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We are pleased to reproduce on the following pages, in our occasional series of *'Citation Classics'*, the article by Simons *et al.* entitled *"Improvement of phosphorous availability by microbial phytase in broilers and pigs"*, which appeared in the *British Journal of Nutrition* in September 1990. Interrogation of the *Science Citation Index* indicates that this is the most highly cited article published by the *BJN* during the 1990's; by September 2004 it had received a total of 300 citations. This figure is, of course, based only on those journals that are included in the *Science Citation Index* database and excludes citations in books and monographs, the true extent to which it has been cited is therefore even greater than the 300 citations recorded.

Paul Trayhurn
Editor-in-Chief

Reference

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Improvement of phosphorus availability by microbial phytase in broilers and pigs

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Techniques have been developed to produce microbial phytase for addition to diets for simple-stomached animals, with the aim to improve phosphorus availability from phytate-P in plant sources. The activity of the crude microbial phytase showed pH optima at pH 5·5 and 2·5. The enzyme was able to degrade phytate in vitro in soya-bean meal, maize and a liquid compound feed for pigs. When microbial phytase was added to low-P diets for broilers the availability of P increased to over 60 % and the amount of P in the droppings decreased by 50%. The growth rate and feed conversion ratio on the low-P diets containing microbial phytase were comparable to or even better than those obtained on control diets. Addition of microbial phytase to diets for growing pigs increased the apparent absorbability of P by 24%. The amount of P in the faeces was 35% lower.

Phosphorus availability: Microbial phytase: Broilers: Pigs

Feeds for poultry and pigs mainly contain ingredients of plant origin. About two-thirds of the phosphorus in these products is present as phytate-P which has a low availability in simple-stomached animals. It therefore contributes to the P pollution problems in areas having an intensive livestock production such as The Netherlands.

Many fungi, bacteria and yeasts produce the enzyme phytase which is needed for the hydrolysis of phytate to inositol and inorganic phosphate. Nelson *etal.* (1968) were the first to add phytase, produced by a culture of *Aspergillus ficuum*, to liquid soya-bean meal. The feed was incubated for 2-24 h at 50°. After drying, it was fed to 1-d-old chicks. The birds showed a considerable increase in bone ash percentage over controls receiving no inorganic phosphate. In a second experiment (Nelson *et al.* 1971), a preparation of phytase produced by *Aspergillus ficuum* was added to a complete diet for chickens. In this case the enzyme was shown to be active in the gastrointestinal tract. The supplemented diet containing 3·0 g total P/kg and 1·8 g/kg phytate-P, gave similar tibia ash percentages as a control diet containing 1·6 g P/kg from disodium phosphate. They concluded that chicks utilized phytate-P as well as supplemental inorganic P.

To our knowledge comparable results with diets for pigs containing added microbial phytase have not been reported. Cromwell & Stahly (1978) performed an experiment in

which a dried live yeast culture of *Saccharomyces cerevisiae* was added to a maize-soya-bean-meal diet for pigs (15 g/kg). They concluded that the yeast culture probably did not improve the availability of phytate-P because growth rate, feed conversion ratio (intake ÷ gain) and bone strength were not influenced. Similar conclusions were presented by Chappie *et al.* (1979) from an experiment in which 20 g live yeast culture/kg feed were substituted for maize in a diet for growing pigs. Growth rate, feed intake and feed conversion ratio were measured. However, an improvement in growth rate was observed in another experiment with pigs from 65 to 100 kg live weight on diets with 35 g total P/kg (Shurson *et al.* 1984). These researchers were also unable to improve phytate-P utilization by including a yeast phytase in the maize-soya-bean meal diet in the balance studies and feeding trials with piglets.

Theoretically, the P content of feeds originating from plant materials should be sufficient to meet the requirements of poultry and pigs. The objectives of the present study were: (1) To further characterize the effects of phytase in vitro. The pH optimum of the enzyme was determined as well as the efficacy of the enzyme in liquid feed and feedstuffs such as soya-bean meal and maize. In addition the resistance of the enzyme to pelleting was determined. (2) To verify and extend the results of Nelson *et al.* (1971). Availability trials were carried out with broilers receiving diets containing phytase. (3) To show whether the same enzyme preparation would be effective both in broilers and pigs. Digestibility trials were carried out with fistulated pigs receiving diets containing the same enzyme preparation as applied for the experiments with broilers.

MATERIALS

Enzyme production

Organism and growth conditions. Aspergillus ficuum strain NRRL 3135 was obtained from the Northern Region Research Laboratory, USDA, 1815 North University Street, Peoria, Illinois, USA. Fungal spore preparations were made following standard techniques. Spores and subsequently cells were transferred through a series of batch fermentations in Erlenmeyer flasks to a 10 litre fermentor. After growth in batch culture contents of this fermentor were used as inoculum for a final 500 litre batch fermentation.

Media used contained (g/1): maize starch (BDH Chemicals Ltd, Poole, Dorset) 91, glucose. $1H_2O$ 38, $MgSO_4.7H_2O$ 0-6. KC1 0-6, $FeSO_4.7H_2O$ 0-2, KNO_3 12. The pH was maintained at 4-6 ± 0-3 by automatic titration with either 4 M-sodium hydroxide or 4 M-sulphuric acid.

Cells were grown at 28° at an automatically controlled dissolved oxygen concentration of 25% air saturation. Phytase production reached a maximum level after 10 d of fermentation.

