Arie K. Kies

Phytase studies in pigs and poultry
Effect on protein digestion and energy utilization

Fytasestudies in varkens en pluimvee
Effect op eiwitvertering en energiebenutting

PROEFSCHRIFT

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op maandag 6 juni 2005
des namiddags te een uur dertig in de Aula.

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1. Levels of dietary phytate normally present in pig and poultry feeds have the potential to complex with most of the dietary protein and affect protein digestion.
   This thesis.

2. Phytase saves dietary protein and energy.
   This thesis.

3. The value of phytate as a food component depends on its degree of degradation.
   Harland, B.F. and E.R. Morris, 1995. Phytate: a good or a bad food component? Nutr.Res. 15:733-754.

4. To prevent or cure osteoporosis, physicians often prescribe a high dose of calcium. Often, insufficient consideration is given to the dietary calcium : phosphorus ratio and to the decreased phosphorus absorption at a higher level of calcium intake. Consequently, the treatment might be counterproductive.
   Heany, R.P. and B.E.C. Nordin, 2002. Calcium effects on phosphorus absorption: implications for the prevention and co-therapy of osteoporosis. J.Am.Coll.Nutr. 21:239-244.

5. Many books and articles mention only the values of physiological variables under standard conditions. This is confusing, because those conditions are rarely of physiological relevance.

6. "More optimal" solutions, or the "most optimal" solution to a problem, call in question the quality of those solutions.

7. After the first year of life, everyone ages a year, every day.

8. To obtain an "AH-erlebnis", propositions should not be numbered, but lettered.

Propositions belonging to the thesis
Phytase studies in pigs and poultry. Effect on protein digestion and energy utilization.
Arie K. Kies
6 June 2005, Wageningen

1. De hoeveelheid fytaat die normaal in varkens- en pluimveevoer voorkomt kan in potentie het meeste voereiwit complexeren en de eiwitvertering te beïnvloeden.
   Dit proefschrift.

2. Fytase bespaard voereiwit en –energie.
   Dit proefschrift.

3. De waarde van fytaat als voedingscomponent hangt af van zijn mate van degradatie.
   Harland, B.F. and E.R. Morris, 1995. Phytate: a good or a bad food component? Nutr.Res. 15:733-754.

4. Ter behandeling of voorkoming van osteoporose, schrijven artsen vaak een hoge dosis calcium voor. Hierbij wordt onvoldoende rekening gehouden met de calcium : fosfor verhouding in de voeding, en met verlaging van de fosforabsorptie door de hoge calciuminname. De behandeling kan daardoor contraproductief werken.
   Heany, R.P. and B.E.C. Nordin, 2002. Calcium effects on phosphorus absorption: implications for the prevention and co-therapy of osteoporosis. J.Am.Coll.Nutr. 21:239-244.

5. In veel boeken en publicaties worden alleen de waarden van fysiologische variabelen onder standaardcondities genoemd. Dit werkt verwarrend, omdat deze condities zelden fysiologisch relevant zijn.

6. "Optimalere" oplossingen of de "optimaalste" oplossing van een probleem, roept vraagtekens op over de kwaliteit van die oplossingen.

7. Na het eerste levensjaar wordt iedereen elke dag een jaartje ouder.

8. Voor een "AH-erlebnis" zouden stellingen niet moeten worden genummerd, maar beletterd.

Stellingen behorend bij het proefschrift
Phytase studies in pigs and poultry. Effect on protein digestion and energy utilization.
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6 June 2005, Wageningen

Aan
Annie en Ab

Preface

The basis for this thesis finds its origin in February 1995, when Constant van Lookeren Campagne (business unit Agri Ingredients of DSM Food Specialties), Piet Simons ("The Spelderholt Poultry Institute") and I, met in Beekbergen to discuss developments in animal nutrition. One of the topics we talked about was the effect of phytase on the performance of broilers. Piet was convinced that animals in his experiments performed better with phytase; even better than animals receiving diets not limiting in phosphorus. This was an interesting idea. So I looked into a pile of results and concluded that, indeed, in many experiments, the performance of broilers receiving phytase was better than could be attributed to the increased phosphorus availability. This finding was tested in some performance studies, but we also wanted to explain why phytase improves animal performance. A small number of studies on protein (amino acid) digestibility and energy utilization were already performed. They were used to make the first version of the "matrix-values" for phytase. This is a list of values attributed to phytase, not only for phosphorus and calcium, but also for digestible protein and amino acids. It was first presented in a Symposium in Arnhem, in March 1997.

The basis for these matrix-values was rather limited. The number of protein digestibility studies was expanded, therefore, which resulted in an update of these values in 2000. This is still the presently used recommendation. The update could be made for amino acid digestibility in pigs and poultry. Also, the effect of phytase on energy metabolizability in poultry was quantified. For the effect of phytase on energy utilization in pigs, however, few data were available. It appeared that energy digestibility was not affected in pigs. This resulted in the hypothesis that phytase might affect post-absorptive energy utilization. Experiments were run at Wageningen University, and some interesting results were obtained. We thought it was desirable to write a publication, but the question was: "who will write it?". The answer was the prelude to this thesis. Even though the incubation time for the thesis was ten years, I first started to seriously write it in 2003. I did not start from scratch, but writing it in a quite short time next to my job was no piece of cake. I could not have done that without the help from many people. I sincerely thank all who have contributed to this thesis in any way. Specifically, I want to thank the following people.

My understanding of how to proceed with our work on the nutritional value of phytase was enhanced by discussions with dozens of people around the world; researchers of institutes and universities and nutritionists of feed companies. Many thanks for the discussions and for sharing your ideas.

Working in a company without animal research facilities means that studies need to be performed externally. This has advantages and disadvantages. An advantage is that one is not bothered by the day-to-day problems associated with the experiments. A disadvantage is that it is not possible to make observations, to correct the execution of the experiment if required, or to get all results in the preferred form. Luckily, I have been able to work with some of the best scientists imaginable. A big "thank you" to the teams of the University of Alberta, Canada (Willem Sauer, Jim He, Yongcheng Zhang, Shengfa Liao and their co-workers); the University of Sydney, Australia ('Ravi' Ravindran, Ganesharanee Ravindran, Wayne Bryden,

Peter Selle and their co-workers); Massey University, New Zealand (Patrick Morel); Division Nutrition and Food, Animal Sciences Group, Wageningen University & Research Center, Lelystad (Age Jongbloed, Paul Kemme, Leon de Jonge, Leon Šebek, Hans van Diepen and their co-workers); and Wageningen University (Walter Gerrits, Johan Schrama, Marcel Heetkamp, Koos van der Linden, Tamme Zandstra, Martin Verstegen and their co-workers).

Results of some of the studies are not included in this thesis, but were of key importance for either my understanding of the mode of action of phytase, or for designing the included experiments. Thanks are due to the teams of Massey University, New Zealand ('Ravi' Ravindran, Wouter Hendriks and their co-workers); the former TNO-ILOB (Ben Schutte, Johan de Jong, Jan Dirk van der Klis and their co-workers); and the Animal Sciences Group of the WUR, Beekbergen (Piet Simons, Koos van Middelkoop, Jan van Harn and their co-workers).

For a long period I worked with fantastic colleagues in the business unit Agri Ingredients: Ria, Constant, Hans and Rob and later also Helen, Sheila, David, Frank, Hagen, Hans, Hans, Johan and Luc. Krijn Rietveld was enthusiastic about the idea to start writing this thesis; Fedde Sonnema made it possible to finalize it, despite sale of the feed enzyme business. Niek Persoon of DFS-R&D allowed me to use one day per week to work on this thesis during nine months, which speeded up the writing a lot. To Karl van Hemert no statistical problem appeared too difficult. To all: many thanks. Especially, I am indebted to Guus Klein Holkenborg, who was for years my "right-hand", and the most critical person to discuss this work with.

The studies described in this thesis were performed for the former Feed Enzyme Alliance of DSM Food Specialties-Agri Ingredients and BASF A.G., Germany. I thank the management of these companies for their agreement to use these results. DSM Food Specialties is also acknowledged for making the training and supervision of this work financially possible.

Of course, writing a thesis is more than collecting results of a number of experiments. Discussions with, and help and support from my promotor, Martin Verstegen, were indispensable. Walter Gerrits did a perfect job as the critical co-promotor. Mariet was an enthusiastic supporter (as always!). Many thanks, all of you!

With Ben Schutte I discussed for many hours about the work reported in this thesis. He also critically evaluated my manuscripts. Also thanks to Anny, who kept us going on with lots of coffee and excellent lunches. Thanks to Ben and Jaap for acting as paranimfs, and taking care for many of the worries of the days to the promotion.

Clare Sloan: many thanks for correcting my English into proper English. Un grand remerciement à Frédéric (Fredo) Bouesnard pour transférer des pensées sur le sujet de ma thèse dans l'image ornant la couverture du livre.

Writing a thesis in your spare-time requires the full understanding and support of your friends and family. Sorry for being not very sociable for a few years! Above all thanks to Annemarie: you made it all possible in the first place. For next year: holidays together again!

Voorwoord

De oorsprong voor dit proefschrift ligt in februari 1995, toen Constant van Lookeren Campagne (business unit DSM Food Specialties-Agri Ingredients), Piet Simons (instituut voor pluimveeonderzoek "Het Spelderholt") en ikzelf in Beekbergen bijeenkwamen om ontwikkelingen in de veevoeding te bespreken. Eén van de onderwerpen die op tafel kwamen was het effect van fytase op de prestaties van kuikens. Piet was ervan overtuigd dat in zijn proeven de dieren die fytase kregen beter presteerden; beter zelfs dan dieren die voldoende fosfor in het voer kregen. Dit was een interessant idee, dus dook ik in een stapel onderzoeksresultaten. De conclusie was dat inderdaad in veel proeven prestaties van kuikens beter was dan kon worden toegeschreven aan de (door fytase verbeterde) fosforbeschikbaarheid. Dit werd in een aantal proeven getest, maar we wilden ook weten hoe dat te verklaren was. In een paar studies was het effect van fytase op de eiwit- en aminozurenvertering en de energiebenutting al gemeten. Op basis hiervan werd de eerste versie van de "matrixwaarden" gemaakt. Dit is een lijst met de voederwaarde van fytase, niet alleen voor fosfor en calcium, maar ook voor verteerbaar eiwit en aminozuren. Het werd voor het eerst gepresenteerd in maart 1997, tijdens een Symposium in Arnhem.

De basis voor deze matrixwaarden was nogal smal. Daarom werden aanvullende eiwitverteringsstudies uitgevoerd, wat in 2000 resulteerde in vernieuwde matrixwaarden. Deze waarden worden nog steeds geadviseerd. De vernieuwing betrof de waarden voor de aminozurenvertering in pluimvee en varkens. Ook kon het effect van fytase op de energiebenutting in pluimvee worden gekwantificeerd. Een dergelijke waarde kon niet worden berekend voor varkens. Er waren weinig data en daaruit bleek geen effect op de energieverteerbaarheid. Daarom werd verondersteld dat er mogelijk een verschil optreedt in de energiebenutting na de vertering. Proeven daarnaar werden uitgevoerd bij Wageningen Universiteit. De resultaten waren interessant en we vonden het de moeite waard ze te publiceren. Het antwoord op de vraag "wie gaat het schrijven?" bleek de opmaat naar dit proefschrift. Hoewel al tien jaar geleden begonnen werd met het beschreven werk, startte het schrijven pas in 2003. Natuurlijk had ik al veel gegevens en waren enkele artikelen al af, maar om in zo'n korte tijd een proefschrift te schrijven, naast mijn gewone werk, was geen gesneden koek. Het zou ook onmogelijk zijn geweest zonder de hulp van velen. Ik bedank iedereen die, in welke vorm dan ook, een bijdrage aan dit proefschrift leverde. Speciaal dank aan de volgende mensen.

Mijn begrip hoe het werk naar de voedingswaarde van fytase aan te pakken werd vergroot door discussies met velen over de gehele wereld: onderzoekers van instituten en universiteiten en nutritionisten van veevoerproducenten. Allen zeer bedankt hiervoor.

Een bedrijf zonder eigen dierproeffaciliteiten is voor dergelijke proeven aangewezen op externe instituten. Dit heeft voor- en nadelen. Een voordeel is dat je je geen zorgen hoeft te maken over de dagelijkse beslommeringen die dergelijke proeven met zich brengen. Nadelen zijn dat je zelf dingen niet ziet, de proef niet kunt corrigeren (indien nodig), en soms de resultaten niet op de manier krijgt die je graag zou willen. Gelukkig kon ik werken met een aantal van de beste onderzoekers die men zich kan wensen. Daarom mijn grote dank aan de onderzoeksgroepen van de University of Alberta, Canada (Willem Sauer, Jim He, Yongcheng

Zhang, Shengfa Liao en hun medewerkers), de University of Sydney, Australië ('Ravi' Ravindran, Ganesharanee Ravindran, Wayne Bryden, Peter Selle en hun medewerkers), Massey University, Nieuw Zeeland (Patrick Morel), Division Nutrition and Food, Animal Sciences Group, Wageningen University & Research Center, Lelystad (Age Jongbloed, Paul Kemme, Leon de Jonge, Leon Šebek, Hans van Diepen en hun medewerkers) en Wageningen Universiteit (Walter Gerrits, Johan Schrama, Marcel Heetkamp, Koos van der Linden, Tamme Zandstra, Martin Verstegen en hun medewerkers).

