mixing of cottonseed meal prior to weighing it into flask, inadequate mixing by swirling of fluid and concentrate, or high protein content may account, in part, for the variation observed. Other inconsistencies occurred for different concentrates, but no general trend was evident (Table 4). The pH of incubation fluid at 24 h for cottonseed meal (6.80), two samples of rice bran (6.62 and 6.43), wheat middlings (6.43), and for hominy after 12 h (6.38) did not indicate abnormal patterns of fermentation although there was some variation among samples. Although this method (23) was developed for use with forages, concentrates appeared to have been fermented well, based on pH values obtained

TABLE 3. Concentration of total and phytate (Phy) P in concentrates commonly fed to dairy cows.

Concentrate	n[1]	Total P	Phy P	Phy P	(18)[2]
		(mg/kg of DM)		(% of total P)	
Cottonseed meal	2	12,220	8480	69.4	70
Dried distillers grains	2	8990	2880	32.0	43
Ground corn	3	2980	1900	63.8	66
Hominy	3	6380	4680	73.4	. . .[3]
Peanut meal	2	6650	3800	57.1	. . .
Rice bran	2	15,780	12,560	80.7	86
Soybean meal	2	7320	5020	68.6	58
Wheat middlings	3	13,400	10,480	78.2	74

[1]Number of samples analyzed in triplicate.

[2]Reported concentrations of P in phytate as percentage of total P (18).

[3]No data available.

over the 24 h. Slower disappearance of P in phytate from concentrates may have resulted, in part, from high protein content and chemical changes induced during processing. Fontaine et al. (7) reported that, when protein concentration was high, P in phytate was less available for animal use. Release of P in phytate also was slower for soybean meal at 6 h (50.2%). Chemical bonds formed between protein and P in phytate must be broken before phytase can liberate inorganic P from phytate (7).

The rapid loss of P in phytate from incubated solids for concentrates evaluated, except for cottonseed meal (>88% by 8 h; Table 4), agreed with in vivo estimates in sheep (22). When P in phytate was quantified in solid contents of rumen and reticulum from sheep slaughtered 1 and 8 h postfeeding, none was detected in the 8-h sample postfeeding. However, because fluids in the various compartments of the digestive tract were not analyzed, phytate may possibly have been liberated from ingredient but was unhydrolyzed in solution.

Hydrolysis of P in phytate in incubation solution also was determined in duplicate samples of filtrate after 1, 2, 6, 12, and 24 h of incubation and after 24 h of incubation in McDougall's buffer alone. Least squares means of concentrations in filtrate were expressed as milligrams per kilogram of original

TABLE 4. Least squares means of disappearance of P in phytate (Phy) from solids relative to hours of incubation in vitro expressed as percentage of initial P in Phy.[1]

	Initial Phy P									
	WM1	WM2	WM3	WM4	RB1	RB2	HOM	SBM	DDG	CSM
	10,130	10,120	10,100	10,140	12,550	13,360	3900	5020	2210	7340
(h)	(%)									
1	. . .[2]	. . .	. . .	66.4	. . .	85.1	90.6	. . .	. . .	35.2
2	35.9	84.6	79.9	60.7	80.6	85.3	52.5	36.3	71.3	48.7
4	57.5	87.8	83.1	59.6	77.3	78.4	63.5	50.2	96.1	17.8
6	66.1	94.9	75.0	97.0	92.6	82.6	99.0	50.2	95.4	57.0
8	99.7	95.9	88.4	90.0	96.8	90.8	98.8	99.5	95.4	39.6
10	99.9	96.0	96.9	93.6	98.8	94.4	98.6	99.9	98.9	66.5
12	99.9	98.2	98.9	96.5	99.6	98.1	99.4	99.9	98.7	71.5
24	100.0	99.8	99.9	99.9	99.9	99.8	99.8	100.0	99.3	99.9
24[3]	. . .	58.6	. . .	40.6	. . .	57.3	40.1	. . .	81.0	14.7

[1]Concentrates: WM = wheat middlings, RB = rice bran, HOM = hominy, SBM = soybean meal, DDG = dried distillers grains, CSM = cottonseed meal. Numbers after concentrate abbreviations represent separate incubations of similar concentrates. Incubation media was rumen fluid in McDougall's buffer (1:4, vol/vol) (23).

[2]No data available.

[3]Sample incubated in McDougall's buffer alone for 24 h.

TABLE 5. Least squares mean concentrations of P in phytate (Phy) in solution during in vitro incubations.[1]

	Initial Phy P					
	WM2[2]	WM4	RB2	HOM	DDG	CSM
	10,120	10,140	13,360	3900	2210	7340
(h)						
1	. . .[3]	10,060	17,710	1620	. . .	4750
2	8240	7530	18,030	960	1320	4290
6	5160	7540	13,062	180	920	4720
12	. . .	200	6	0	. . .	590
24	40	80	0	0	70	0

[1]Incubation media consisted of rumen fluid and McDougall's buffer (1:4, vol/vol) (23).

[2]Concentrates: WM = wheat middlings, RB = rice bran, HOM = hominy, DDG = dried distillers grains, CSM = cottonseed meal. Numbers after concentrate abbreviations represent separate incubations of similar concentrates.

[3]No data available.