Downstream processing. Fermentation broth was subjected to filtration followed by germ-free filtration. Phytase and other proteins were precipitated from the filtrate by adding acetone to a final volume of 600 ml/1 under continuous stirring. The precipitate was dried in a vacuum at 35°. After grinding the dry powder, the enzyme product was used as such for application experiments.

Phytic acid

Phytic acid was obtained from Sigma (St Louis, MO). The batch was found to contain 150 g water/kg. The other reagents used were of analytical grade and were obtained from Merck (Darmstadt, West Germany).

Table 1. *Composition of basal diet in experiments with broilers*

Ingredients	Contents (g/kg)
Yellow maize	280
Sorghum (low tannin)	200
Sunflower-seed meal (solvent extracted)	80
Soya-bean meal (solvent extracted, 488 g protein/kg)	350
Soya-bean oil	68·5
Vitamins*	5
Minerals*	15
Limestone	1
Synthetic methionine	1
Chromic oxide	0·5
Total	1001
Metabolizable energy (MJ/kg)	13·1
Lysine	12·9
Methionine + cystine	9·1
Calcium	6·0 (6·0, 6·6)†
Total phosphorus	4·5 (4·7, 4·7)†
Organic phytate-P	3·0 (3·1, 3·1)†

* Amount supplied (mg/kg diet): 3·6 retinol, 0·05 cholecalciferol, 5 vitamin E, 1·5 vitamin K_3, 1 thiamin, 5 riboflavin, 1 pyridoxine, 30 nicotinic acid, 7·5 D-pantothenic acid, 0·015 vitamin B_{12}, 0·5 folic acid, 350 choline chloride, 75 ethoxyquin, 9·5 g $CaCO_3$, 2·5 g NaCl, 0·26 g $FeSO_4$, 0·24 g $MnSO_,$, 45 mg $CuSO_4$, 60 mg $ZnSO_4$, 105 mg KI mixture.

† Values in parentheses were analysed for Expts 1 and 2 respectively.

Diet content and pelleting

Two experiments were carried out to study the effects of microbial phytase on P availability of feeds in broilers. The composition of the basal broiler diet is given in Table 1.

Control diets without the addition of phytase were used and these contained the following amounts (g/kg) of Ca, total P and phytate-P respectively: diet 1, 6·0, 4·5, 3·0; diet 2, 7·5, 6·0, 3·0; diet 3, 9·0, 7·5, 3·0. No graded feed phosphate was added to diet 1 (basal diet). Ca and P from a mixture of anhydrous dicalcium phosphate and monoammonium phosphate (5:1, w/w) were added to diets 2 and 3. All experimental diets were obtained by additions to the basal diet (diet 1).

In experiment 1 the optimum of the phytase dose was studied by adding 250, 500, 750, 1000 and 1500 units phytase/kg to the basal diet. A second experiment was performed to examine the effect of a dose higher than 1500 units phytase/kg on P availability, growth rate and feed conversion ratio in broilers. To the basal diet were added 375, 750, 1500 or 2000 units phytase/kg.

In an experiment with pigs, two diets (Table 2) were used because the effect of phytase in vivo may differ with the type of diet. The first diet was based on maize and solvent-extracted soya-bean meal; the second diet was more like a practical diet, as used in The Netherlands, containing mainly tapioca meal and hominy feed. Limestone was added to the feeds to provide 5·5 g Ca/kg diet. To one part of both diets 1000 units phytase/kg diet were added after which the diets were stored at 4°. The diets were offered twice daily in a meal form and were not pelleted.

The major components of the liquid feed for pigs, used for the in vitro experiment, were (g/kg) tapioca 400, maize-gluten-feed meal 150, peas *(Pisum sativum)* 120 and solvent-extracted soya-bean meal 90. The feed also contained (g/kg): Ca 6·4, P 5·1, phytate 2·7, dry matter 300-350. The pH of the mixture was 5·5 at the beginning of the incubation.

Table 2. *Composition of the diets for pigs (g/kg)*

	Maize soya-bean meal		Practical	
Maize	859-5		—	
Soya-bean meal (solvent extracted; crude libre < 3-5%)	124-fi		124-6	
Tapioca meal	—		421-0	
Hominy feed USA	—		3361)	
Sunflower-seed meal (solvent extracted)	—		80-0	
Soya-hcan oil	—		260	
Limestone	11-8		83	
Minerals and vitamins*	4-1		4-1	
Total	1000		1000	
Phytasc	—	+	-	4-
Dry matter	*$63*	863	KS2	882
Ash	35	35	65	66
Crude protein (nitrogen x 6-25)	136	136	158	156
Crude fat	34	32	47	47
Crude libre	25	27	64	66
Calcium	5-2	5-0	5-6	5-7
Magnesium	1-5	1-5	2-5	2-5
Phosphorus	3-3	3-3	4)	4-1
Potassium	6·0	5·9	10·0	10·0
Phylic acid	5-6	8-5	8-6	K-4
IP_5	1-5	1-7	2-4	2-6
IP,	1-2	1-2	1-6	1-fi

IP_6, myo-inosiml pcntakispriosphale; 1P,, myo-inositol lelrakisphospliale.

'Amount supplied (mg/kg diet): 2-4 retinol. 004 cholecalciferol, B vitamin E, 4 riboflavin. 20 nicotinic acid. 8 panlothenic acid, 0-02 vitamin B,,,. 125 cholinc chloride. 125 antioxidant, 2-5 g NaCl, 043 g $FeSO_,$. 50 mg MnO, 155 mg $ZnSO_4$. 40 mg $CuSO_4$, 2 mg Kl, *001* mg Se.