Resultaten van enkele studies die niet zijn opgenomen in dit proefschrift waren van groot belang voor een goed begrip van de werking van fytase, of voor het goed opzetten van de gerapporteerde proeven. Dank daarom ook aan de onderzoeksgroepen van Massey University, Nieuw Zeeland ('Ravi' Ravindran, Wouter Hendriks en hun medewerkers), het voormalige TNO-ILOB (Ben Schutte, Johan de Jong, Jan Dirk van der Klis en hun medewerkers) en de Animal Sciences Group van de WUR, Beekbergen (Piet Simons, Koos van Middelkoop, Jan van Harn en hun medewerkers).

Een aantal jaren werkte ik in de business unit Agri Ingredients met fantastische collega's: Ria, Constant, Hans en Rob en later ook Helen, Sheila, David, Frank, Hagen, Hans, Hans, Johan en Luc. Krijn Rietveld was enthousiast over het idee een proefschrift te gaan schrijven, Fedde Sonnema maakte het mogelijk om het af te maken, ondanks de verkoop van de voerenzymen business. Niek Persoon van DFS-R&D stond mij toe om gedurende negen maanden één dag per week aan dit proefschrift te werken, wat het schrijven sterk versnelde. Karl van Hemert ging geen statistisch probleem uit de weg. In het bijzonder dank aan Guus Klein Holkenborg, jarenlang mijn "rechterhand" en de meest kritische persoon om dit werk mee te bespreken.

De proeven beschreven in dit proefschrift werden uitgevoerd voor de voormalige Feed Enzyme Alliance van DSM Food Specialties-Agri Ingredients en BASF A.G, Duitsland. Ik bedank het management van deze bedrijven voor hun toestemming de resultaten te gebruiken. DSM Food Specialties bedank ik ook voor de financiële bijdrage aan de opleidingskosten.

Natuurlijk is een proefschrift meer dan een verzameling resultaten van een aantal proeven. Discussies met en hulp en aanmoediging van mijn promotor, Martin Verstegen, waren onmisbaar. Walter Gerrits deed prima werk als kritische co-promotor. Zoals altijd was Mariet een enthousiaste supporter. Allen zeer bedankt!

Met Ben Schutte discussieerde ik urenlang over de gerapporteerde resultaten. Ook keek hij kritisch naar de manuscripten. Ook dank aan Anny, die ons op de been hield met veel koffie en prima lunches. Ben en Jaap bedankt om op te willen treden als paranimf, en om veel van de zorgen voor de promotiedag weg te nemen.

Clare Sloan: zeer bedankt voor de correctie van mijn Engels in echt Engels. Un grand remerciement à Frédéric (Fredo) Bouesnard pour transférer des pensées sur le sujet de ma thèse dans l'image ornant la couverture du livre.

Een proefschrift schrijven in je vrije tijd doet een zwaar beroep op het begrip van vrienden en familie. Excuus dat ik een paar jaar niet erg sociaal was! Bovenal dank aan Annemarie: jij maakte het in de eerste plaats mogelijk. Volgend jaar weer samen op vakantie!

Abstract

Phytase is applied for improving digestibility of phosphorus in pig and poultry diets. Independently, phytase also improves animal performance. The mechanisms to explain this effect were investigated and quantified. Protein can be complexed with phytate, especially under the acid conditions that occurs in the stomach of animals. Dietary phytase supplementation prevents formation of such complexes or, if such complexes are formed, helps to release protein faster and to a larger extent from phytate. Consequently, protein digestibility may increase. This effect was confirmed in a meta-analysis of digestibility experiments, both in poultry and pigs. The higher protein digestibility explains, only in part, the improved performance. In poultry, the apparent metabolizable energy level increased with dietary phytase, mainly as the result of higher protein and fat digestion. Because in literature no effect of phytase on energy digestibility in pigs was shown, post-absorptive energy utilization was investigated. Using indirect calorimetry, no clear effect of phytase could be shown on energy partitioning. Phytase improved, however, energy utilization during the first two weeks post-weaning of *ad libitum* fed piglets. This may indicate that adaptation of piglets is somewhat facilitated by phytase. In an experiment with restrictedly-fed piglets, three weeks post-weaning, energy digestibility increased with phytase, but not energy metabolizability. A number of observations indicated, however, that energy metabolism of the piglets was affected. Processes that increase or decrease heat production balance each other out. Phytase increased digestibility of minerals considerably, including the monovalent cations sodium and potassium. Mineral absorption and excretion are, in part, active processes, increasing heat production. Using a mathematical model, this effect was estimated at about 1% of energy requirements for maintenance. A lower energy requirement may result from a reduced produc-tion of endogenous protein. In growing pigs, dietary phytase supplementation decreased gastric mucin production. Possibly, the formation of inositol mono-, di- or tri-phosphates may act positively on the growth of animals, but this remains to be confirmed. In conclusion, phytase improved digestibility of amino acids, both in poultry and pigs. It also improved energy metabolizability in poultry. Energy utilization in pigs is probably affected, but the mechanism needs further clarification and quantification.

Contents

Introduction

PHYTATE

About 65% of phosphorus (P) in vegetable feedstuffs is present in phytate. Phytate is a salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate). Phytate accumulates in discrete regions of grains and seeds during the ripening period and during maturation. These regions are not the same for all seeds. In corn 88% of phytate is stored in the germ, whereas in wheat 87% is stored in the aleurone layer. In most dicotyledonous seeds, the largest part is stored in the cotyledons (Reddy, 2002). For plants, phytate is the primary storage for P, inositol and cationic minerals (Reddy et al., 1982). Phytate is negatively charged under physiological conditions, thus can complex cations like Ca, Zn, Fe and K. The negative charge makes phytate a strong organic chelator. During germination, these nutrients are made available to the plant at the required moment by the action of phytate-degrading enzymes (Reddy et al., 1982; Scott and Loewus, 1986; Lásztity and Lásztity, 1990; Loewus, 2002; Reddy, 2002).

The history of phytate goes back to 1855, when Hartig isolated a fraction from plants that mainly contained this material. Andersen (1914) described the chemical structure of phytic acid (cit. Reddy et al., 1982). Bruce and Callow (1934; cit. Taylor, 1979) reported that animals utilize phytate-P less efficiently than inorganic P. From that time on, the relationship between phytate and mineral availability (notably P) has been studied widely in animals. Soon, it was accepted that phytate-P is not readily available to monogastric animals. Moreover, it became clear that phytate inhibits mineral availability (Taylor, 1979).

Monogastric animals like pigs and poultry lack the enzyme system to degrade phytate in the gastro-intestinal tract. Diets for these animals consist largely of feedstuffs of vegetable origin, thus P-digestibility is usually low. Also between such feedstuffs P digestibility varies. For pigs, P digestibility is only 14% in phytate-rich rice bran. In peas, which contain a relatively small amount of phytate, this value is 45%. P digestibility for poultry shows values similar to those for pigs (CVB, 2000). In order to fulfill the animal's requirement for the essential mineral P, a source of highly digestible P (e.g. an inorganic phosphate) needs, therefore, to be included in its diet. As the quantity of P retained in the animal's body is more or less constant, a high excretion of non-utilized P into manure is the result. This may lead to environmental pollution in areas with intensive pig and poultry production. Phosphate overfeeding of lakes and rivers, locally or downstream, may result in an excessive growth of algae and a depletion of oxygen in the water. A reduction in the aqueous fauna (e.g. fish) may be the consequence.

PHYTASE

Phosphate can be hydrolyzed from the phytate-molecule by phytase, an enzyme of the group of phosphatases. Suzuki et al. (1907, cit. Reddy et al., 1982) were the first to extract phytase (from rice bran). Phytases are present in plants, animals, fungi and bacteria. Two types of phytase are known: 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). The first ester bond hydrolyzed by these enzymes is at the D-3 and the L-6 position, respectively. Most microorganisms produce 3-phytase. 6-phytase is mainly found in plants, although some microorganisms produce it too (Misset, 2003).

Plants that contain high levels of phytase, e.g. rye, wheat and barley, may be used to improve P-digestibility (Pointillart et al., 1987; Pointillart, 1991). Their phytase levels are, however, often variable (Eeckhout and De Paepe, 1994; Barrier-Guillot et al., 1996). Per unit activity, vegetable phytase is less efficient than microbial phytase (Eeckhout and De Paepe, 1992b; Frapin and Nys, 1994; Waremko et al., 2001).

Nelson et al. (1968) showed that microbial phytase supplemented to chicken feed increased P availability. But it was not considered possible to produce the enzyme in an economical way until the 1980's. Political pressure to reduce environmental pollution and the introduction of "manure-legislation", led in The Netherlands to the formation of a consortium that investigated means of reducing animals' P-output. This consortium included research organizations (Poultry Institute "Het Spelderholt", Institute of animal nutrition "IVVO" (both now part of the Animal Sciences Group, Wageningen University and Research Center) and TNO), the feed industry and Gist-brocades (now DSM). One of the main research objectives was the development of an economic usable phytase product. The recent development of modern biotechnology made this possible. Researchers from TNO and DSM cloned the phytase gene from an *Aspergillus niger* (*ficuum*) and brought it to overexpression in a production organism, also an *Aspergillus niger* (Van Hartingsveldt et al., 1993). The product developed, Natuphos®, was first commercialized in 1991. It allows the reduction in P-excretion of animals by about 30%.

Phytases from different sources differ in their biochemical characteristics. The *Aspergillus niger* phytase mentioned has two pH optima: around 5.5 and 2.5. At pH 5.5 the activity is maximal (at 37°C). This phytase can hydrolyze the phosphate groups from the substrates phytate (IP_6) and the "lower" inositol phosphates: *myo*-inositol 1,2,4,5,6-pentaphosphate (IP_5) to *myo*-inositol 2-monophosphate (2-IP_1). Phosphate and inositol are the final products. The enzyme has, however, a low affinity for 2-IP_1 (Misset, 2003). The ability of phytase to release P from phytate is called "P-equivalency". This indicates the quantity of phytase that can replace 1 g P from monocalcium phosphate (MCP-P). For pigs and broilers, P-equivalency is 500 FTU. FTU is the abbreviation for FyTase (phytase) Unit. One FTU is the amount of enzyme that liberates, in one minute, 1 µmol orthophosphate from a 5.1 mM sodium phytate solution at pH 5.5 and at 37°C (Engelen et al., 1994).

The combined characteristics of phytase and conditions in the gastro-intestinal tract (residence time of feed and phytase, pH value, grade of phytate accessibility and phytase degradation) determine that, in pigs, the stomach is the main site of phytase activity. Jongbloed et al. (1992) and Yi and Kornegay (1996) confirmed this experimentally. The crop, proventriculus and gizzard are the main sites of phytate degradation in poultry (Liebert et al., 1993; Yu et al., 2004). A limitation to phytate hydrolysis is the time available to the enzyme for this hydrolysis. Retention time in the stomach of pigs, and in the crop, proventriculus and gizzard of poultry, varies widely, depending in part on feed composition and feed-particle length. Especially the passage of the liquid fraction, including the dissolved components, is generally high (Argenzio, 1993a; Van der Klis, 1993).

PHYTATE-NUTRIENT COMPLEXES – THEIR DEGRADATION BY PHYTASE

Phytate contains six ortho-phosphate groups. At pH 4 the phytate anion has six negative charges, at pH 6 six or seven (Champagne, 1988; Bebot-Brigaud et al., 1999). There is, consequently, a strong ability to complex positively charged ions. Phytate can form complexes with cationic minerals, but also with protein and starch, as depicted in a model of the phytate-complex (**Figure 1**).

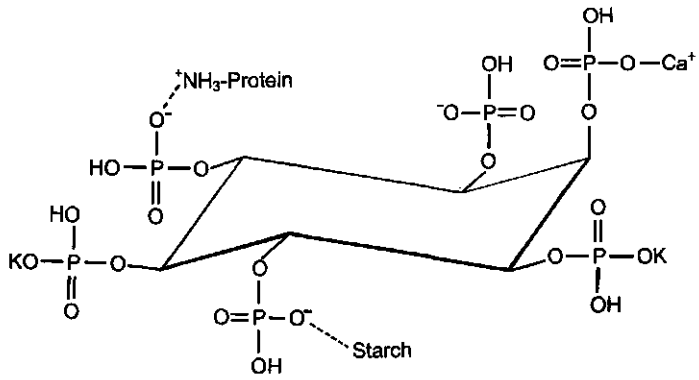

Figure 1. A possible model of phytate at slightly acidic pH.

