DIGESTIBILITY OF PHYTIN PHOSPHORUS AND INTESTINAL PHYTASE IN DAIRY COWS AND SHEEP

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The experiments described in the previous paper[17] showed that the feeding of turnip roots or raw beet-tops produces a severe hypophosphataemia in dairy cows. In these cases, however, supplements of rice bran or wheat bran, which are concentrates containing phytin phosphorus in abundance, prevented the disorders caused by the hypophosphorosis.

HARRISON et al., HOFF-JØRGENSEN et al.[8,9,10] and other authors[11,25] reported that phytic acid phosphorus is poorly utilized by animals; the feeding of concentrates containing phytate in abundance produces metabolic disorders of bone caused by the decrease of calcium retention, because the acid forms insoluble salts of calcium, magnesium, iron and other metals in the small intestine. On the contrary, FINGERLING, MATHUR, and TILLMAN and BRETHOUR have reported that phytic acid phosphorus is absorbed from the intestinal canal according to the dietary conditions in ruminants.

The most reliable determination of the digestibility of phytin phosphorus can be obtained from dietary experiments with labelled phytate[24] and from the detection of phytase activity in the intestinal canals. The experiments described in the present paper show that the major part of dieted phytin phosphorus is hydrolyzed into soluble inorganic phosphate in the small intestine of the ruminants, and that the hydrolysis is catalysed by a phytase which is contained in the intestinal contents. Furthermore, the author describes several experiments concerning fundamental characteristics of the enzyme.

MATERIALS AND METHODS

Experimental animals

Four castrated adult sheep were used for the dietary experiment. Daily collections of the feces were made with a vinyl sac coated with cotton placed under the anus. Each animal was fed the experimental diet daily during successive 14-day preliminary and 7-day collection periods. The constituents of experimental diet are shown in table 1; the daily intake of phytate phosphorus and other phosphorus compounds is shown in table 2.

The gastrointestinal content, from which the specimens of phytase and alkaline

TABLE 1. *Constituents of Experimental Rations*

RATION	MOISTURE	CRUDE PROTEIN	PHOSPHORUS	
			Total	Phytic
	%	%	mg %	mg %
Wheat bran	12.9	16.5	1305	830
Beet-tops	80.5	4.75	81	Trace
Hay	—	9.1	550	Trace

TABLE 2 *Digestibility of Phytin Phosphorus*

RATION	WHEAT BRAN AND HAY		WHEAT BRAN AND BEET-TOPS		HEAT TREATED WHEAT BRAN AND HAY	
	Sheep				Controlled	Examined
	No. 1	No. 2	No. 3	No. 4		
Intake (g/day/10 kg of body weight)						
Dry matter	269	139	311	293	97	97
Crude protein	31	25	53	45	11	11
P, acid soluble	1.69	1.69	2.19	2.03	0.63	0.63
P, phytic	1.25	1.25	1.25	1.25	0.47	0.47
P, inorganic	0.25	0.18	—	—	0.09	0.09
Excretion (g/day/10 kg of body weight)						
P, acid soluble	1.71	1.39	1.64	1.35	0.48	0.60
P, phytic	0.06	0.02	0.06	0.02	0.01	0.02
P, inorganic	1.49	1.31	1.46	1.23	0.45	0.51

phosphatase were prepared, was estimated from 25 dairy cows of the Sapporo slaughter house and 5 farm sheep. The samples were taken from the gastrointestinal canals immediately after the animal was slaughtered. In these samples, 5 cases of the cows and 3 cases of the sheep were used for the estimation of the pH of the gastrointestinal content.

Preparation of the enzyme solution

An aliquot of intestinal content collected from the anterior sac of rumen, abomasum, and small intestine respectively was centrifuged to remove the bulk of solid ingesta from each. After a few drops of toluol were added, the supernatant was dialyzed against tap water for 48 hours and further against distilled water for 24 hours, thereafter the volume of the dialyzed solution was measured. An aliquot of the solution was used for the estimation of the enzymatic activity.