The pelleting experiments were performed on a CPM pelleting press (type CL 3; California Pellet Mill Corp., Crawfordsville, IN). The production capacity of this monoroll press is 200 kg/h. The pellets had a 5 mm diameter. The meal was common pig feed. The meal was heated with sleam to a temperature of 50 or 65° before pelleting.

METHODS

Extraction and analysis of phytic acid and other inositol phosphates

The chymus and feed samples were freeze-dried and ground over a 0-5 mm sieve.

Feed samples were taken as such and about 2 g of each sample were accurately weighed and shaken with 20 ml 0-8 M-hydrochloric acid for 2 h at room temperature. The slurry was centrifuged at 3000 rcv./min (l800#) for 10 min. The supernatant fraction was separated by decantation and filtered over a paper filter.

The filtrate (1-0 ml) was mixed in a 10 ml volumetric flask with 0-25 ml 2-8 M-NaOH, 0-75 ml of a solution of sodium acetate (167 g/l) and 10 ml EDTA solution (40 g/1, adjusted to pH 6 with 2H M-NaOH), The mixture was allowed to stand at room temperature for 15 min. The contents of the volumetric flask were made to volume with distilled water.

The resulting mixture had a pH of about 6. If necessary, the solution can further be diluted with sodium acetate solution (15 g/1, pH 6). After filtration through a Millipore filter (0-45 micrometer) 50 //I of the solution were injected onto the column of the liquid chromatograph.

Analysis of myo-inositol pentakisphosphate (IP_5) and tetrakisphosphate (IPJ was performed according to Phillippy & Johnston (1985), but for IP_5 0-08 M-HNO_3 was used and for IP_4 0·06 M-HNO_3 was used.

Assays for phytase and phosphate

Phytase activity. The assay is based on the production of free phosphate from phytate. The activity was determined in (a) enzyme preparations, (b) feed supplemented with phytase, and (c) contents of the gastrointestinal tract. Assays were carried out at 40° in a shaking water-bath. The reaction was initiated by adding a 0-1 M-sodium acetate buffer adjusted to pH 5-5 and containing phytic acid at a final concentration of 1 g/1. A time-course of 60 min was taken by terminating the reaction after 15, 30 and 60 min by adding 6 M-HC1 to a final concentration of 1 M. Samples to which HC1 had been added before the addition of phytic acid served as a blank for the contents of free phosphate.

After termination of the reaction samples were centrifuged for 10 min at 1800^. The amounts of free phosphate were determined in the clear supernatant fractions as described below. Activities were calculated from time-intervals during which activity was linear with time. One unit of phytase is the activity that liberates 1 //mol phosphate from phytic acid in 1 min.

Determination of free phosphate. Free phosphate was determined by means of an AutoAnalyzer method, using a Technicon AutoAnalyzer II. A sample from an in vitro experiment was filtered and 10 ml of the filtrate was mixed with 90 ml 1-2 M-HC1. Part of the mixture was injected into the AutoAnalyzer. The reagent used for the analysis of phosphate was the molybdovanadate reagent, described by Olsen & Sommers (1982). The preparation of the reagent was somewhat modified. Solution A: 50 g ammonium paramolybdate ($(NH_4)_sMo_7O_{24}.4H_2O$) were dissolved in 800 ml warm distilled water. The solution was cooled to room temperature and diluted to 1 litre with distilled water. Solution B: 2-5 g ammonium metavanadate (NH_4VO_3) were dissolved in 500 ml boiling distilled water. The solution was cooled and 20 ml concentrated nitric acid were added. The mixture was diluted to 1 litre with distilled water. Solution C: 333 ml concentrated HNO_3 were carefully added to 600 ml distilled water. The mixture was diluted to 1 litre with distilled water. Solution A was poured into solution B. Solution C was added to the mixture of A and B.

The acid diluent for the AutoAnalyzer was prepared by adding 40 ml of concentrated sulphuric acid to 800 ml of distilled water and the mixture was diluted to 1 litre with distilled water. To the acid diluent and to the water a detergent (aerosol) was added at a concentration of 0-5 ml/1. The AutoAnalyzer was calibrated by using standard solutions in the range of 5-30 mg P/l.

Miscellaneous analyses. Faeces and droppings were air- and freeze-dried and analysed for dry matter, Ca, P and chromium (Jongbloed, 1987). A composite sample of feeds was made, freeze-dried and analysed for dry matter, Ca, P, phytic acid (Oshima *et al.* 1964) and phytase activity.

Liquid chromatography

Liquid chromatographic system. The chromatograph consisted of two modified LDC-Milton Roy minipumps from Biotronik (Munich, West Germany). One pump was used for the eluent and one for the reagent. The single-headed pumps were provided with pulse-damping devices as are commonly used in automated amino acid analysers. The anion-exchange column used was a Dionex HPIC AS-3 (5-0 mm i.d. x 250 mm). A Dionex HPIC AG-3 guard column was employed. The injector had been supplied by Pharmacia (Uppsala, Sweden). The injection volume was 50 //I. The reaction coil was made of Teflon tubing with an internal diameter of 0-3 mm and a length of 3 m. It was kept in an oil bath at 50°. A variable u.v./visual detector (300 nm) of Kratos was used.