Under acidic conditions (pH < 5), cationic minerals are mono-coordinated, bound to phosphate at the 2 (2-P) or 5 (5-P) position. From pH 5 to 9, divalent cations are bound mono- or bi-coordinated, to 2-P and 1-P or 3-P, or to 5-P and 4-P or 6-P. At pH > 9, they are bound bi-coordinated, mainly to 2-P and 1-P or 3-P (Bebot-Brigaud et al., 1999). Under acidic conditions, proteins and phytate mainly form binary complexes. At pH 8-9, which is for pigs and poultry of little significance, ternary protein-cation-phytate complexes can be formed (Cosgrove, 1966; Cheryan, 1980; Selle et al., 2000). Complexed protein can be of dietary origin (Ravindran et al., 1995; Selle et al., 2000) and of endogenous origin, e.g. digestive enzymes (Camus and Laporte, 1976; Singh and Krikorian, 1982; Knuckels and Betschart, 1987; Knuckels, 1988). Also starch and fatty acids can bind to phytate, the latter via ternary complexing to a cation or protein (Cosgrove, 1966; Thompson and Yoon, 1984; Lásztity and Lásztity, 1990). When phosphate is hydrolyzed from phytate, the binding between phytate and the complexed cations, proteins or starch is also severed. These nutrients can then be dissolved and are available for gastro-intestinal absorption.

Phytate contains a lot of P that is not available to the animal, and since it also inhibits the availability of some other nutrients, it is called an anti-nutritional factor (ANF). The definition of ANF ('non-fibrous, natural substances causing negative effects on growth and health in man and animals'; Huisman, 1990) is here adapted to 'non-fibrous, natural substances causing negative effects on growth, health, or nutrient utilization in animals'. Possible anti-nutritional effects of phytate are presented in **Table 1**.

Table 1. Phytate: possible anti-nutritional effects and consequences.

Phytate characteristic	(Dietary) effect
Contains much P (28.2% in phytic acid).	Low P availability. Inorganic phosphates added to feed; much P in manure. Buffering capacity of the feed affected.
Complexing to cations (Ca, Zn, Fe, Cu,...).	Low solubility of cations. Decreased availability. Acid-base balance affected, which affects energy metabolism.
Complexing to proteins: – Native proteins. – Dietary proteins (*de novo* complexing within g.i.t.[1]). – Endogenous proteins (digestive enzymes; mucus).	Proteins are not maximally solubilized. Proteins are bound to phytate, and not/less soluble. Less digestive enzymes available in the g.i.t. Production and secretion of digestive enzymes and/or mucus increased. ⇒ Lower availability of protein. ⇒ Digestion of protein, starch and lipids lower. Loss of endogenous proteins. Energy costs of extra produced endogenous proteins.
Complexing to starch and lipids.	Less starch and/or lipids are solubilized in the g.i.t. Lower energy utilization.

[1] gastro-intestinal tract

In many studies it has been shown that dietary phytase supplementation reduces the anti-nutritional properties of phytate. Many papers described the improved P digestibility by phytase. Düngelhoef and Rodehutscord (1995) and Kornegay (2001) reviewed this effect. The effect on the digestibility of minerals, like Ca, Zn, Fe and Cu, has been investigated in pigs (Pallauf et al., 1992a; Adeola, 1995; Jongbloed et al., 1995) and poultry (Simons et al., 1990; Yi et al., 1996a). Officer and Batterham (1992a) and Mroz et al. (1994) showed that protein digestibility in pigs improved with phytase supplementation; Van der Klis and Versteegh (1991) found similar results in poultry. Phytase may increase the digestibility of starch (Thompson and Yoon, 1984) and of fat (Ravindran et al., 2000), and thus of dietary energy utilization.
Increased mineral digestibility permits the feed compounder to reduce dietary levels of these minerals, and, consequently, their output into the environment. Similarly, increased protein digestibility and energy utilization permits the feed compounder to reduce inputs (e.g., protein), and to reduce output to the environment (e.g., nitrogen and the quantity of manure). A better utilization of feed increases the efficacy of animal production, with a reduction of manure output to the environment, and optimizes the economy of animal production.

MICROBIAL PHYTASE – EFFECT OF DIETARY APPLICATION

It might be expected that the reduction of the anti-nutritional properties of phytate by phytase will result in improved performance of pigs and poultry. This hypothesis was tested in several studies, by adding phytase to diets not limiting in digestible P. A review of the literature (Kies, 1997; Kies et al., 1997) pointed out that feed conversion efficiency of piglets was improved

markedly (**Figure 2**). At an inclusion level of 500 FTU/kg, the calculated improvement in performance was 3%. A similar improvement in performance was observed in broilers fed diets not limiting in P (Schutte and Kies, 1995; Kies and Schutte, 1997).

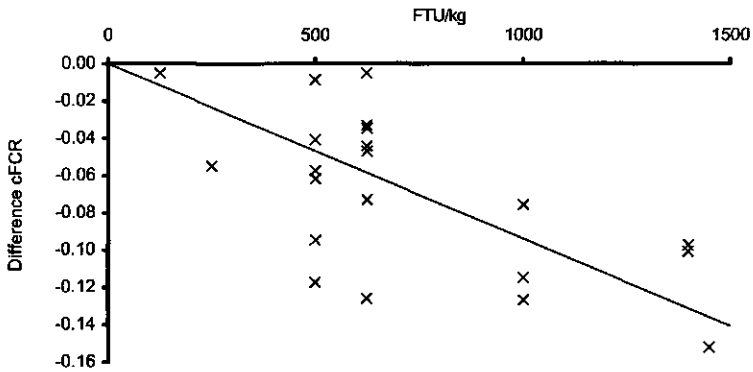

Figure 2. Effect of dietary microbial phytase on corrected feed conversion ratio (cFCR)[1,2] in piglets (6-30 kg), using diets not limiting in digestible P[3,4].

[1] The feed conversion ratio (feed : gain) was corrected for differences in growth rate, assuming 25 g growth/d is equal to 0.01 FCR-unit.

[2] The response was expressed as 'difference cFCR', the difference of the calculated cFCR with that at 0 FTU/kg. Mean cFCR was 1.51 at 0 FTU/kg.

[3] Data from: Beers and Jongbloed (1992), Pallauf et al. (1992b), Barnett et al. (1993), Hoppe et al. (1993), Van der Peet-Schwering (1993), Yi et al. (1996d), Kornegay and Qian (1996) and Campbell (1993-1996). 17 sub-trials (defined by the basal diet) were used. Basal diets were not limiting in digestible phosphorus level according to the authors or the recommendations of Jongbloed et al. (1994).

[4] Regression line: Difference cFCR = A_i + 0.000094 * FTU/kg, with: A_i = calculated intercept per (sub-) trial (i=1-17), and FTU/kg = added microbial phytase (R^2= 0.94).

The improved performance due to phytase could be explained by:

1. Increased P digestibility. The assumed P requirement does not necessarily give maximum performance. It was shown, however, both in pigs (Van Kempen et al., 1976; Jongbloed, 1987) and broilers (Huyghebaert, 1996) that a digestible phosphorus level slightly above requirement has little effect on performance.

2. Increased solubility of cationic minerals (e.g., Ca, Zn and Fe). Because (micro-) minerals, except Ca, P and Na, are usually added to feeds in excess of the animals' requirement, it is unlikely that improved mineral availability results in an enhanced animal performance.

3. Increased amino acid digestibility. The effect of phytase on protein digestibility was quantified (Kies, 1997). Increased amino acid digestibility explains the improved performance in part. The contribution of the increased lysine digestibility to the improved performance was estimated by comparing the result with the effect of dietary lysine supplementation on performance (cFRC) of piglets (Kies, 1997). It was estimated that the improved lysine digestibility by phytase (0.10 g/kg feed containing 500 FTU) could

explain about 10% effect of phytase on performance (Figure 2). For the effect of phytase on all amino acids together, roughly 10-25% of the improved cFCR could be explained (Kies, 1998).

4. Increased energy utilization. Phytate may inhibit digestion of starch and fat. But in studies of Eeckhout and De Paepe (1992a), O'Quinn et al. (1997) and Sands et al. (2001) no positive effect of dietary phytase addition on energy digestibility was observed in pigs. Energy utilization could, however, also be improved due to amelioration of postabsorptive energy processes. For example, endogenous protein production could be decreased due to phytase supplementation. Endogenous protein production is a process that requires much energy. Prior to the start of the projects described in this thesis, this was not investigated.

HYPOTHESES

The primary hypotheses that are investigated in this thesis are:

- Dietary phytase facilitates the extent to which amino acids are released from protein by endogenous enzymes.
- Dietary phytase improves the utilization of energy due to an increased digestibility, an increased metabolizability, an improved level or rate of fat- and/or protein deposition, or a reduced requirement of the animal for maintenance (e.g. via an altered acid-base balance or a reduced endogenous protein production).

Secondary hypotheses tested are:

- Dietary phytase supplementation results in an increased absorption and excretion of minerals, and consequently in a change to the acid-base balance. Mineral absorption and excretion may cost energy.
- Dietary phytase facilitates a faster adaptation of newly weaned piglets to their new environmental conditions.
- Dietary phytase reduces the energy required for gastro-intestinal tissues and absorptive processes. By binding digestive enzymes, phytate could increase the need for enzyme production via a negative feedback mechanism (Singh and Krikorian, 1982; Selle et al., 2000). Phytate degradation would result in lower production of endogenous proteins.

CONTENTS OF THIS THESIS

Chapter 1 describes the complexation of phytate and protein. The effect of phytase on the rate and extent of hydrolysis of the phytate-protein complex and on the efficacy of protein degradation by digestive proteases from the phytate-protein complex was studied *in vitro*.

Chapter 2 describes a study performed with broilers. In the experiment, the effect of dietary phytase supplementation on protein digestibility and on energy metabolisation was measured in a diet limited in lysine content.

Chapter 3 presents a meta-analysis that was performed on the effect of phytase on amino acid digestibility in poultry and pigs. This analysis was based on literature data. Also the effect on metabolizable energy (ME) for poultry was estimated. An update of the values calculated by Kies (1997), and their economic impact, is presented.

Chapter 4 describes a study to the effect of phytase on energy utilization in newly weaned piglets fed ad libitum, using indirect calorimetry.

In Chapter 5 a similar study in restrictedly fed piglets, three weeks post weaning, is described. In this study, digestibility and a number of blood parameters and organ weights were measured. Because some positive effects of phytase, e.g. increased mineral absorption, can have a negative effect on energy requirement, a model is proposed to estimate energy costs of (re-) absorption of nutrients and mineral deposition in bone.

Chapter 6 describes a study on the effect of graded, dietary doses of phytase on mineral absorption in piglets.

In Chapter 7, the effect of phytase on ileal mucin loss in grower pigs is described. Mucin is an important source of endogenous protein production (and loss). Pigs were fed diets with a high or a low phytate level, to measure its influence on mucin production. The energy cost related to altered mucin production was estimated.

Finally, all findings are discussed in Chapter 8. Metabolic costs involved with dietary phytate, and its reduction by phytase, were estimated, and possible physiological and physical explanations are given.

Chapter 1

Interaction between protein, phytate and microbial phytase; *in vitro* studies

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Submitted.

ABSTRACT

The interaction protein-phytate was investigated *in-vitro* at pH ranging from 2 to 10, using proteins extracted from five common feedstuffs and from casein. The appearance of natural soluble protein-phytate complexes in the feedstuffs, the formation of complexes at different pHs, and the degradation of these complexes by pepsin and/or phytase were investigated. Complexes of soluble proteins and phytate in the extracts appeared to be in small amounts, with the possible exception of rice pollards. Most proteins dissolved almost completely at pH 2, but not after addition of phytate. Phytase prevented precipitation of protein with phytate. Pepsin could release protein from a precipitate at a rate that was increased by phytase. Phytase released protein from a protein-phytate complex, but did not degrade it. Addition of both pepsin and phytase resulted in protein breakdown and release from the complex. It appears that protein-phytate complexes are mainly formed at low pH, as occurs in the stomach of animals. Phytase prevented the formation of the complexes, and aided in dissolving them at a faster rate. This might positively affect protein digestibility in animals.

INTRODUCTION

Phytates, salts of *myo*-inositol 1,2,3,4,5,6-hexa*kis* di-hydrogen phosphate or phytic acid, are found commonly in vegetable feedstuffs (Eeckhout and DePaepe, 1994; Ravindran et al., 1995). On average about 67% of P in such feedstuffs is present in phytate. Monogastric animals, like pigs and poultry, degrade phytate poorly. To increase the consequent low P digestibility, products containing microbial phytase were developed as a feed additive. Today, they are widely used in animal feeds. The enzyme hydrolyses phosphate groups from phytate, increasing P digestibility.