Preparation of the enzyme powder

The partially purified enzyme powder was used for the comparison of enzymatic characteristics between the phytase and alkaline phosphatase of small intestinal contents and the phytases of rice and wheat bran. The dialyzed enzyme solution of intestinal

content was prepared by the method described above. The extraction of the enzymes from rice and wheat bran and the dialysis of the extracted solution was carried out by the method of PEERS. Partial purifications of the extracted enzyme solution were carried out as follows: One part of the enzyme solution was poured with vigorous stirring into 9 parts of ice-cold ethanol, allowed to stand 30 minutes, and then the bulk of the supernatant was decanted. The precipitate was collected on a filter paper and allowed to dissolve with a small volume of distilled water. The ethanol-precipitation procedure was repeated twice. Then, the obtained enzyme solution was dialyzed against tap water to remove ethanol. Two parts of the dialyzed solution were poured into one part of ice-cold acetone with vigorous stirring. After the precipitate was decanted, two parts of the filtrate were poured into one part of ice-cold acetone with vigorous stirring. The precipitates were dissolved with distilled water and the precipitating procedure with acetone was repeated again. The obtained precipitates were washed with acetone and dried *in vacuo*.

Preparation of sodium phytate

About 1 kg of rice bran was added into 8 L of 1.5% HCl solution, allowed to stand over-night with occasional stirring, and the bulk of insoluble material was removed by filtration. One hundred and twenty g of barium acetate were added into 3 L of the filtrate, containing phytin, with vigorous stirring and the formed white precipitate of barium phytate was deposited by still standing. The precipitates were collected on a filter paper, washed by suspension in water and filtration with a filter paper; the washing procedure was repeated 3 times. The washed barium phytate was dissolved in 2 L of 3% HCl solution and allowed to decolorize with three 80 g portions of active carbon. So the decolorized solution was added an equal volume of 96% ethanol, allowed to stand over night, and the precipitate was collected on a filter paper. After the procedure was repeated 2 or more times, the precipitate, purified barium phytate, was washed with ether and dried *in vacuo*. Twenty g of the purified barium phytate was dissolved in 1 L of 1.5% HCl solution and 25 ml of saturated ferric chloride solution were added with vigorous stirring to form the precipitate of ferric phytate. Then, the precipitate was collected on a filter paper and washed repeatedly with distilled water. The washed precipitate was suspended in 800 ml of distilled water and 100 ml of 20% sodium hydroxide solution were added to form sodium phytate. The brownish precipitate of ferric hydroxide was removed by filtration. The resultant colorless solution was poured into twofold volume of ethanol with stirring, and allowed to stand until the deposition of sodium phytate was completed. The deposited material was dissolved in 100 ml of distilled water, 200 ml of 96% ethanol were added and the preparate was allowed to stand as described above. This procedure was repeated 3 times. The final ethanol-mixture was warmed by a water bath to remove ethanol thoroughly. Sodium phytate was deposited from the solution in a refrigerator. The yield was about 70%.

Found,

Phytic Phosphorus	13.3 %	
Na	20.5	
H_2O	29.0	(Dried at 100°C, for 5 hours in an oven)
Orthophosphate Phosphorus	Less than 0.001 %	

Enzyme assay

Reaction mixtures were prepared as follows:

Buffer solution	5.0 ml
Substrate solution	2.5
Activator (inhibitor or H_2O)	1.0
Enzyme solution	1.5

For the comparison of the phytase activities in the contents of the various compartments of the gastrointestine, acetate buffer, pH 5.8, was used in preparation of the reaction mixture. The final pH of the reaction mixture prepared with acetate buffer, viz., 5.8, is about 6.3, which appears to be the optimum pH of the intestinal phytase as reported below. The buffer solutions used in the other experiments were as follows:

pH 3.5~ 5.8	0.1 M acetate buffer
pH 6.8~ 8.8	0.05 M barbiturate buffer
pH 9.2~10.5	0.05 M carbonate-bicarbonate buffer

Besides these solutions, acetate-barbiturate buffer[15] was used for the determination of the optimum pH of the enzyme.

The actual pH of the prepared reaction mixture was checked from lot to lot.

Phytase unit

In the present paper, one phytase unit was defined as the amount of enzyme releasing 1 mg of orthophosphate phosphorus from 0.5×10^{-3} M sodium phytate solution, containing 0.001 M magnesium chloride, at pH 6.3, 38°C and in 60 minutes.