Liquid chromatography conditions. Eluent A was an aqueous solution of sodium nitrate and EDTA. Sodium nitrate (2-5 g) was dissolved in 1 litre water containing 0-5 ml of an EDTA solution (40 g/1). The pH was adjusted to 6-0 with 2-8 M-NaOH. Eluent B was an aqueous solution of 0-1 M-sodium nitrate adjusted to pH 3-5 with eluent C. Eluent C was an aqueous solution of nitric acid (9 ml/1).

The elution programme was: eluent A for 5 min, eluent B for 5 min, eluent C for 13 min and eluent A for 7 min. The eluent flow was 1 ml/min. The postcolumn reagent was a solution of 2-2 g iron(III)perchlorate nonahydrate and 12-8 ml perchloric acid (ml/1) in 1-0 litre water. The reagent was stored in a brown flask. A flow-rate of 0-5 ml/min was applied.

EXPERIMENTAL PROCEDURES

In vitro degradation

The degradation of the phytate in feeds by the microbial phytase was studied in experiments in vitro. The enzyme was added to ground maize and to a sample of ground soya-bean meal. The degradation of phytate was studied at two pH values (2-5 and 5-5) and at 40°. Therefore, the ground feedstuffs (4 g) were mixed with 8 ml buffer solution (0-1 tri-acetate buffer solution resulting in a pH of 5-5, or 0-1 M-citrate buffer solution resulting in a pH of 2-5). A series of pelleting experiments were performed with feed to which microbial phytase had been added. As part of the pelleting process, the meal was steam-heated to 50° or 65°. The energy uptake by the press was chosen Mow[1] or "high'.

Experiments with broilers

Two experiments were carried out to study the effects of microbial phytase on P availability of feeds in broilers.

Male broiler chicks (1-d-old; Hybro) were housed in two-tier battery cages (0-45 m^2). The ambient temperature was 32° during the first 2 d and was decreased by 4° in the first week. Every following week the temperature was decreased by 2°. Broilers were reared in a 1 h light and 3 h dark regimen.

Birds were vaccinated against Newcastle Disease at 1 d of age using Clone 30 vaccine. During the experiments the broilers were fed on the experimental diets *ad lib.* (Table 1). The diets were not pelleted.

Growth and feed conversion ratios were measured during the experimental periods. Apparent availability of total P was measured in a 3d period, during which feed consumption was measured as dry matter intake and excreta were collected quantitatively. Apparent availability of P is defined as the difference between intake of P and excretion of P with the excreta.

Post-mortem examination was carried out on all broilers which died or were obviously ill, to determine the cause of death or illness.

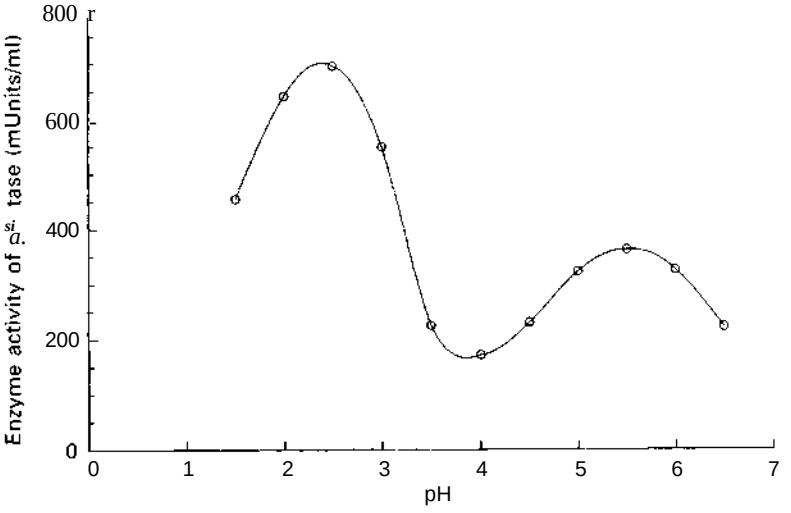

Fig. I. The activity of crude microbial phytasc determined at several pH values.

Expt I. The experiment was carried out with 176 broilers in sixteen battery cages (eleven per battery cage) until the age of 24 d. The eight treatments (diets) were replicated two times and were assigned randomly to the cages within each tier. The availability of P was measured from 21 to 24 d of age.

Expt 2. The experiment was carried out with 168 broilers in fourteen battery cages (twelve per battery cage) until the age of 4 weeks. The seven treatments (diets) were replicated twice and were assigned randomly to the cages within each tier. The availability of P was measured from 21 to 24 d of age.

Experiment with pigs

In this experiment six pigs of the Large White breed were used in the live weight range of 35-70 kg. Throughout the experiment the animals were housed individually in pens of 2-00 x 1-45 m and were not tied up. At a live weight of 37 kg these animals were fitted with two simple T-cannulas, one approximately 180 mm beyond the pylorus and one 200 mm proximal to the ileo-caecal junction. In the animals the apparent digestibilities of P and Ca were measured using Cr-NDR (chromium absorbed to neutral detergent residue) as an indigestible marker. The amount of Cr-NDR was weighed out separately (4-0 g/kg feed) and added just before feeding in the trough and mixed with feed and water. The water was added to the feed just before feeding time in the proportions 2-5:1 (w/w). To avoid feed refusals the amount of feed supplied was 2-3 times maintenance requirement = 293 kJ net energy for fattening/kg body-weight^{075} or 418 kJ metabolizable energy/kg body-weight^{075}.