At pH values of 1 to 6, which is the normal acidity in the stomach of pigs and in the crop, proventriculus and gizzard of poultry, phytate appears as an ion with 3 to 6 negative charges (Bebot-Brigaud et al., 1999). As a result, complexes are formed with cations such as K, Ca, Mg and Zn. Proteins can also bind to the phytate anion. This can be as a binary protein-phytate complex, where protein is bound directly to phytate, or as a ternary protein-phytate complex. In the latter case protein is bound to a mineral ion, which itself is bound to phytate. The first form is mainly found at a pH of 5 and lower whereas the second form is mainly found at pH values above 7 (Cheryan, 1980).

Phytate-protein complexes may be found, naturally, in plants (Ravindran et al., 1995). They can also be formed *de novo* in the gastro-intestinal tract of animals. The complexed proteins may, therefore, be of dietary or of endogenous origin, e.g. digestive enzymes (Camus and Laporte, 1976; De Rham and Jost, 1979; Singh and Krikorian, 1982). A free amino acid like lysine may also bind to the phytate ion (Rutherfurd et al., 1997). If these protein-phytate complexes are insoluble in the aqueous environment of the gastro-intestinal tract, it is more difficult for proteolytic enzymes to hydrolyze these proteins. Consequently, protein digestion may be inhibited.

When phytase hydrolyzes the phosphate groups from phytate, complexed cations and proteins are also liberated. This may increase the availability of protein for the animal. In several experiments with pigs and poultry, the addition of phytase to the diet showed an improved digestibility of protein (Mroz et al., 1994; Selle et al., 2000; Kies et al., 2001). Although the effect is often quite small (Adeola and Sands, 2003), it may have a considerable impact in practice. Small changes in protein and amino acid digestibility can reduce feed costs considerably, due to a reduced need for addition of the first limiting amino acids.

The objective of current *in vitro* experiments was to investigate mechanisms for the effect of phytase on protein digestion. Studied was whether different feedstuffs contain natural soluble protein-phytate complexes. It was also intended to study the formation of such complexes under conditions similar to those in the stomach of monogastric animals, and the effect of phytase thereon. In addition the effect of phytase on the hydrolysis of protein from a phytate-protein complex by pepsin was investigated.

MATERIALS AND METHODS

Materials The experiment involved *in vitro* studies with six feedstuffs: corn, canola meal, rice pollards, soybean meal, sunflowerseed meal and casein. Apart from casein, these are commonly used in animal feeds. Samples were obtained from feed compounders, air-dried at 70 °C, and ground to pass a 1 mm sieve. From these feedstuffs protein extracts were prepared as follows:

1. 2.5 g of feedstuff was extracted with 25 ml water for 30 min at room temperature. After centrifugation (3000 g; 30 min), the supernatant was removed ("extract 1"). The remaining residue was extracted with 25 ml 0.1 M NaOH during 30 min at room temperature. After centrifugation (3000 g; 30 min), the supernatant was removed ("extract 2"). The remaining residue was extracted with 25 ml 70% ethanol during 30 min at room temperature. After centrifugation (3000 g; 30 min), the supernatant was removed ("extract 3").
2. For study 3, only casein and soybean meal were used. One extract was prepared by a slightly modified method. 10 grams of air-dry feedstuffs were mixed with 100 ml 0.1 M NaOH over a 2-hour period. The supernatant was removed by centrifugation (3000 g; 30 min). The pH of the solution was adjusted to 4.7 (1 N HCl) to precipitate the proteins. The precipitate was freeze-dried. The concentrations of protein and phytate were measured in this material, which was used for the studies ("extract 4").
3. For study 6, only casein was used. 10 ml solutions were constituted (0.1 M citrate buffer (pH 2.4) containing 25 mg casein). Where required, 1.25 mg phytic acid (PA) was added. The suspension was prepared one hour before the incubation with enzymes started, and was kept at 37°C.

Sodium phytate and porcine pepsin were obtained from Sigma Chemical Co., St. Louis, MO, USA (numbers P-8810 and P-6887, respectively). The amount of phytate in solutions was calculated as PA. pH of solutions was obtained using buffers: 0.1 M citrate buffer or (for pH 8 or higher) 0.1 M borate buffer. The pH was adjusted with HCl or NaOH. All reagents used were of analytical grade. Microbial phytase (3-phytase, EC 3.1.3.8, from *Aspergillus niger*; Natuphos®) was obtained from DSM Food Specialties, Delft, The Netherlands.

Methods Protein contents of feedstuffs and extracts were measured using the Kjeldahl method (AOAC, 1984). Relative protein content in solutions was measured with the Bio Rad Protein Assay. Phytic acid analyses were performed by HPLC, using an OmniPac PAX-100 column and suppressed conductivity detection (Dionex). Size Exclusion Chromatography (Bio-Gel P-100; Bio Rad) was used to separate free and protein-bound phytate, using water as the eluent (Okubo et al., 1976). Separation and quantification of soluble protein was done using electrophoresis, according to their molecular weight. A homogenous gel, type 12.5 (Pharmacia, Uppsala, Sweden), was used. Running conditions were 600 V, 50 mA, 30 W. After one hour, Coomassie Blue R 250 was used to fixate the gel and for coloring. The coloring is necessary for detection. Phytase was analyzed according to Engelen et al. (1994). Phytase activity is expressed in FTU, where one FTU is defined as the phytase activity that

liberates 1 µmol orthophosphate from 5.1 mM sodium phytate per minute at 37°C and at pH 5.5. All measurements were performed in duplicate.

Experimental procedures

Study 1. The extent of binding of proteins with phytate in nature was studied by measuring protein and phytate contents in the extracts no. 1, 2 and 3. In extract 1, binding between protein and phytate was measured using Size Exclusion Chromatography.

Study 2. Binding of protein and phytate in aqueous solution was studied at different pH's. Extract 2 was used, because (with the exception of rice pollards) this contained a high level of protein and a low level of phytate. The quantities of protein and PA in the solutions (10 ml) are reported. For the phytate addition, natural PA was taken into account. The quantity was chosen to obtain maximal precipitation; only results with this dose are shown.

Study 3. In this study, the ratio of protein (extract 4) to PA in the protein-phytate complex was measured dependant upon pH (2 and 3) and the protein : PA ratio (5, 10 and 20:1, w/w). After precipitation, the amounts of protein and PA in solution were measured, from which the quantities in the precipitate were calculated.

Study 4. The effect of phytase on the formation of protein-phytate complexes was studied by adding 2.91 FTU phytase to the phytate solution (0.5 mg PA), before adding that to the protein extract (extract 2).

Study 5. Phytate (0.5 mg) was added to extract 2 (the quantity used was chosen to obtain a final ratio protein : PA of 10:1) at pH 2, to form a protein-phytate precipitate. To study the release of protein from the complex, pepsin (8 FIP-U), pepsin and phytase (4 FTU), or nothing was added to the solution. Solubility of protein and phytate were measured after incubating the mixture at 37 °C for 30, 60, 120, 180, or 240 minutes. One FIP-Unit is defined as the quantity of pepsin that changes the absorption at 280 nm with 0.01 unit/min at pH 2.0 and at 37°C from the TCA-soluble fraction, using hemoglobin as the substrate.

Study 6. To the casein-phytate suspension pepsin (8 FIP-U), phytase (0.08 FTU), or both were added. Also, solutions of only the enzymes in buffer were tested. After several time intervals (1, 2, 3, 4, 5, 6 and 24 hours) over the incubation period (37 °C), a sample of the total suspension was taken. These samples were divided into two equal parts. In one part the soluble phase was separated using ultra centrifugation (30000 g; "solution"). The other part was used as such ("suspension"). Proteins were determined by electrophoresis as described previously.

RESULTS

Soluble protein extracts of the different feedstuffs (study 1) contained between 53% (canola meal) and 82% (rice pollards) of the total protein present in the raw materials (**Table 1**). Recovery of phytate was low in sunflowerseed and canola meals, and high in corn. Overall, extract 2 contained most of the soluble protein, and extract 1 most of the soluble phytate. In extract 1, proteins and phytate were not bound. No clear correlation was observed between protein and phytate contents in the different extracts.

Table 1. Protein and phytate contents in different extracts of feedstuffs, total amounts of crude protein (CP; g/kg) and phytate (as phytic acid, PA; g/kg), and the percentage of soluble protein and phytate in these extracts relative to the content of the feedstuffs (study 1).

		Extract				Soluble[1]
Feedstuff	Parameter	1	2	3	Total	(%)
Casein	CP	18	552	55	880	71
	PA	-[2]	-	-	-	-
Corn	CP	8	46	18	99	73
	PA	6.9	2.2	-	8.8	103
Canola meal	CP	46	106	27	336	53
	PA	3.7	2.5	-	32.6	19
Rice Pollards	CP	49	56	10	140	82
	PA	19.1	11.0	-	98.0	31
Sunflowerseed meal	CP	27	128	21	300	59
	PA	4.9	-	-	35.4	14
Soybean meal	CP	41	257	39	470	72
	PA	12.1	1.3	-	15.8	85

[1] In fractions 1 through 3, relative to total.
[2] Not detectable.

Acidity of the solutions and phytate addition had a large impact on the solubility of protein (study 2; **Table 2**). At pH 2, and at pH 8 or higher, protein dissolved almost completely, whereas most of it precipitated at pH 3 to 5. For most feedstuffs, addition of phytate decreased solubility of protein under acid conditions, especially at pH 2. The disappearance of dissolved phytate from the solution confirms that protein-phytate complexes were formed. The exception was rice pollards: its protein precipitated at pH 2, independent of phytate addition.

Table 2. *Relative amount of protein (%) in solution with or without addition of* phytate at different pH's (study 2).

		pH					
Feedstuff	Addition	2	3	4	5	8	10
Casein (13.75)[1]	-[2]	100	3	1	85	92	90
	+	1	0	1	91	86	83
Corn (4.4)	-	100	42	36	34	97	84
	+	28	33	32	33	98	86
Canola meal (2.65)	-	100	81	71	76	93	99
	+	63	78	73	74	97	100
Rice Pollards (1.4)	-	22	39	38	38	96	100
	+	16	33	36	35	91	97
Sunflowerseed meal (3.9)	-	100	20	20	22	88	98
	+	26	17	16	21	98	93
Soybean meal (2.36)	-	91	60	17	71	87	100
	+	2	23	16	61	87	100

[1] mg crude protein in solution per 10 ml sample (extract 2)
[2] -: no phytate added; +: phytate added (amounts per sample: casein 1.0 mg; corn 0.5 mg; canola meal 0.2 mg; rice pollards 0 mg; sunflowerseed meal 0.2 mg; soybean meal 0.5 mg).

Precipitation of protein-phytate complexes depended on the ratio of protein to phytate in the solution (study 3). Protein precipitated at pH 2 when this ratio was 10 : 1 (**Table 3**). With casein, but not with soybean meal extract, some precipitate was formed at a ratio of 20 : 1. At pH 3 most protein precipitated, also at a ratio of 20 : 1. The ratio of protein : phytate in the precipitate was about 10 : 1 with both feedstuffs at pH 2. This ratio was higher at pH 3 than at pH 2. This indicates that less phytic acid is required to form a precipitate at pH 3 than at pH 2. Contradictory to pH 2, at pH 3 the protein : phytic acid ratio in the precipitate increased with increasing quantity of phytate added to the solution. Also, for the other four feedstuffs, protein: phytate ratios of about 10 :1 and 20 : 1 were found in the precipitates formed at pH 2 and 3, respectively.

Table 3. Relative amount of protein and phytic acid (%) in the precipitates obtained at pH 2 or 3, with different amounts of added phytate (expressed as phytic acids; PA) and protein (CP). Experiment with extract 4 from casein and soybean meal (study 3).

		Amount added		In precipitate		
Feedstuff	pH	CP (mg)	PA(mg)	CP (%)	PA (%)	Ratio (w/w)
Casein	2	25	5	99	48	10
			2.5	98	90	11
			1.25	36	56	13
	3	25	5	99	42	12
			2.5	98	66	15
			1.25	95	100	19
Soybean meal	2	23.6	5	92	50	9
			2.5	87	88	9
			1.25	0	0	-
	3	23.6	5	95	39	11
			2.5	93	57	15
			1.25	90	67	25

Addition of phytase to the phytate solution before addition to the protein extract (study 4) prevented the formation of a protein precipitate to a large extent (**Table 4**). Phytate is hydrolyzed by phytase, which prevents the formation of protein-phytate complexes.