Method of estimation

MCCANCE and WIDDOWSON's method with slight modification was used to determine the phytate phosphorus. Orthophosphate phosphorus was determined by the method of FISKE and SUBBAROW. Acid soluble phosphate was extracted with aliquots of 10% trichloroacetic acid and cinerated with 10 N sulfuric acid and concentrated hydrogen peroxide solution. The orthophosphate content of the cinerated solution was estimated by the method described above. Alkaline phosphatase activity was estimated by the method of BESSAY et al., and the estimation of pH was carried out by the use of a glass electrode pH meter (Toyorika Co. Ltd., Model GA).

RESULTS

I. Digestibility of phytin phosphorus

As shown in table 2, the sheep, which were dieted with 1.25 g of phytin phosphorus per 10 kg of body weight daily as wheat bran, excreted only 0.02 and 0.06 g of phytin phosphorus in feces per day. This finding reveals that dieted phytin phosphorus had been hydrolysed in the gastrointestinal canals of the sheep. Feeding of wheat bran which contained 1.25 g of phytin phosphorus together with beet-tops daily gives the same results. They show that the feeding of beet-tops did not affect the digestibility of phytin phosphorus in the animals examined. Further, the sheep whose daily intake of phytin phosphorus was 0.47 g per 10 kg of body weight as heat-treated wheat bran and hay, excreted only 0.02 g

of phytin phosphorus in feces per day. This finding shows that the hydrolysis of phytin phosphorus in the gastrointestinal canals of sheep was carried out by a hydrolysing enzyme originating from the digestive organs or alimentary microflora, because the plant enzyme had been inactivated by the heating.

II. Estimation of phytase and alkaline phosphatase activity in the contents of gastrointestinal canals

Phytase activity

As shown in table 3, the major part of phytase activity occurs in the contents of the small intestine, especially upper and middle parts (duodenum and jejunum), ranging 15~60 units/dl in the cows and 5~45 units/dl in the sheep. The activity decreases gradually in the content of the under part of the small intestine and cecum. In the content of the rumen, abomasum and colon, the enzyme exerts only a feeble action, if any, on the substrate.

TABLE 3. *Phytase Activity in the Contents of the Gastrointestine* (unit/dl)

ANIMAL	EXP. NO.	RUMEN	ABOMASUM	SMALL INTESTINE, PART OF			CECUM	COLON
				Upper	Middle	Under		
Dairy cows *	1	0	0	35	20	5	2	0
	2	0	0	15	38	10	3	0
	3	0.01	0	45	30	10	5	0
	4	0	0	25	40	5	2	0
	5	0	0	60	55	10	5	0
Sheep	1	0.02	0	5	25	7	0	0
	2	0	0	15	20	5	0	0
	3	0	0	30	25	10	5	0
	4	0	0	10	45	15	3	0
	5	0	0	20	35	13	0	0

* Each experimental datum shows the mean value of phytase activity of 5 cases examined.

Alkaline phosphatase activity

As shown in table 4, alkaline phosphatase activity is detectable in the content of the rumen, but the activity is feeble. In the content of the upper and middle parts of small intestine, the activity is higher than in the other parts of alimental canals examined, ranging 1,250~9,800 units in the cows and 900~5,800 units in the sheep. The activity decreases in the contents of the under part of the small intestine, cecum and colon gradually, but the activities there are higher than in rumen and abomasum.

The distributions of the activities of phytase and alkaline phosphatase upon the contents of the gastrointestinal canals of the dairy cows and the sheep are almost the same as described above. Feeble or negative activity of phytase upon the rumen and cecum contents, which have large numbers of microflora, is suggestive that the enzyme originated

TABLE 4. *Alkaline Phosphatase Activity* in the Contents of the Gastrointestine*

ANIMALS	EXP. NO.	RUMEN	ABOMASUM	SMALL INTESTINE, PART OF			CECUM	COLON
				Upper	Middle	Under		
Dairy cows**	1	0.4	0.9	4400	5600	700	12.8	5.6
	2	1.5	1.5	6800	4500	830	18.5	3.6
	3	0.3	0.3	1250	3800	650	10.5	6.2
	4	6.5	2.1	8700	9800	1050	30.5	10.5
	5	5.2	1.5	5500	6500	780	21.0	5.7
Sheep	1	4.5	2.1	1250	3600	550	30	5
	2	6.7	1.0	2700	5800	1050	25	7
	3	3.5	0.5	1500	3300	330	20	15
	4	8.2	4.2	3300	2100	980	10	4
	5	1.5	0.3	900	4300	780	40	18

* Expressed in BESSAY's units.