For the experiment a cross-over design was used. A maize -soya-bean meal diet without and with phytase was supplied in two periods to two groups of three pigs. The mean body-weight in both periods was 46 and 52 kg respectively. Afterwards a more practical diet was supplied in the same way to the same pigs. The mean body-weight was 60 and 66 kg respectively. The collection of faeces started 8 d after change of maize-soya-bean meal diets and 9 d after changing to the practical diet. At 4 weeks after surgery the first collection period was started. During two consecutive days per animal, grab samples of fresh faeces were gathered and added together.

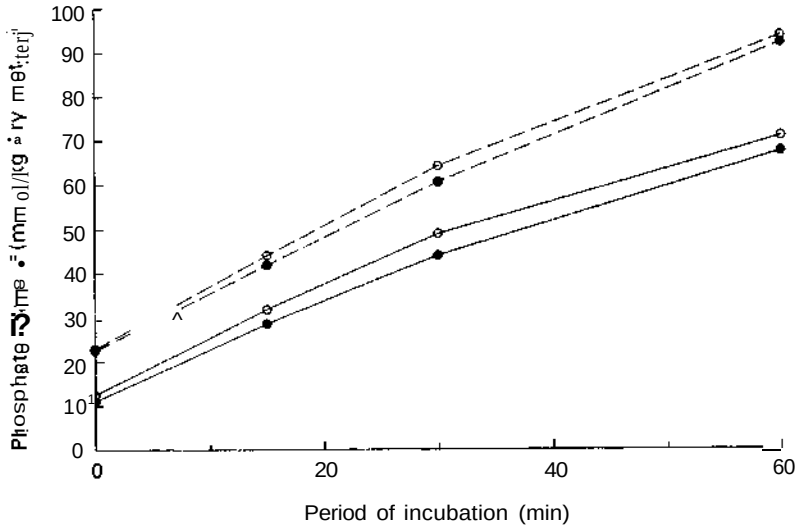

Fig. 2. Increase in the amount of free phosphate from the degradation of phytatc in samples of maize and soya-bean meal by microbial phytase (1000 units/kg). Values as a result of period of incubation and pH. Maize: (•——•) pH 2-5; (O——O) pH 5-5; soya-bean: (O — O) pH 2-5; (•——•>, pH 5-5.

Statistical analyses

The experimental data of the broiler experiments were examined by analyses of variance using non-linear regression models, which included:

model 1 (Expts 1 and 2)

$$Y_{ij} = N + T_i + Bl_j + e_{ij},$$

model 2 (Expts 1 and 2; diets 1, 4, 5, 6 etc.)

$$Y = A + BR^x + e,$$

where N is the mean level, T is the effect of treatment, Bl is the effect of tier, e is the error contribution with average 0 and variance rr^2, A, B and R are parameters ($0 < R < 1$) and x is the dose of phytase (100 units/kg).

The results obtained with the pigs were analysed using analysis of variance according to the following model:

$$Y_{ijk} = N + A_i + P_j + T_k + e_{ijk},$$

where A is the effect of animal and P is the effect of period. There was no evidence of P x T interaction.

RESULTS

In vitro experiments

The activity of the crude microbial enzyme was determined at several pH values. The activities found are shown in Fig. 1. The enzyme showed maximal activities at pH 5-5 and at pH 2-5.

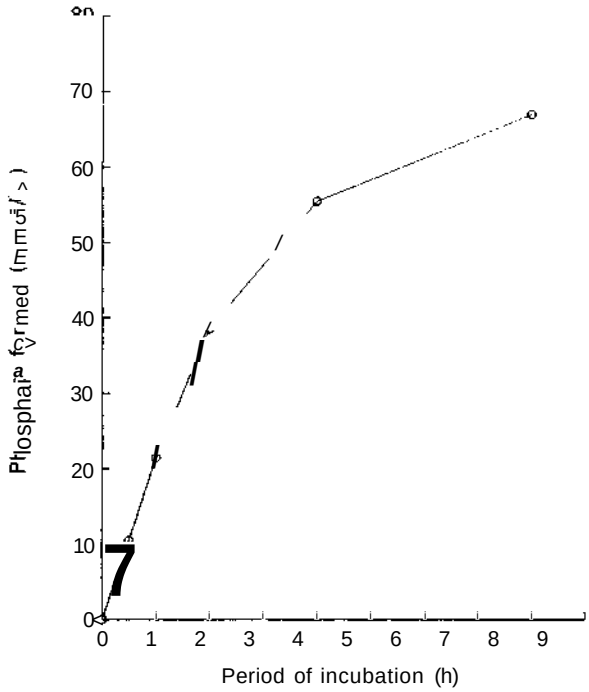

Fig. 3. Increase in the amount of free phosphate from the in vitro degradation of phytate in liquid feed for pigs by microbial phytase (1000 units/kg feed) at room temperature. Values as a result of period of incubation.

Table 3. *Phytase activity of pelleted feed produced under various pelleting, conditions*

Temperature of the meal before the press (°)	Temperature of the pellets after pelleting O	Energy input (Watt h/kg)	Phytase activity in the pellets (units/kg)*	Remaining activity (%)
50	78	20	240	96
50	81	25	234	94
65	84	17	208	83
65	87	23	115	46

* The phytase activity of the meal before the pelleting process was 250 units/kg.