Incubation of soybean protein-phytate precipitate with pepsin at pH 2 (study 5) slowly reduced the amount of precipitated protein (**Figure 1A**). When phytase was also added, protein dissolved faster from the precipitate. In addition, the extent of protein dissolution was larger. Without the addition of pepsin, the amount of protein precipitated was stable at 100%. At pH 3 (**Figure 1B**) the extract already showed a partial precipitation of its protein without phytate addition (Table 2). For that reason, the quantity of precipitated protein without addition of phytate decreased at pH 3 (Figure 1B). When phytate was added, the dissolution rate of precipitated protein was reduced, but when phytase was also added this rate was higher than for the control.

Table 4. Relative amount of protein (%) in solution with or without addition of phytate to feedstuffs extract (extract 2), in combination with phytase at pH 2 and 3 (study 4).

	pH 2			pH 3		
Feedstuff	P[1]	PP[2]	PPP[3]	P	PP	PPP
Casein (13.75)[4]	100	1	93	3	0	4
Corn (4.4)	100	28	100	42	33	42
Canola meal (2.65)	100	63	95	89	81	82
Rice Pollards (1.4)	22[5]	16	57	39	33	47
Sunflowerseed meal (3.9)	100	26	90	34	23	28
Soybean meal (2.36)	100	2	99	60	32	60

[1] P: protein extract only
[2] PP: protein extract with phytate (amounts per sample: casein 1.0 mg; corn 0.5 mg; canola meal 0.2 mg; rice pollards 0 mg; sunflowerseed meal 0.2 mg; soybean meal 0.5 mg).
[3] PPP: protein extract with phytate and phytase (2.91 FTU)
[4] mg crude protein in solution (3.1 ml) per sample
[5] *probably due to the high content of phytate in the protein extract*

Degradation of protein from the protein-phytate precipitate by pepsin, with or without addition of phytase, was studied in study 6. The electrophoresis-gels after 1 and 2, 3 and 4, 5 and 6, and 24 and 1 hours incubation, are shown in **Figure 2**, panes A, B, C and D, respectively. The bands indicate the size of proteins in suspension and in solution. Pepsin and phytase themselves do not form bands (Figure 2A, lanes 2 and 3), thus are not interfering with the results. After one hour, protein bound to phytate resembles pure casein (Figure 3A, suspension, lanes 4, 5, 7 and 8 vs. lane 1). When phytate was not added, pepsin hydrolyzed protein (lane 6, suspension) and proteins appeared in solution. Phytate formed a stable complex with casein: even after 24 hours protein was in suspension, whereas no protein appeared in solution (Figure 2D, lane 4). Addition of phytase liberated part of the protein from the protein-phytate complex, but did not degrade the protein into smaller units (lane 5). In contrast, addition of pepsin alone degraded the protein of the complex into smaller units, but did not

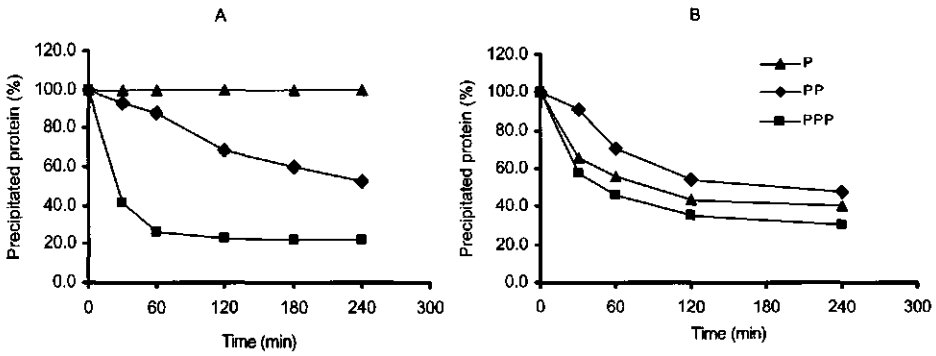

Figure 1. Relative amount of protein in a precipitate formed at pH 2 (from soybean meal protein extract), after addition of pepsin (8 FIP-U). P is the control, no addition of phytate or phytase. To PP phytate was added. To PPP, phytate and phytase (4 FTU) were added. Measurements at pH 2 (A), or at pH 3 (B) (study 5).

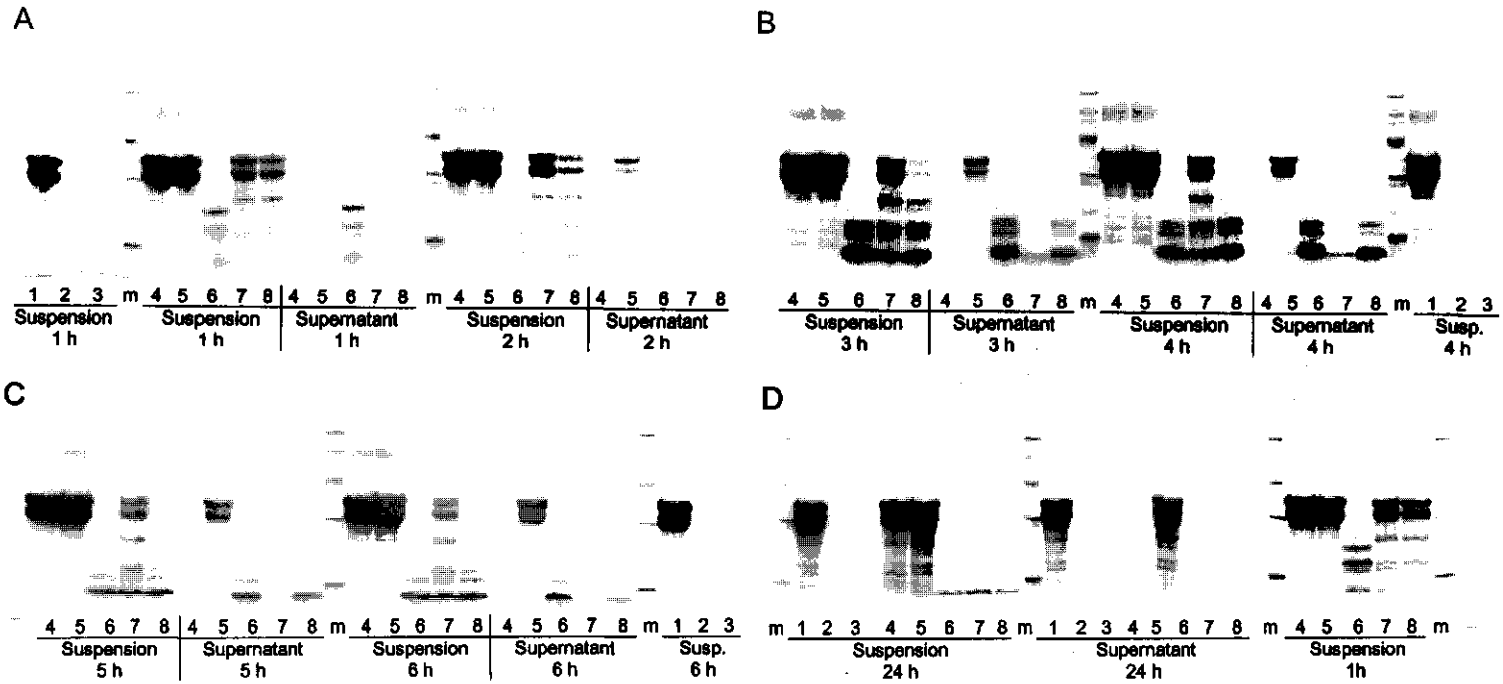

Figure 2. Study 6: electrophoresis-gel of casein. Size of proteins in suspension and in solution is shown. After 1 or 2 hours of incubation (A), 3 or 4 hours (B), 5 or 6 hours (C), or 24 or 1 hours (D; the latter is for comparison).

Treatments:
Lane 1: buffer and casein; Lane 2: buffer and phytase; Lane 3: buffer and pepsin.
Lane m: marker.
Lanes 4 – 8: buffer and casein with addition of phytate (4), phytate and phytase (5), pepsin (6), phytate and pepsin (7), and phytate, phytase and pepsin (8).

liberate these units from the complex until small units (ca. 12 kD) were formed (lane 7 vs. lane 4). Combined effects: liberation of the protein from the protein-phytate complex and degradation of the protein into smaller units, were observed when both phytase and pepsin were added (lane 8).

DISCUSSION

Protein-phytate complexes may already exist in the plant, or they might be formed when protein and phytate react within the gastro-intestinal tract of monogastric animals. In the present study, no clear correlation between the amount of protein and phytate in extracts 1-3 (water, dilute NaOH and ethanol extracts) was observed. In extract 1, phytate levels were relatively high with soybean meal, corn and rice pollards. Size exclusion chromatography indicated that no protein-phytate complexes were present in this extract. Only in rice pollards was recovery of phytate in extract 2 high. This feedstuff, therefore, may contain natural, soluble protein-phytate complexes. A low recovery of both protein and phytate, such as in canola meal, does not permit to draw a conclusion on the existence of natural protein-phytate complexes. These results suggest that complexes of soluble proteins and phytate do not exist, or exist in small amounts only, in the investigated feedstuffs, with the possible exception of rice pollards.

In many plants phytate is stored in globoids. Globoids are particles that are usually incorporated in the protein bodies of plant-cells (Scott and Loewus, 1986). Consequently, phytate is usually stored in tissues that are rich in protein, e.g., the germ or aleurone layer. This may lead to the assumption that protein is bound to phytate. The similar solubility behavior of both proteins and phytate also suggests this (Cheryan, 1980). But globoid crystals contain often only a small amount of protein (Reddy, 2002). This makes a direct binding between protein and phytate less obvious. Our results agree with this, though we studied soluble proteins only.

The complexing of protein with phytate has been shown in different in vitro studies (De Rham and Jost, 1979; Cheryan, 1980). In vivo, the possible influence of protein-phytate complexing was studied in a number of experiments, by means of digestibility studies. In those studies diets were supplemented with phytase, which degrades phytate. With phytase supplementation, ileal amino acid digestibility increased in pigs (Officer and Batterham, 1992a; Mroz et al., 1994) and poultry (Namkung and Leeson, 1999; Ravindran et al., 2000). The improvement is not always significant (Adeola and Sands, 2003), but in a meta-analysis the phytase effect was significant (Kies et al., 2001).

From study 1 it appears unlikely that a large quantity of natural, soluble protein-phytate complexes exist in the feedstuffs studied. Thus, insoluble protein-phytate complexes that are digested with phytase addition must have been formed within the gastro-intestinal tract of monogastric animals. From our results it is very likely that such insoluble complexes are formed in the stomach. At pH 2, most proteins were dissolved, but most precipitated after addition of phytate, with the exception of canola meal. Distal from the proximal duodenum, the pH is usually higher than 4. No protein-phytate complexes were formed at such pH values.

More than 85% of the protein precipitated when the ratio protein : phytate was 10 : 1 (at pH 2), or 20 : 1 (at pH 3; Table 3). The protein : phytate ratio in the complex was lower at pH 2 than at pH 3. Probably, phytate has more negative sites available for binding to protein at pH 3, than at pH 2 (Bebot-Brigaud et al., 1999). A higher number of negative sites result in an increased rate of formation of phytate-protein agglomerates. The "expansion" of this agglomerate continues when additional phytate is available, because the level of phytate in the complex increases with increasing phytate addition at pH 3. At pH 2, the possible binding sites may be saturated with protein-bonds, when the precipitate is formed. Therefore, the relative amount of phytate in the protein-phytate complex varies little with increasing phytate addition. As an approximate mean, practical corn-soybean meal diets for monogastric animals contain about 20% crude protein and about 1% phytate (expressed as phytic acid), a ratio of 20 : 1. Therefore, the formation of protein-phytate complexes described above may occur in the stomach of animals, assuming a mechanism similar to that in the extracts used in the present study.

The ratio of protein : phytate in the precipitates (Table 3) was higher than those measured by Lásztity en Lásztity (1990). They found a maximal phytate load in protein-phytate precipitates (pH 2 – 5.5) of 126, 90 and 30 mol per 100 kg protein, for soy glycenin, sunflower seed globulin and wheat gluten, respectively. This means the protein : phytate ratios were 1.2 to 5 (w/w). This large difference from our results may be explained by the different characteristics of the proteins used, and by the test conditions.

In these tests, at pH 3 the protein : phytic acid ratio was higher in the precipitate of soybean meal than of casein. This is probably related to the amino acid composition. Basic amino acids may link best to the phytate ion. Soybean meal contains (on a protein basis) a higher level of arginine, and about equal proportions of lysine and histidine, as compared to casein (CVB, 2000).

Phytase supplementation prevented the formation of protein-phytate complexes to a large extent (study 4). If these complexes had been formed already, pepsin dissolved protein from the complexes at a higher rate and to a larger degree when phytase was also added (Figure 1). Protein present in precipitates was hydrolyzed into smaller parts by pepsin (Figure 2). The pieces were only dissolved from phytate when they were smaller than about 12 kD. When phytase was added to the protein-phytate precipitate together with pepsin, proteins were liberated from the complex, and hydrolyzed into smaller fragments at a faster rate than without phytase. Phytase itself, however, did not hydrolyze protein (Figure 2).