** Each experimental datum shows the mean value of phytase activity of 5 cases examined.

not from these microflora but from another source (s), such as intestinal mucosa or digestive juice. Recently the author has detected the enzyme activity from intestinal mucosa[18].

III. Comparison of the phytases prepared from intestinal content, rice bran and wheat bran

Optimum pH

The pH-activity curves of phytases prepared from intestinal content, rice bran, wheat bran and alkaline phosphatase of intestinal content are shown in the figure. These show the optimum pH of activity to be about 4.6 for rice bran phytase, 5.2 for wheat bran, and 9.0~9.5 for alkaline phosphatase of intestinal content respectively, while that of phytase of intestinal content is 6.3. These differences indicate that each of the tested enzyme has its own independent nature.

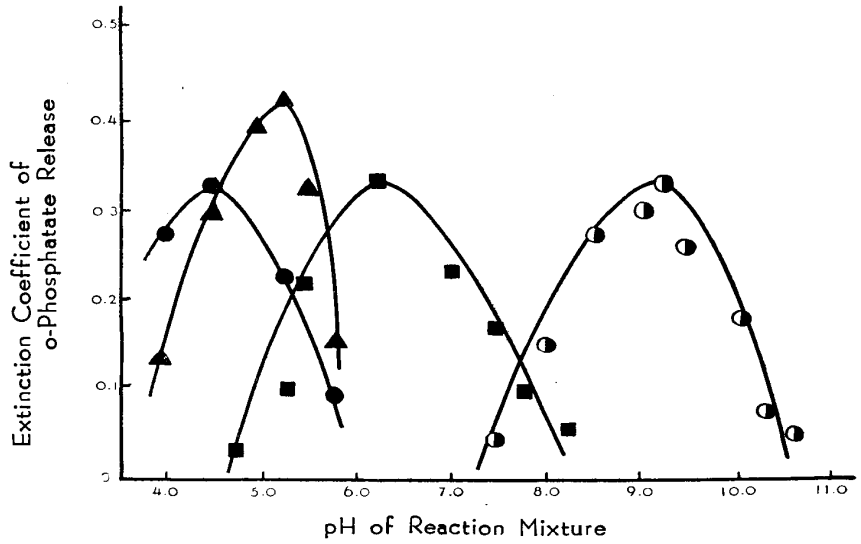
Optimum temperature

As shown in table 5 the three enzymes tested have the same optimum temperature 55°C. These enzymes show activity at 55°C to be about twice as great as at 37°C.

Effect of various salts on the activity of the phytases

This series of experiments was carried out as usual except that no magnesium chloride was added. The final concentrations of tested salts were as recorded in table 6. It is seen that sodium acetate, sodium citrate and sodium cyanide partially inhibit the activity of the enzyme of intestinal content whereas mercuric chloride and calcium chloride completely inhibit it. Calcium ions probably act by removing the phytate ions from the solution, because the white precipitate of calcium phytate formed in the reaction mixture

FIGURE. *The Effect of pH on the Activity of the Phytases Prepared from the Small Intestinal Contents and Brans of Wheat and Rice and of Alkaline Phosphatase of Small Intestinal Contents*



Notes:
- ●——● Rice bran phytase
- ▲——▲ Wheat bran phytase
- ■——■ Phytase of small intestinal content
- ◑——◑ Alkaline phosphatase of small intestinal content

TABLE 5, *Effect of Temperature on the Activity of the Phytases Prepared from the Small Intestinal Content, Wheat Bran and Rice Bran*

TEMPERATURE	SMALL INTESTINAL CONTENT	WHEAT BRAN	RICE BRAN
°C	% of maximal activity		
30	30	42	31
37	40	52	42
45	50	69	52
50	86	81	69
55	100	100	100
60	74	56	42

when the calcium ions were added. Postassium oxalate has no effect on the activity of the enzyme. On the contrary, magnesium chloride, especially at a concentration of 0.001 M, apparently activates the enzyme.