The degradation of the phytate in samples of soya-bean meal and in ground maize by the microbial phytase was studied in experiments in vitro. The results are shown in Fig. 2. The soya-bean meal contained 12-1 g phytic acid/kg dry matter. After complete hydrolysis of this phytic acid, llOmmol free phosphate/kg might be formed. About 93 mmol free phosphate/kg were formed after an incubation time of 1 h at 40°.

The maize contained 6-94 g phytic acid/kg dry matter. After a complete hydrolysis of the phytic acid, 63 mmol free phosphate/kg might be formed. About 69 mmol free phosphate/kg were found after an incubation of 1 h. This suggests that the phytic acid had

Table 4. *Expt 1. The effect of microbial phytase on the apparent availability of total phosphorus and calcium, P in manure and the performance of broilers**

Diets no.	Ca (g/kg)	P (g/kg)	Added phytase (units/kg)	Availability (%) 21-24 d P	Availability (%) 21-24 d Ca	Amount of P in manure (g/kg dry matter feed intake)	Growth 0-2 weeks (g)	Feed conversion ratio 0-2 weeks	Growth 0-24d (g)	Feed conversion ratio 0-24 d
I	6	4-5	0	49-8[a]	47-2"	2-7[a]	166[a]	1·69[a]	338"	1·85[a]
2	7-5	6	0	45-6"	48-9[a]	3-8"	247"[r]	1·48"	592""	1·61"
3	9	7-5	0	44-6"	46-9[a]	4-9'[1]	288"[e]	1·38"	683"	1·55[1]"'
4	6	4-5	250	56-5''	57-1"	2-3"	238"	1·46"	566"	1·59"
5	6	4-5	500	59-6[cd]	59-3"[c]	2·[de	266""	1·40"	623[r]	1·56"°
6	6	4-5	750	59-5"[1]	60-3"[r]	2-l"[f]	293[e]	1·37"	675"	1·55""
7	6	4-5	1000	62-5"[p]	64-3""	2-0°	291°	1·38"	690"	1·52[i]
8	6	4-5	1500	64-5"	68-1"	1-9"	298*[1]	1·34"	733"	1·50"
Statistical significance of difference: *P*<				0001	0-001	0-001	0001	0-01	0001	0001
SED				1-46	2-53	0089	9-7	0-064	14-1	0-029

SED, standard error of difference between two means.
* For details of diets, see Table 1.
a.t),i.d.e Values within the same column with different superscript letters were significantly different: $P < 0.05$.

Table 5. *Expt 2. The effect of microbial phytase on the apparent availability of total phosphorus, P in manure and the performance of broilers**

Diet no.	Ca (g/kg)	P (g/kg)	Added phytase (units/kg)	P availability (%) 21-24 d	Amounts of P in manure (g/kg dry matter feed intake)	Growth 0 2 weeks (g)	Feed conversion ratio 0-2 weeks	Growth 0–4 weeks (g)	Feed conversion ratio 0-4 weeks
1	6	4-5	0	51-6[a]	2-5"	234[a]	1-48"	788"	1-59
Ł	7-5	6	0	46-2"	3-8"	294"	l-41[atl]	1066"	1-58
3	9	7-5	0	41-4[f]	5-0[b]	315[1""]	1-37"[1]	1081"	1-59
4	6	4-5	375	60-0[d]	2-1"	338"[C]"	1-32[1""]	1101"	1-57
5	6	4-5	750	61-7"	2-0"	346"[1]	1-31[C]"	1087"	1-58
6	6	4-5	1500	62-3"	2-0"	365"	1-29"'	1139"	1-54
7	6	4-5	2000	62-6"	2-0"	359[0d]	1-25"	1125"	1-56
Statistical significance of difference: *P* <				0001	0001	0-01	0-05	001	NS
SED				1-89	0-130	19-5	0-040	53-0	0-037

SED, standard error of differences between two means; NS, not significant.

* For details of diets, see Table 1.

a.D. i-ii Values within the same column with different superscript letters were significantly different.

Table 6. *Results of non-linear regression analysis in broiler experiments*
(Estimated values with their standard errors)

		Expt 1			Expt 2		
		A	*B*	*R*	*A*	*B*	*R*
Availability	E	64-8	-14-6	0-831	62-4	-10-8	0-676
P 21-24 d (%)	SE	1-83	1-91	0-051	0-13	0-22	0-016
Growth	E	300-1	-134-1	0-739	359-3	-125-0	0-647
0-2 weeks(g)	SE	4-4	6-1	0-026	5·1	9·1	0064
Feed conversion ratio	E	1-359	0-330	0-638	1-274	0-204	0-715
0-2 weeks	SE	0011	0019	0-043	0018	0031	0099
Growth	E	718-6	-375-3	0-734	—	—	—
0· 24 d (g)	SE	17-3	24-5	0038	—	—	—
Feed conversion ratio	E	1-524	0-325	0-565	—	—	
0-24 d	SE	0-014	0026	0075	—	-	—
Growth	E	—	—	—	1118-8	-330-6	0-484
0-4 weeks (g)	SE	—	—	—	15-9	30-7	0-184
Feed conversion ratio	E	—	—	—	1-545	0-045	0-911*
0^4 weeks	SE	—	—	-	0045	0042	0·190

E, estimate of parameters *A, B* and *R* (for details, see p. 532).
* Non-linear regression line is not reliable, % variance accounted for 28-6.