For the digestion of protein by animals, the importance of the increased rate of protein hydrolysis into smaller fragments, and the solubility of these fragments, is presently unknown. Pepsin hydrolyzes protein at a lower rate from a protein-phytate precipitate than from soluble protein. This does not mean, however, that the digestibility of these protein fragments is reduced to zero. When the pH rises, in the small intestine, probably a large part of the protein-fragments is dissolved. Proteolytic enzymes in the small intestine may further degrade these fragments. The digestion of amino acids depends on a dynamic system, which includes pH, resi-

dence time in the different compartments of the gastro-intestinal tract, concentration and degree of solubility of proteins, and concentration of proteolytic enzymes.

Dietary phytate increases the formation of insoluble protein-phytate complexes in the stomach. So there is a risk that phytate affects digestibility negatively. Phytase prevents formation of these complexes, or aids to degrade them faster and further. It may, therefore, improve protein digestibility. This mechanism could explain the small increase in protein digestibility (about 1-2%-units) observed in many experiments (Kies et al., 2001). Also the binding of proteolytic enzymes to phytate may explain a part of this effect.

It is concluded that in the feedstuffs studied in the present experiments, only a small amount of natural soluble protein-phytate complexes is present. Insoluble protein-phytate complexes are formed at low acidity, as found in the stomach of monogastric animals. Protein degradation from such complexes by pepsin is at a lower rate than from soluble protein. Protein digestibility may, therefore, be slightly reduced. Dietary phytase supplementation prevents the formation of protein-phytate complexes, or aids in dissolving them faster. Phytase, therefore, may affect protein digestibility positively.

Chapter 2

Microbial phytase improves performance, apparent metabolizable energy, and ileal amino acid digestibility of broilers fed a lysine-deficient diet

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ABSTRACT

An experiment was conducted to examine the effects of adding microbial phytase or lysine on the performance of broilers fed a phosphorus (P)-adequate, but lysine-deficient diet. A wheat-soybean meal-sorghum-based diet, containing 1.00% lysine and 0.45% nonphytate P, was either supplemented with graded levels of lysine (0.06, 0.12, or 0.18%), or with graded levels of phytase (125, 250, 375, 500, 750, or 1000 phytase units (FTU)/kg diet). Each diet was fed to six pens of 10 chicks each from day 7 to 28 post-hatching.

Addition of lysine linearly increased ($P < 0.001$) weight gain and gain : feed ratio of broilers. The response in weight gain to added phytase reached a plateau at 500 FTU/kg diet (quadratic effect, $P < 0.001$). Phytase had no effect on gain : feed to 250 FTU/kg diet and then increased (quadratic effect, $P < 0.05$) with further additions.

Addition of increasing levels of dietary phytase improved ($P < 0.001$) the digestibilities of nitrogen and all amino acids. Assuming that the observed responses in weight gain and gain : feed to added phytase were due to the release of lysine alone and by solving linear or non-linear response equations for lysine and phytase levels, the lysine equivalency value was calculated to be 500 FTU/kg diet = 0.074% lysine.

Phytase also increased the apparent metabolizable energy (AME) level; the response reached a plateau at 750 FTU/kg diet (quadratic effect, $P < 0.001$). These results showed that both amino acid (lysine) and energy responses are responsible for the performance improvements observed when phytase was added to a wheat-soybean meal-sorghum-based diet.

INTRODUCTION

The value of microbial phytase in releasing phytate-bound P and improving P bioavailability of plant ingredients for poultry is well documented (Coelho and Kornegay, 1996). Phytate, in its natural state, is also complexed with various cations, protein, lipids (Cosgrove, 1966) and starch (Thompson and Yoon, 1984). The significance of phytate in the utilization by poultry and swine of nutrients other than P, however, has received little attention until recently. By releasing these phytate-bound nutrients and improving their utilization, dietary microbial phytase supplementation would be expected to have protein/amino acid and energy effects in monogastric animals. This hypothesis has been confirmed in a number of studies in poultry. Generally, positive effects of supplemental phytase were shown on amino acid digestibility (Yi et al., 1996b; Biehl and Baker, 1997; Sebastian et al., 1997; Ledoux et al., 1999; Ravindran et al., 1999a, 2000) and on AME (Farrell et al., 1993; Farrell and Martin, 1998; Ledoux et al., 1999; Namkung and Leeson, 1999; Selle et al., 1999; Ravindran et al., 2000).

The observed improvements in protein and amino acid digestibility due to phytase are of considerable practical significance and need to be quantified to enable their inclusion in least-cost diet formulations. In the present study these effects were quantified in a basal diet that was lysine-deficient but available P-adequate. The effect of graded dietary levels of phytase or lysine on performance, apparent ileal amino acid digestibility and AME of broilers was investigated. On the basis of the results, equations were generated for performance responses obtained with supplemental phytase and lysine. These were used to calculate the equivalency value of phytase for lysine. This approach was similar to that employed by Yi et al. (1996c) to calculate P equivalency values for phytase for broilers fed P-deficient diets.

MATERIALS AND METHODS

Dietary treatments Ten experimental diets, containing varying levels of lysine or microbial phytase, were formulated. Feed ingredients sufficient for the feeding trial were obtained in bulk and were analyzed for phytate P, total P, Ca, N and amino acids prior to feed formulation. Monocalcium phosphate and limestone were analyzed for total P and Ca. A wheat-sorghum-based diet containing 91 % of the recommended level of lysine for broiler starters (NRC, 1994) served as the basal diet (diet 1). Composition of the basal diet is shown in **Table 1**. This diet met or exceeded recommended requirements for all amino acids, except lysine (NRC, 1994), and contained recommended levels of non-phytate P (0.45%). The phytate-P level in the diet was 0.30%. The Ca : non-phytate P ratio was maintained at 2.3 : 1. Celite, a source of acid-insoluble ash (AIA), was added at 2 % as a digesta marker. Wheat was steam-pelleted prior to mixing the diets, to lower the inherent phytase activity.

Diets 2 to 4, containing 96, 102 and 107% of the recommended total lysine levels (NRC, 1994), were formulated by the addition of L-lysine monochloride to the basal diet (diet 1). The other five diets (diets 5 through 10) were used for testing graded levels of phytase. To the basal diet (diet 1) 125, 250, 375, 500, 750, or 1000 FTU/kg was added. The microbial phytase (Natuphos® 5000 Granulate; DSM Food Specialties, Delft, The Netherlands; obtainable from BASF AG, Germany) contained 5,000 phytase units (FTU) / g phytase activity. One FTU is the quantity of enzyme that releases 1 μmol of inorganic P/min from 5.1 mM sodium phytate at

pH 5.5 at 37 °C. All diets were supplemented with a xylanase product (Natugrain® Blend; DSM Food Specialties, Delft, The Netherlands). Addition of xylanase preparations in wheat-based diets is a routine practice to ameliorate the adverse effects of wheat nonstarch polysaccharides on broiler performance (Ravindran et al., 1996c). In diets with added phytase, non-phytate P and calcium levels were lowered by adjusting the inclusion rates of monocalcium phosphate and limestone and by taking into account the P equivalency for phytase (500 FTU = 1 g P from monocalcium phosphate) as recommended by the manufacturer. Sorghum levels were increased in the phytase diets in place of monocalcium phosphate and limestone.

TABLE 1. Composition of the basal diet (diet 1)[1]

Ingredient	Percentage	Ingredient	Percentage
Wheat	33.05	Celite	2.00
Sorghum	22.26	Monocalcium phosphate	1.64
Soybean meal	22.50	Limestone	1.66
Canola meal	5.00	DL-methionine	0.29
Rice polishings	2.60	L-threonine	0.07
Corn gluten meal	2.50	Vitamin-mineral premix[2]	0.50
Vegetable oil	4.70	Salt	0.30
Dextrose	0.75	Choline chloride	0.20
Calculated analysis[3], %		Determined values, %	
Total P	0.75	Crude protein	19.62
Phytate P	0.30	Lysine	0.97
Non-phytate P	0.45	Threonine	0.93
Calcium	1.03	Tryptophan	0.23
Calcium: non-phytate P	2.3:1	Phenylalanine + tyrosine	1.98
AME, MJ/kg	13.1	Valine	1.46
Crude protein	19.75	Isoleucine	1.11
Lysine	1.00[4]	Leucine	1.80
Methionine + cysteine	0.93	Histidine	0.49
		Arginine	1.26

[1] Diets 2 to 4 were formulated by addition of L-lysine monochloride to provide 1.06, 1.12, or 1.18% lysine. Diets 5 to 10 were formulated by the supplementation of 125, 250, 375, 500, 750, or 1000 FTU/kg diet. All diets contained xylanase (Natugrain® blend granulate; 80 mg/kg).

[2] Supplied per kilogram diet: *trans*-retinol, 3.3 mg; cholecalciferol, 87.5 μg; dl-*a*-tocopheryl acetate, 20 mg; menadione, 2 mg; thiamine, 1.5 mg; riboflavin, 8 mg; calcium pantothenate, 15 mg; niacin, 30 mg; pyridoxine, 5 mg; folic acid, 2 mg; cyanocobalamin, 15 μg; biotin, 100 μg; Mn, 75 mg; Zn, 50 mg; Cu, 5 mg; Mo, 1.6 mg; Co, 300 μg; I, 1 mg; Fe, 20 mg; Se, 100 μg; choline chloride, 300 mg; ethoxyquin, 125 mg.

[3] Calculated based on analyzed values for individual ingredients, with the exception of AME, which was obtained from unpublished values from Camden (W.L. Bryden, unpublished data).

[4] All amino acids, except lysine, are supplied to meet or exceed the recommended requirements for broiler starters (NRC, 1994).

Birds and Conduct of the Trial Experimental procedures were approved by the University of Sydney Animal Care and Ethics Committee and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

One-day-old male broiler chicks (Cobb) were obtained from a commercial hatchery and fed a commercial broiler starter diet (23 % crude protein) to day 7. On day 7, the birds were indivi-

dually weighed, and the heaviest and lightest were discarded. Six hundred chicks of uniform weights were randomly allotted to 60 pens (10 chicks/pen) in electrically heated, raised, wire-floored starting cages in an environmentally controlled room. Each of the 10 dietary treatments was randomly assigned to six pens of ten chicks each. The diets, in mash form, were fed from days 7 to 28. Feed and water were available *ad libitum*. On day 18, all birds were transferred to 60 cages with facilities for excreta collection.

Measurements Individual body weights and pen feed intake were recorded at weekly intervals. Mortality was recorded daily. During the third week of the trial, feed intake and excreta output were measured quantitatively per pen over four consecutive days (days 24 to 27) to obtain AME data. The excreta was collected daily at 0900 h, dried for 24 h at 80 °C in a forced-air oven, and pooled within a pen for analysis. Care was taken to avoid contamination from feathers, scales and debris. The dried excreta were allowed to equilibrate to atmospheric conditions before being weighed. Representative samples were taken and ground to pass through a 0.5-mm sieve.

On day 28, all surviving birds were killed by intracardial injection of sodium pentobarbitone. The small intestine was immediately exposed, and digesta contents were collected from the lower half of the ileum, pooled within a pen and processed as described previously (Ravindran et al., 1999a). Toe ash has been shown to be a good indicator of P status and accurate in determining P availability of diets for poultry (Potter, 1988). Therefore, toe samples were obtained by severing the left middle toe through the joint between the second and third tarsal bones from the distal end. The toes of all birds within a pen were pooled, and the composite samples were dried to a constant weight at 100 °C and then ashed in a muffle furnace at 600 °C for 6 h. Toe ash was expressed as a percentage of dry weight.

Chemical analysis The gross energy levels of diet and excreta samples were determined using an adiabatic bomb calorimeter (Gallenkamp) standardized with benzoic acid. Nitrogen content was determined by the method of Sweeney (1989) using an FP-428 nitrogen determinator (LECO Corp., St. Joseph, MI, USA). Amino acid concentrations in the diet and ileal digesta samples were determined using a Shimadzu amino acid analysis system (Shimadzu Corp., Kyoto, Japan) after acid hydrolysis (Ravindran et al., 1999a). Tryptophan contents were determined following alkaline hydrolysis of samples according to the procedures of Ravindran and Bryden (1996). Separate sulfur amino acid analyses were not carried out. The AIA contents of diet and ileal digesta samples were measured after ashing the samples and treating the ash with boiling 4 M hydrochloric acid (Siriwan et al., 1993). All diets were analyzed for phytase activity using the procedures described elsewhere (Selle et al., 1996).