DM (Table 5). Variations caused by sample and replication (sample) were 258,403 and 89,055, respectively. Total recovery of P in phytate (that remaining in solid residue plus that in filtrate) was greater than initial P for wheat middlings at 1 and 2 h; rice bran at 1, 2, and 6 h; and cottonseed meal at 1 h. Reasons for this finding are not known. Importantly, variation for measurement of P in phytate in the filtrates was greater than for solids with SE of 130 to 250 mg of P/kg of DM. By 6 h of incubation, concentration of P in phytate in solution had decreased for all concentrates, and, by 12 h, virtually all P in phytate (>95%) had been hydrolyzed.

In Vivo

Disappearance of P in phytate from solids and from solution in vitro indicated that there should be high availability of this source of P for absorption in the lower digestive tract of the cow (12). This was evaluated by measuring P in phytate in fecal samples collected during wk 9 of a digestion trial (14). Concentration of P in phytate in the ration fed to the cows was .17% of total dietary DM. Trace amounts of P in phytate were recovered in the feces. Digestibility of P in phytate was greater than 99%, based on actual differences between intake and excretion and by use of Cr. Use of ADL as an indigestible marker indicated that 94 to 98% of P in phytate was digested. Slightly lower values obtained from use of ADL are consistent with previous findings and probably occurred because less than 100% of ADL was recovered from contents of the gastrointestinal tract. Decreased marker recovery underestimates digestibility of the feed. Overall, these results agreed with those in which greater than 98% of P in phytate was hydrolyzed in the digestive tract of high producing dairy cows fed a ration supplemented with three different sources of Ca and containing .22% of dietary DM as P in phytate (2).

Twenty fecal samples from cows on southern Florida dairies fed diets with high concentration of P in phytate (.38% of dietary DM) also were analyzed. Cows on those dairies were fed throughout the year concentrates that contained varying amounts of cottonseed hulls, malt sprout pellets, hominy, whole cottonseeds, wheat middlings, soybean meal, rice bran, and corn meal. Forages fed were corn silage, sorghum silage, or green chop. Digestibility of P in phytate, estimated using ADL as an indigestible marker, ranged from 79.8 to 98.8%; mean digestibility was 94%. Data are included (Table 6) to indicate variability observed. Thus, P in phytate was hydrolyzed in vivo even when cows were fed a high concentration of P in phytate and a total of 60 to 120 g of P daily.

Results of the current study and those of Clark et al. (2) indicate a rapid and high degree of hydrolysis of P in phytate of rations fed to lactating dairy cows. The rate and extent of hydrolysis in vitro may be less than maximal (Tables 3 and 4) because a diluted sample of rumen fluid was incubated with the concentrates. Phytase of rumen microorganisms (22) and that associated with the concentrates (20,

TABLE 6. Total and phytate (Phy)-bound P and acid detergent lignin (ADL) concentrations in feces of dairy cows fed a diet containing .38% Phy P (percentage of dietary DM) on commercial dairies.

Cow	Total P	Phy P	ADL[1]	Digestion coefficient[2]
	(% in feces)			
1	1.19	.17	13.5	79.8
2	.99	.11	14.3	87.7
3	.46	.03	15.4	96.9
4	.89	.10	15.0	89.3
5	.80	.04	13.4	95.2
6	1.03	.04	14.6	92.3
7	1.05	.05	14.4	94.4
8	1.00	.01	13.5	98.8
9	.59	.03	16.2	97.0
10	1.05	.03	13.6	96.5
11	.71	.04	12.6	94.9
12	.69	.02	14.2	97.7
13	.72	.12	13.1	85.3
14	1.35	.04	16.3	96.8
15	.75	.03	16.4	97.6
16	.65	.04	16.5	96.8
17	.92	.04	17.6	97.0
18	1.13	.04	17.8	97.0
19	.83	.06	17.8	95.6
20	1.10	.07	15.8	94.2

[1]Mean percentages of ADL in feed were 6.1 for cows 1 to 13 and 5.0 for cows 14 to 20.

[2]Digestion coefficient determined as 100 – [(percentage of ADL in feed ÷ percentage of ADL in feces × percentage of Phy P in feces ÷ percentage of Phy P in feed)] × 100.

21) are responsible for the hydrolysis in vitro and in vivo. Apparently, the size of the feed particles incubated in vitro and in the digestive tract of lactating cows was small enough and did not limit hydrolysis. The liberation of P from phytate occurred for both solid and fluid fractions, indicating that the P was available for absorption in the small intestine (10). Some reduction in both the rate and extent of hydrolysis of phytate can occur because of changes in the concentrates from processing (7, 16). However, there was no indication in vivo that other ration components fed to the lactating cows had prevented complete digestibility, although it cannot be ascertained that all hydrolysis, as measured by fecal concentration of P in phytate, had occurred in the upper digestive tract so that complete absorption resulted. Although high concentrations of Ca in the diet may reduce hydrolysis because of formation of Ca phytate, which precipitates and is less well hydrolyzed (17, 22), increasing diet concentrations of Ca from .6 to .9% did not reduce phytate hydrolysis (2).

As used in the current study, the in vitro method showed that the P in phytate of individual concentrates high in proportion of P in phytate was rapidly and essentially fully hydrolyzed after 12 to 24 h in the incubation solution. These results were consistent with the almost complete disappearance of P in phytate in vivo; only traces were found in fecal matter. With further testing, the in vitro system may prove to be an effective method to evaluate the interaction of various concentrates on the rate and extent of hydrolysis of phytate that might be expected to occur in vivo. We conclude that essentially all P in phytate in concentrates commonly used to formulate rations for high producing dairy cows should be considered to be available for absorption and used to meet their daily requirements.

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