The phytase of the wheat bran is activated by potassium oxalate but not by magnesium

TABLE 6. *The Effect of Various Salts on the Phytase Activity of Small Intestinal Content and Brans of Wheat and Rice*

SALTS ADDED	FINAL CONCENTRATION	ENZYMES OF		
		Small intestinal content	Wheat bran	Rice bran
	M	Extinction coefficient of o-phosphate released		
Control		0.200	0.188	0.370
Sodium acetate	0.15	0.170	0.154	0.120
Potassium oxalate	0.01	0.200	0.202	0.220
Sodium citrate	0.01	0.120	0.188	0.120
Sodium cyanide	0.01	0.020	0.125	0.070
Sodium fluoride	0.01	0.220	0.075	0.370
Mercuric chloride	0.002	0.050	0.006	0.070
Calcium chloride	0.01	0.020	0.113	0.150
Magnesium chloride	0.01	0.240	0.188	0.220
	0.001	0.280	0.188	0.330
	0.0001	0.220	0.183	0.320

chloride. Sodium acetate, sodium cyanide, sodium fluoride and calcium chloride partially inhibit the activity of the enzyme, and mercuric chloride completely inhibits it.

The phytase of the rice bran is inhibited by all the tested salts except sodium flouride and magnesium chloride which had not any definable effect on the enzyme examined.

IV. Estimation of the pH value of intestinal content in the dairy cows and the sheep

As shown in table 7, the pH values of the gastrointestinal contents of both species examined are as follows: The rumen contents are neutral, ranging 6.3~7.5 in the cows

TABLE 7. *The pH Values of the Gastrointestinal Contents of Dairy Cows and Sheep*

ANIMALS	EXP. NO.	RUMEN	ABOMASUM	SMALL INTESTINE, PART OF			CECUM	COLON
				Upper	Middle	Under		
Dairy cows	1	6.8	3.5	6.5	7.1	7.5	7.8	7.5
	2	7.1	5.5	6.8	7.3	8.1	8.5	7.8
	3	6.4	4.0	7.1	7.5	7.9	8.1	7.9
	4	7.5	5.8	6.7	7.2	7.8	7.8	7.2
	5	6.3	4.5	6.5	7.2	7.9	8.2	7.5
Sheep	1	6.8	3.3	—	—	—	—	—
	2	6.3	4.9	6.8	6.9	8.3	7.9	7.8
	3	6.4	4.3	7.0	7.0	7.9	7.7	7.7

and 6.3~6.8 in the sheep; the abomasum contents are acidic, ranging 3.5~5.8 in the cows and 3.3~4.9 in the sheep; the contents of the under part of small intestine and cecum are alkaline, showing 8.5 as the highest value; and the contents of colon decrease in pH values to neutral again. It is interesting that the pH values of the contents of the upper part of small intestine, ranging 6.5~7.1 in both species, agreed with the optimum pH of the phytase prepared from the same part of intestine.

Discussion

Owing to the formation of insoluble metal salts such as calcium phytate, magnesium and ferric phytate, it has been generally concluded that phytic acid phosphorus was not utilized by animals, and further that the feeding of the acid decreased the absorption of calcium, magnesium, iron and other metals. On the contrary, phytin, the complex of calcium and magnesium phytate, is generally recognized as a compound in which the constituent phosphorus seems to be available and the inhibitory action on the absorption of calcium, magnesium, iron and other metals to be less than that which phytic acid effected. Palmer and Mottram showed that phytin has an antirachitogenic action and that the rachitogenic activity of cereals is caused by reducing of Ca : P ratio, because the activity was depressed by an addition of calcium lactate to the cereals. The evidence that the existence of organic acids, especially hydroxy acids, in the ingesta showed an antirachitogenic action by means of shifting the pH range to cause deposition of calcium phytate is reported by Møllgaard. It is interesting that ruminants are producing much of such acids in their gastrointestine in nature. Fingerling, Mathur and Gowda et al. reported that the phytin phosphorus appears to be utilized by ruminants while Tillman and Brethour presented isotopical confirmation of that opinion.