Calculations The AME values of the diets were calculated using the following formula. Appropriate corrections were made for differences in moisture content.

$$AME_{diet}\ (MJ/kg) = \frac{(\text{feed intake} \times \text{gross energy}_{diet}) - (\text{excreta output} \times \text{gross energy}_{excreta})}{\text{feed intake}}$$

Apparent ileal nitrogen and amino acid digestibilities were calculated, using AIA as the indigestible marker, as follows:

$$\text{Apparent digestibility (\%)} = \frac{(\text{amino acid / AIA})_d - (\text{amino acid / AIA})_i \times 100}{(\text{amino acid / AIA})_d}$$

where $(\text{amino acid / AIA})_d$ and $(\text{amino acid / AIA})_i$ are the ratios of amino acid or nitrogen to acid-insoluble ash in the diet and the ileal digesta, respectively.

Statistical analysis The data were analyzed by the General Linear Models procedure of SAS® (Release 6.04; SAS Institute, Cary, NC, USA) with pen means as the experimental unit. Linear and quadratic effects of lysine (diets 1 to 4) and supplemental phytase (diet 1 and diets 5 to 10) on gain, feed intake, gain : feed ratio, toe ash, AME and ileal digestibility of nitrogen and amino acids were tested using orthogonal polynomials.

Linear and nonlinear response functions of body weight gain, feed intake and gain : feed that best fit the data were derived for lysine levels (diets 1 through 4) and for phytase levels (diet 1 and diets 5 through 10). The models used were as follows:

Linear function $Y = a + bX$
Nonlinear function $Y = a(1-be^{-kX})$

where Y = the response measurement, and X = lysine (percentage of diet) or phytase added (FTU/kg diet). The nonlinear or linear response equations with the higher r^2 value for added lysine and the equations for added phytase were set equal and solved using the procedures described by Yi et al. (1996c). Linear and non-linear response functions of AME and ileal digestibility of nitrogen and amino acids that best fit the data were also derived for phytase levels (diet 1 and diets 5 through 10).

RESULTS

Mortality during the trial was within acceptable levels (less than 2 %) and was not related to dietary treatments. The analyzed phytase activities indicated that the determined values agreed well at lower dosages, but there was an overestimation at dosages of 500 FTU/kg diet and above. The determined supplemented microbial phytase activity in diets 5, 6, 7, 8, 9 and 10 were 123, 253, 378, 688, 1068 and 1363 FTU/kg diet, respectively. The analyzed amino acid contents of diet 1 (lysine-deficient basal diet) confirmed that this diet met or exceeded the recommended requirements for all essential amino acids (NRC, 1994), except lysine (Table 1). The determined value for lysine agreed closely with the calculated value.

Performance Data The influence of dietary treatments on broiler performance is summarized in **Table 2**. Addition of graded levels of lysine, to a wheat-soybean meal-sorghum basal diet containing 1.00% lysine, linearly ($P < 0.001$) increased weight gain and gain : feed ratio of broilers. Feed intake increased up to the 1.12% dietary lysine and then declined (quadratic effect, $P < 0.05$) with further addition.

TABLE 2. Performance and toe ash contents[1] of broilers fed a lysine-deficient wheat-soybean meal-sorghum diet containing varying levels of lysine and phytase from 7-28 days of age.

	Treatment					
Diet no.	Lysine, %	Phytase, *FTU/kg*	Weight gain, *g/bird*	Feed intake, *g/bird*	Gain : feed, *g/kg*	Toe ash content, %
1	1.00[2]	0	823	1,475	558	12.9
2	1.06	0	850	1,489	571	12.9
3	1.12	0	884	1,514	584	13.1
4	1.18	0	899[3]	1,468[4]	612[3]	13.2
5	1.00	125	832	1,492	558	13.0
6	1.00	250	847	1,516	559	13.3
7	1.00	375	857	1,509	568	13.0
8	1.00	500	864	1,497	577	13.2
9	1.00	750	867	1,492	581	13.4
10	1.00	1,000	861[5]	1,494	576[6]	12.9
SEM			5.8	13.3	3.1	0.18

[1] Each mean represents six pens of 10 birds each.
[2] Represents 91% of the recommended lysine level for broiler starters (NRC, 1994).
[3] Lysine effect (linear, $P < 0.001$).
[4] Lysine effect (quadratic, $P < 0.05$).
[5] Phytase effect (linear, $P < 0.001$; quadratic, $P < 0.001$).
[6] Phytase effect (linear, $P < 0.001$; quadratic, $P < 0.05$).

The response in weight gain to the addition of graded levels of phytase reached a plateau at 500 FTU/kg diet (quadratic effect, $P < 0.001$). In the case of gain : feed, added phytase had no effect up to 250 FTU/kg diet and then increased (quadratic effect, $P < 0.05$) with further addition of phytase. Feed intake was not influenced ($P > 0.05$) by the addition of phytase.

Using the procedure described by Yi et al. (1996c), this data set was used to estimate lysine equivalency values for microbial phytase. Linear and nonlinear functions that gave the best fit (the highest r^2) to the data were derived for lysine and phytase. The equations were then set equal and solved. Estimates were obtained for weight gain and gain : feed, but equations for feed intake were found to be poor fits and are further not considered.

For weight gain (Y), the following equations and solution were obtained:

$Y = 404.29 + 420.25 \times$ dietary lysine (%) $\quad (r^2 = 0.98)$

$Y = 868.2\ (1 - 0.0556e^{-0.00359 \times FTU/kg}) \quad (r^2 = 0.92)$

Solution: % lysine $= 1.1039\ (1 - 0.1041e^{-0.00359 \times FTU/kg})$.

The dietary lysine level is the calculated total lysine level, and the phytase level is the calculated added phytase. When resolved, the level of lysine equivalent to 500 FTU/kg diet addition is 1.0848 %. Therefore, released lysine = lysine equivalency estimate – lysine in basal diet (i.e., 1.00%). The gain response from 500 FTU/kg diet is therefore equal to 0.0848 % lysine. Because the recommended inclusion rate of Natuphos® phytase in broiler diets is 500 FTU/kg diet, this level was considered in the calculation of lysine equivalency.

Similarly for gain : feed ratio (Y_1) responses, this resulted in the following equations:

$Y_1 = 282.2 + 272.8 \times$ dietary lysine (%) $(r^2 = 0.97)$
$Y_1 = 587.9\ (1 - 0.0574e^{-0.00157 \times FTU/kg})$ $(r^2 = 0.79)$
Solution: % lysine $= 1.1206\ (1 - 0.1104e^{-0.00157 \times FTU/kg})$.

When resolved, the level of lysine equivalent to 500 FTU/kg diet addition is 1.0641%. The gain : feed response from 500 FTU/kg diet is therefore equal to 0.0641% lysine.

The average of the above two estimates was 0.0744 %. Based on the assumption that the observed responses in weight gain and gain : feed to added phytase were due to the release of lysine alone, the lysine equivalency value of the enzyme can be considered as 500 FTU/kg diet = 0.0744% lysine.

Toe ash data Although the diets were formulated to contain recommended levels of non-phytate P, to discount any possible P effects, the toe ash contents of the birds were determined. The results summarized in Table 2 show that treatments had no effect on toe ash contents.

Ileal Digestibility of Nitrogen and Amino Acids The influence of microbial phytase on the apparent ileal nitrogen and amino acid digestibilities is presented in **Table 3**. Addition of increasing levels of dietary phytase caused linear ($P < 0.001$) improvements in the digestibilities of nitrogen and all amino acids. The increases in the digestibility of arginine, lysine, aspartic acid and glutamic acid also contained quadratic components ($P < 0.05$ to 0.01). The

TABLE 3. Apparent ileal digestibility of N and amino acids[1] of a lysine-deficient wheat-soybean meal-sorghum diet for broilers as influenced by varying levels of microbial phytase.

Diet No	Diet 1	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	SEM	*P*-value[2]
Phytase, *FTU/kg*	0	125	250	375	500	750	1000		
Nitrogen	78.1	78.7	78.9	79.8	81.2	81.0	82.2	0.51	< 0.001
Arginine	82.1	82.4	82.8	84.9	85.6	85.2	86.2	0.45	< 0.001
Histidine	79.6	79.7	80.3	82.8	82.4	83.4	82.8	0.76	< 0.001
Isoleucine	76.1	75.2	76.5	76.8	79.5	79.3	79.6	0.56	< 0.001
Leucine	76.4	76.9	77.0	79.2	79.2	79.6	81.3	0.82	< 0.001
Lysine	79.4	81.2	81.6	82.5	83.0	83.4	84.1	0.46	< 0.001
Phenylalanine	77.1	77.2	78.3	78.1	80.8	80.2	82.5	0.58	< 0.001
Threonine	74.9	75.6	76.8	76.3	78.5	77.9	79.6	0.66	< 0.001
Tryptophan	76.2	76.6	75.8	78.0	79.4	79.2	79.7	0.47	< 0.001
Valine	76.9	75.1	76.3	74.3	80.8	80.0	81.8	0.59	< 0.001
Alanine	77.2	76.8	76.9	79.7	80.1	82.4	82.6	0.70	< 0.001
Aspartic acid	77.2	77.3	78.0	78.7	80.7	81.9	81.0	0.43	< 0.001
Glutamic acid	80.8	81.1	82.1	84.7	85.5	85.4	85.7	0.58	< 0.001
Glycine	76.8	77.4	76.7	79.8	80.1	79.4	81.6	0.47	< 0.001
Serine	76.3	77.1	76.2	78.0	79.4	80.3	81.3	0.63	< 0.001
Tyrosine	76.1	75.4	76.6	76.5	78.7	77.9	79.1	0.52	< 0.001
Overall mean	77.5	77.7	78.1	79.4	80.9	81.0	81.9	0.36	< 0.001

[1] Each mean represents six pens of 10 birds each.
[2] Linear effect. Quadratic effect was not significant, except for arginine, lysine and aspartic acid ($P < 0.05$) and glutamic acid ($P < 0.01$).

magnitude of response to added phytase varied depending on the level of supplementation and the amino acid considered. The increases in amino acid digestibility were minimal at 250FTU/ kg diet addition. For most amino acids, the highest responses were observed at the addi-tion of 1000 FTU/kg. The relative improvements in the digestibility of all essential amino acids were 4.4 and 5.6% with 500 and 1000 FTU/kg dietary addition, respectively.

AME data The AME content of the diet was not influenced by lysine levels (**Table 4**) but increased with increasing levels of supplemental phytase. The maximum effect was obtained at 750 FTU/kg (quadratic effect, $P < 0.001$). Addition of 500 FTU/kg improved the AME value by 2.3% (14.2 vs. 14.5 MJ/kg dry matter).

TABLE 4. Apparent metabolizable energy (AME)[1] of a lysine-deficient wheat-soybean meal-sorghum diet for broilers as influ-enced by varying dietary levels of lysine and microbial phytase.

	Treatment		
Diet no.	Lysine, %	Phytase, *FTU/kg*	AME, *MJ/ kg dry matter*
1	1.00[2]	0	14.2
2	1.06	0	14.3
3	1.12	0	14.2
4	1.18	0	14.2
5	1.00	125	14.2
6	1.00	250	14.3
7	1.00	375	14.4
8	1.00	500	14.5
9	1.00	750	14.7
10	1.00	1,000	14.6[3]
SEM			0.1

[1] Each mean represents six pens of 10 birds each.
[2] Represents 91% of the recommended lysine level for broiler starters (NRC, 1994).
[3] Phytase effect (linear, $P < 0.001$; quadratic, $P < 0.001$).

DISCUSSION

The relevance of phytate-protein complexes in lowering protein utilization in monogastric animals and the potential of microbial phytase to release the phytate-bound protein have attracted considerable attention in recent years (Kies and Selle, 1998). Although the P equivalency values for microbial phytase are well established (Coelho and Kornegay, 1996), corresponding data on amino acid release are limited. The present study was designed to estimate the lysine equivalency value of phytase in a lysine-deficient basal diet. Phytase will release lysine from phytate-protein complexes and result in an improved performance of broilers. Based on this assumption, the addition of 500 FTU/kg of a wheat-soybean meal-sorghum diet was calculated to be equivalent to 0.074 % lysine as measured by responses in body weight gain and gain : feed ratio. The diets 3 and 4 might not have been deficient in lysine during the last week according to the NRC (1994) recommendation, which is 1.00% lysine for 3 to 6 wk-

old broilers. Because there was still a clear improvement in gain and gain : feed on these diets, this recommendation is probably too low for the chicks in this experiment.