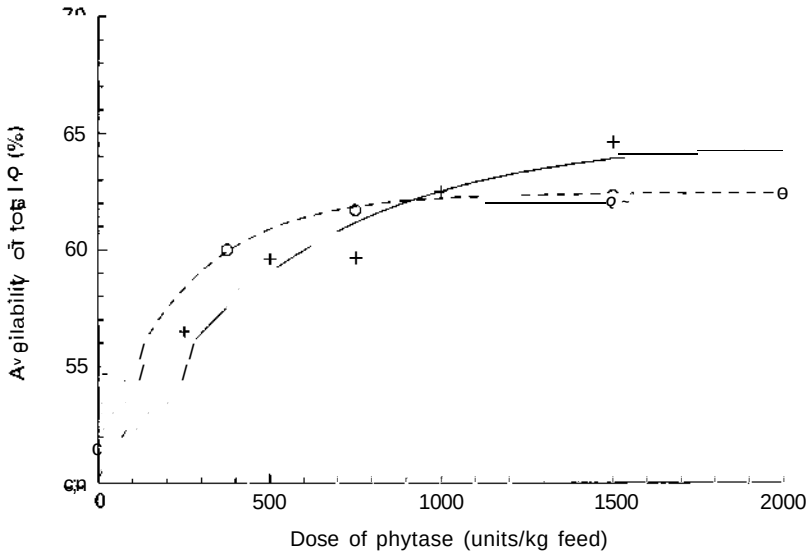

Fig. 4. Apparent availability of phosphorus in broiler diets with 0-45 phosphorus and various additions of phytase; result of non-linear regression analysis (+). Expt 1, 21-24 d; (O), Expt 2, 21-24 d.

been degraded completely, with some phosphate having been liberated from other phosphate sources by the crude enzyme preparation.

The application of phytase in liquid feed for pigs was studied in vitro at room temperature. The increase in the concentration of or/Ao-phosphate after the addition of 1000 units of microbial phytase/kg is shown in Fig. 3. The feed contained 7-3 g phytic

acid/kg dry matter. After complete hydrolysis of this phytic acid, 66-4 mmol free *ortho*-phosphate/kg might be formed. An increase of free phosphate of 66-8 mmol/kg was observed after the incubation for 8 h. It may be concluded that the phytate-bound P was completely liberated. About 80% of the phytate-bound phosphate (55-5 mmol/kg) was liberated after 4 h of incubation.

The pelleting experiments with feed to which microbial phytase had been added showed significant inactivation of phytase activity when temperatures of the pellets after pelleting exceeded 84° (Table 3).

Broiler experiments

The results of two experiments with broilers are given in the Tables 4-6 and Fig. 4. A low apparent availability of total P was measured with diet 1 (Tables 4 and 5). Addition of inorganic graded feed phosphate (and Ca) to this basal diet decreased the apparent availability of P significantly. The apparent availability of P was significantly improved by adding different levels of microbial phytase. When the availability of P improved the availability of Ca increased (Table 4) and the amount of P in the droppings decreased. Both non-linear regression lines for apparent availability of total P are reliable since the percentage of variance accounted for 94-5 and 99-8 in Expts 1 and 2 respectively (Table 6). The standard error of the parameters in Expt 2 are much lower than those in Expt 1 due to a much smaller residual variance; no explanation for this could be found.

Fig. 4 shows that an almost maximum apparent P availability in Expt 2 was obtained when 800 units microbial phytase/kg feed were added; in Expt 1 a further increase of the apparent P availability was observed at doses exceeding 800 units/kg. However, the standard error in Expt 1 was much larger than that in Expt 2.

Growth rate and feed conversion ratio with the low-P diets with added microbial phytase were significantly improved, except for the feed conversion ratio from 0 to 4 weeks. Growth rate and feed conversion ratio of the broilers were dependent on the level of microbial phytase. A lower growth rate compared with control diet 3 was found with 250 units phytase/kg in Expt 1 (Table 4). In Expt 1 an improved growth rate compared with diet 2 was obtained with additions of 750, 1000 or 1500 units microbial phytase/kg. During the period 0-24 d, 1500 units phytase/kg gave a significantly better growth compared with all the other groups (Table 4). In Expt 2 the best growth at 4 weeks of age was also obtained with 1500 units phytase/kg (diet 6), although this was not significantly different from diets 2, 3, 4, 5 and 7 (Table 5). Table 4 shows that from 0 to 24 d in Expt 1, diets 7 and 8 (1000 and 1500 units phytase/kg respectively) yielded a significantly improved feed conversion ratio compared with diets 1, 2 and 4 (0 or 250 units phytase/kg). From 0 to 2 weeks in Expt 2, additions of 750 units phytase or more improved the feed conversion ratio compared with diets 1 and 2 (Table 5). As can be seen in Tables 4 and 5, an increase in the level of phytase improved the feed conversion ratio. This is even more apparent from the non-linear regression lines. The non-linear regression lines of the growth rate and feed conversion ratio, with the exception of the feed conversion ratio line until 4 weeks of age, were reliable since the percentage of variance accounted for 91 -4-99-8 in both experiments (Table 6). This means that a higher dose of phytase in the diets improved both the growth rate and feed conversion ratio significantly.

Diet 1 caused a rather high mortality. No difference in mortality occurred between the various groups receiving the other diets.