Since phytic acid or phytate is a stable compound, it is natural to think that if orthophosphate is released from the compound in the gastrointestinal canals, some phosphoesterases, for example phytase, must be concerned with them. Raun et al. demonstrated that the washed suspension of rumen microorganisms was able to produce phytase. Mathur and the present author[18], however, found undigested phytate in the ingesta of the abomasum and rumen, suggesting that the microbiotic hydrolysis in the rumen was insufficient. For the determination of the main site of intestinal hydrolysis of the dietary phytate, it is necessary to compare the phytase activities in the contents or juices of the various compartments of the gastrointestine.

In the present paper, the digestibility of phytin by the sheep dieted with heat-treated cereals, i.e. the phytase activity of the diet was inactivated, apparently was equal to the digestibility of raw cereal phytin, suggesting the existence of

phytase activity in the gastrointestinal contents. Actually, as the high potent phytase activity was found in the small intestinal content of the dairy cows and sheep, the author is able to conclude that the hydrolysis of phytate was catalized by the enzyme and the main site of intestinal hydrolysis of the compound was in the small intestine. It is favourable to support this opinion just stated that the reactions of the small intestinal contents, especially in duodenum, were similar to the optimum pH of the enzyme.

The phytase of small intestinal content has the optimum pH value 6.3 which is different from rice bran phytase 4.6, wheat bran phytase 5.2, intestinal phytase of rat 7.8[20], and intestinal alkaline phosphatase 9.0~9.5. RAPOPORT et al. reported that the phytase prepared from the blood of birds and reptiles had the optimum pH 6.6. This is similar to the enzyme we reported in the present paper. The enzyme reported by RAPOPORT et al., however, was inhibited with sodium flouride and potassium oxalate, but the enzyme reported in this paper is ineffective with these salts under the conditions examined, suggesting that the two enzymes are entirely different from each other.

COURTOIS and RAUN et al. demonstrated that a phytase was produced by the microflora in the gastrointestinal content. However, since these results were wanting comparative examination on the levels of the phytase activities of the definite compartments of the gastrointestine, such as the rumen, small intestine, cecum and colon, it seems impossible to presume the actual role of the intestinal microflora in the digestion of phytate. Although the origin of the enzyme is not completely clarified, the author has found the activity of the enzyme in the mucus preparation from duodenum[18]; further study is now in progress. The findings obtained will be published in a separate paper.

It is revealed that the feeding of a large quantity of beet-tops or turnip-roots produces hypophosphateamia in dairy cows[5,12,17]. The author showed biochemically that supplements of rice and wheat bran phytin as a source of phosphorus were effective to prevent the disorder. Nevertheless, there were many doubtful points in relation to the supplement of cereal bran for the dairy cattle as mentioned previously, because the fundamental mechanisms of the digestion of phytin phosphorus have not yet been revealed. However, as the enzymatic bases of the mechanisms are given in the present paper, the author is convinced that the phytin phosphorus of cereal bran supplemented as a source of phosphorus is digestable and available.

SUMMARY

The digestive mechanisms of phytin phosphorus have been studied in dairy cows and sheep. The summary of the results is as follows:

1. Phytin phosphorus of cereal bran has been digested completely in the gastrointestine of sheep. The digestibility is not altered in the heat-treated cereal bran whose own enzyme is inactivated.

2. High potential activity of a phytase was found in the content of the small intestine, but in the contents of the rumen, abomasum, cecum and colon the enzyme exerted only a feeble action, if any, on the substrate.

3. The optimum pH of the enzyme is about 6.3, optimum temperature 55°C. The enzyme is activated by magnesium ion at an optimum concentration of 0.001 M, inactivated by sodium cyanide, mercuric chloride, and calcium chloride, and not effected by potassium oxalate and sodium flouride at a concentration of 0.01 M.

4. The pH values of the contents of the upper small intestine in dairy cow and sheep range 6.5~7.1. These values are most near to the optimum pH of the intestinal phytase among the contents of other parts of the gastrointestine examined.

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