Table 7. *Results of the digestibility trials with pigs*

	Maize-soya-bean meal				Practical			
Microbial phytase... «...	— 6	+ 6	SED	Statistical significance of difference	— 5	+ 5	SED	Statistical significance of difference
Digestibility (%)								
DM	85-2	85-0	1-6	NS	81-0	81-3	0-1	NS
Phosphorus	20	46	4-0	**	34	56	1-6	***
Calcium	44	50	5-2	NS	50	58	1-0	**
P in faeces (g/kg DM)	21-0	13-6	1-3	**	16-3	10-9	0-4	***
Ca in faeces (g/kg DM)	22-8	19-2	1-3	*	17-4	15-4	0-4	*

SED, standard error of difference; DM, dry matter; NS, not significant.
*P < 0-05, **P< 0-01, *** p < 0-001.

Pig experiment

One pig became feverish at about 60 kg live weight and refused feed occasionally. As the results obtained with this pig deviated considerably from those in the other animals, these were omitted from the final calculations.

As can be seen in Table 2, the chemical analyses in the two types of diet were nearly equal, except for the phytase activity. The effect of microbial phytase on the digestibility of dry matter and the apparent absorbability of Ca and P and on the P concentration in the faeces are given in Table 7. The addition of phytase to the diet had no effect on the digestibility of dry matter. The faecal concentration of P decreased by about 35% and the apparent absorbability of P improved substantially (twenty-four percentage units). There was a significant reduction (P < 005) in the faecal Ca concentration, but the apparent absorbability of Ca was increased significantly only in the practical diet.

DISCUSSION

In vitro experiments

The microbial phytase is active over a wide pH range (Fig. 1). This implies that the enzyme will be active in liquid pig feed having a pH of about 6, as well as in the stomach of animals where the pH is between 2 and 5. The enzyme has no activity at pH 7. Phytate in samples of maize and in soya-bean meal is easily degraded by added phytase at pH 2-5 as well as at pH 55 (Fig. 2). In a liquid compound feed for pigs similar results were obtained at a pH of 5-5 (Fig. 3). Metal ions like Ca, magnesium, iron or zinc may form strong complexes with phytic acid (Wise, 1983) which have a poor solubility at higher pH values. Complexes of these metal ions with phytic acid might be less available for degradation by phytase. No indications for such a low solubility of phytate were obtained in the in vitro experiment of liquid pig feed with microbial phytase. This is rather surprising since animal feed usually contains a high level of divalent metal ions.

The results of the pelleting experiments are given in Table 3. A high energy input means that the shear during the pelleting process is high. Under these conditions many plant cells are crushed, which is favourable for the digestion process in animals. The effect of the shear on the phytase activity appears to be small; it is mainly the high temperature which results from the high energy input which will inactivate the enzyme.

The experiments make it clear that the thermal stability of the microbial enzyme is good. The enzyme will remain stable if the proper pelleting conditions are chosen.

Broiler experiments

The low apparent availability of P in broilers fed on phytase-free diets (diet 1) can be explained by the high level of phytate-P that is not available for simple-stomached animals. Additions of microbial phytase to diet 1 improved the availability of P significantly. The amount of P in the droppings is, therefore, significantly lowered. As the availability of P increases, the availability of Ca increases, since both are deposited in the bone at the same time. Possibly the Ca content in the diets with microbial phytase was too low to achieve a maximum availability of P. It was expected from experiments reported by Nelson *et al.* (1971) that growth rate and feed conversion ratio of birds given diets with sufficient microbial phytase would be comparable with those given control diets (2 and 3) which contained supplemental graded feed phosphate (and Ca).

In our experiments growth rate and feed conversion ratio were improved by increasing the level of phytase (Tables 4, 5 and 6); although there were differences in level between both experiments at the same age, comparable results were obtained.

From these two experiments with broilers it may be concluded that an addition of 1000 units microbial phytase to feed provides levels of performance which are as good as or better than those with feed supplemented with phosphate. The improved growth rate and feed conversion ratio with higher levels of phytase might be explained by (a) release of minerals and trace elements from complexes with phytic acid, or (b) by utilization of inositol by the animals after hydrolysis of phytic acid to inositol and inorganic P, or (c) a possible increased starch digestibility as suggested by Knuckles & Betschart (1987) and Cawley & Mitchell (1968), or combinations of a, b and c. An improved feed conversion ratio till 2 weeks of age was obtained with even lower doses of phytase. Why the feed conversion ratio is no longer significantly improved at 4 weeks of age is not clear. A lower requirement of P at 4 weeks of age compared with 2 weeks of age and an adaptation of the birds to utilize more phytate-P in critical circumstances might explain these findings. This aspect warrants further research. When the level of phytase was increased to 1500 units/kg, improved bird performance compared with the control diets was observed.

The increased mortality of the broilers in diet 1 was due to the fact that this feed was deficient in P, since phytic acid-P was not available to the young chicks and the requirement of P in young chicks is much higher than the amount offered.

Pig experiment

The results in Table 7 show that the addition of 1000 units microbial phytase/kg feed has a significantly favourable effect on the apparent digestibility of P. Based on the phytic acid-P content in the feed and the assumption that 80% of the non-phytic acid-P is digestible, it can be calculated that the digestibility of the phytic acid-P in the feeds improves, on average from —12 to 30%. However, when these calculations are based on the sum of IP_6, IP_5 and IP_4, those values oscillated from 9 to 42 % respectively. The latter values should be regarded as more accurate. This implies that about 50 % of the P from phytate-bound P is absorbed.

In earlier experiments by Jongbloed (1987) it was calculated that in maize-soya-bean meal diets no P from phytic acid was absorbed. This is confirmed in the present experiment.

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