The multifaceted effects of phytase in practical diets are being increasingly appreciated, and it is possible that the observed performance responses may reflect the release of P, available amino acids and energy by the added phytase. The absence of a significant influence of phytase on toe ash content (Table 2) indicates that the diets contained adequate amounts of non-phytate P to support bone mineralization and that the observed performance responses were independent of P effects of the enzyme. The digestibility data show that the addition of phytase significantly improved not only the ileal digestibility of lysine but also of other amino acids. The positive influence of microbial phytase on the apparent ileal digestibility of amino acids is in agreement with previous reports. In the present study, the addition of 500 FTU/kg diet increased the mean amino acid digestibility with 3.4% units, which is higher than the increments of 1.3 to 2.3% units observed in previous studies (Yi et al., 1996b; Sebastian et al., 1997; Namkung and Leeson, 1999; Ravindran et al., 2000). Ravindran et al. (1999a) and Selle et al. (2000) discussed possible mechanisms contributing to the observed improvements in amino acid digestibility in detail.

Significant improvements in AME were also observed with phytase addition. The energy effects of phytase in wheat and wheat-sorghum based diets have been reported previously (Ravindran et al., 1999c, 2000), and the present results again confirm these effects. Improvements in the AME or TME (true ME) of poultry diets based on corn (Namkung and Leeson, 1999), sorghum (Farrell et al., 1993; Selle et al., 1999), oats (Farrell and Martin, 1993) and barley (Zhang et al., 1999) have also been reported in the literature. The mechanism of the AME effect is largely unknown, but improved protein digestibility is responsible, at least in part, for these responses. The data of Ravindran et al. (2000) show that phytase may improve energy utilization, independent of its effect on amino acid digestion. It was proposed that mineral-phytate complexes may contribute to the formation of insoluble metallic soaps in the gastrointestinal tract, which is a constraint on lipid utilization. By preventing the formation of mineral-phytate complexes, phytase may reduce the degree of soap formation in the gut and enhance the utilization of energy derived from lipids. Dietary levels of calcium and saturated fats would have particular relevance to this proposed mode of action.

Starch digestibility of poultry diets is not usually considered to be limiting, but another possible facet of the mode of action of phytase is the removal of the adverse effects of phytic acid on starch digestion. It has been demonstrated in humans that manipulation of dietary phytate levels modifies the blood glucose response or glycemic index (Thompson et al., 1987). The glycemic index was negatively correlated ($r = -0.71$; $P < 0.01$) with phytate concentrations in foods (Yoon et al., 1983), which infers that phytate reduces carbohydrate digestibility. Thompson and Yoon (1984) suggested that phytate might affect starch digestibility by interacting with proteins closely associated with starch or by direct binding with starch via phosphate links. It was also suggested that phytate inhibition of amylase might be a factor in the reduced blood glucose responses. Considerable *in vitro* evidence indicates that phytate is a potent, noncompetitive inhibitor of α-amylase activity, probably as a result of phytate complexing with the enzyme or blocking its active sites (Sharma et al., 1978; Knuckles and Bet-

schart, 1987; Li et al., 1993). Desphande and Cheryan (1984) suggested that the capacity of phytate to inhibit amylase may play a physiological role in relation to starch reserves during seed germination.

In the case of wheat, an additional mode of action has been recently proposed to explain improvements in AME with added phytase (Ravindran et al., 1999c). Based on the observation that phytate is an integral component of the cell wall matrix in wheat (Frolich, 1990), it was postulated that microbial phytase may be acting in a manner similar to that of exogenous xylanases, by disrupting cell walls and enhancing contact between digestive enzymes and cell contents.

It is therefore evident that responses in overall amino acid digestibility and AME are responsible for the performance improvements observed when phytase was added to the lysine-deficient diet. The present results, along with other reports (Ledoux et al., 1999; Namkung and Leeson, 1999; Ravindran et al., 1999c, 2000; Zhang et al., 1999), indicate that the amino acid responses to added phytase are generally associated with energy responses. Because it is not possible to separate the amino acid and energy responses, the difficulty in achieving the original aim of estimating lysine equivalency values for phytase becomes evident. Not withstanding this difficulty, data are generated on the dose-response effects of phytase addition on amino acid digestibility and AME values in a wheat-soybean meal-sorghum diet for broiler chickens. The present results also confirm the positive effects of microbial phytase on the digestibility of nutrients other than P.

Chapter 3

The effect of phytase on protein and amino acid digestibility and energy utilization in poultry and pigs

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ABSTRACT

Phytate is a molecule rich in phosphorus (P). However, the P in phytate is of low availability to monogastric animals because they lack the proper enzyme system to hydrolyze phytate. Consequently, there is a high P concentration in the manure of animals fed on diets containing phytate, and this can lead to environmental pollution. Because phytate can complex with minerals, starch, proteins and digestive enzymes, it also has anti-nutritional properties. Limiting the P output of monogastric animals, by increasing the digestibility (pigs) or availability (poultry) of P in the diet, by hydrolyzing phosphate from phytate, was the original reason for developing microbial phytase. It has been shown in many studies that P excretion by pigs and poultry can be reduced by 30% by including phytase in their diets.

The digestibility of other nutrients bound to phytate can also be increased considerably by hydrolysis of the phytate molecule by phytase. A number of studies have been performed in poultry and pigs to determine the effect on amino acid digestibility of adding microbial phytase to the feed. In general, an increase of 1-3% has been reported. It was shown by meta-analysis that these improvements were significant for most amino acids at a phytase supplementation rate of 500 FTU/kg diet. In piglets and broilers an improvement in performance of 1.5-3% was often observed when phytase was included in the diet, even if the diet met the digestible/available P requirement. This improvement in performance cannot be explained by improvements in amino acid digestibility alone. It has been suggested that there is an effect on energy utilization as well, and this has now been confirmed in studies with poultry.

To apply this information in feed compounding, matrix values are proposed for use by the industry in linear programming. Depending on many factors, feed costs can be decreased by up to EUR 3.50/ton (December 2000; US$ 3.00) by the addition of phytase to diets that are not limiting in P. Limiting the total P content in the diet to a lower concentration can increase the economic advantage of adding phytase. The broadness of the impact of this enzyme on the nutritional value of feed makes it a really remarkable enzyme.

INTRODUCTION

Phytase has been used as a commercial feed additive for 10 years. It effectively improves phosphorus (P) digestibility in diets that differ widely in composition, for different species of monogastric animals. Even though Nelson and colleagues reported already in 1968 that the addition of microbial-derived phytase increased the availability of P for poultry, it was not until 1991 that phytase could be used at a commercially attractive price. There were two reasons for this: (1) it was only at this time that scientific developments (recombinant DNA technology) made it possible to produce phytase at a cost that was attractive to the feed industry, and (2) intensive livestock production in some regions of the world, notably The Netherlands, created a demand for phytase.

Phytase was specifically developed as an "environmental" enzyme. Between 60% and 75% of the P in feedstuffs of plant origin is present as phytate. Because monogastric animals lack the enzyme necessary to hydrolyze phytate, under practical feeding conditions their requirement for digestible (pigs) or available (poultry) P can only be met by addition of inorganic phosphates to their diets. A large proportion of P is excreted in feces, creating environmental problems. The addition of phytase to the diet permits hydrolysis of phytate. As a result, it is possible to decrease the P content of the diets and reduce P output in manure. The phytase product Natuphos® was developed as the result from a large research program, initiated within DSM during the 1980s, with the aim to identify a suitable phytase-producing microorganism. Thousands of microorganisms were screened and their phytases tested for activity at different pH values, their resistance to pepsin and their thermostability. Finally, a phytase from *Aspergillus niger* (formerly known as *A. ficuum*) was identified as the best candidate and was purified, cloned and overexpressed in an *A. niger* organism (Van Gorcum et al., 1995).

In this paper the studies performed to assess the effects of microbial phytase on the digestibility of protein and amino acids in feeds for poultry and pigs and on the metabolizable energy content of feeds for poultry are reviewed. Only trials performed with Natuphos® are included, because this product was the choice in almost all trials carried out to study the effect of phytase on protein digestibility and energy metabolizability.

PHYTASE: EFFECT ON DIGESTIBILITY OF P, PROTEIN AND AMINO ACIDS

Effect on phosphorus digestibility (availability) The efficacy of microbial phytase was evaluated with different animal species in close collaboration with the Dutch feed industry. In these studies, and in many other studies thereafter (in the Netherlands and other countries), the main objective was to quantify the amount of P liberated by phytase and the amount of inorganic phosphate that it could replace. An example of the effect of microbial phytase on P availability in broilers is presented in **Table 1**. In this trial (Simons et al., 1990), a diet consisting of corn, sorghum, soybean and sunflower meals was fed. The basal diet was deficient in available P; it contained 4.5 g total P (of which 3.0 g phytate-P) per kg. An inorganic phosphate or microbial phytase was added. A diet containing 7.5 g total P / kg (4.5 g available P / kg) was included as the positive control.

Table 1. Effect of increasing concentrations of dietary microbial phytase[1] on the availability and excretion of phosphorus in broilers (Simons et al., 1990).

Phytase addition, *FTU/kg*	P content, *g/kg*	P availability, *%*	P in manure, *g/kg dry matter intake*
0	4.5	49.8	2.7
0	6.0	45.6	3.8
0	7.5	44.6	4.9
250	4.5	56.5	2.3
500	4.5	59.6	2.1
750	4.5	59.5	2.1
1000	4.5	62.5	2.0
1500	4.5	64.5	1.9

[1] The amount of phytase supplemented to the diet is expressed in phytase units (FTU). One FTU is defined as the amount of enzyme that liberates 1 µmol orthophosphate/min from 5.1 mM sodium phytate, at pH 5.5 and 37°C (Engelen et al., 1994).

From Table 1 it appears that microbial phytase increased the availability of P for broilers considerably. At 1500 FTU/kg diet, P availability increased by 15%, and its excretion was reduced, especially when compared to the positive control diet. The performance of broilers also improved. Weight gain (0 to 24 days of age) increased from 338 g/broiler (negative control) to 683 g (positive control) and to 733 g (1500 FTU/kg). The corresponding feed : gain ratios were 1.85, 1.55 and 1.50, respectively. Thus, P availability increased, P excretion decreased and performance was improved by phytase addition. These results were not unexpected, since the control diet was deficient in available P.

Similar results were obtained in studies with pigs. Beers and Jongbloed (1992) investigated the effect of adding microbial phytase to two basal diets, on P digestibility in growing pigs (20-55 kg). The first diet contained corn and soybean meal as main components, and also tapioca, barley and peas; the second diet additionally contained by-products (hominy feed, sunflower seed meal and rapeseed meal) and had a higher phytate content than the first diet. Six levels of phytase were added, ranging from 200 to 2000 FTU/kg feed. The increase in the digestible P content of the diets is shown in **Figure 1**.

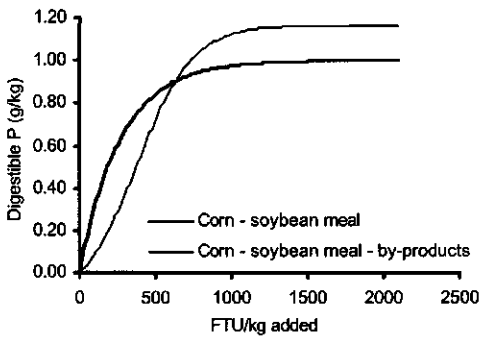

Figure 1. Effect of increasing concentrations of microbial phytase on the digestible P content of pig feeds (Beers and Jongbloed, 1992).

Of interest in Figure 1 is the different shape of the two curves. The digestible P content in the corn-soybean meal diet increases rapidly up to an enzyme inclusion of 400 FTU/kg. At higher inclusions there is only a small further increase. In the diet containing by-products, the increase in digestible P was lower at low additions of phytase, but increased sharply up to a concentration of 1000 FTU/kg; thereafter, no additional effect was observed. In both diets the inclusion rate of 500 FTU/kg increased the digestible P by approximately 0.8 g/kg diet.

In most trials the efficacy of microbial phytase was compared to inorganic P sources. From studies mentioned above, and others, it was concluded that dietary supplementation with 500 FTU is equivalent to 1 g of P from monocalcium phosphate (MCP). This value is similar for pigs and broilers, but for laying hens the value is 300 FTU. These values were recently confirmed in a literature review (Kornegay, 1999).

Effect of phytase on performance When experiments were carried out to establish the P value of phytase, it was often observed that performance was also improved. This is not surprising, because P-deficient basal diets were used in those studies and P deficiency is known to reduce performance (Simons et al., 1990). Responses were, however, often larger than could be attributed to the improved P supply. To study the effect of microbial phytase on performance, using diets not limiting in available P, two broiler trials were performed. Kies and Schutte (1997) offered a corn-soybean meal diet, adequate in available P, to broilers housed in floor pens. Three pens of females (55/pen) and three pens of males (50/pen) were included per treatment. Starter (1-14 days) and grower (14-38 days) diets were fed. The available dietary P content was kept constant by the addition of MCP and / or microbial phytase, assuming that 500 FTU is equivalent to 1 g of MCP-P. No additional effect from adding more than 500 FTU/kg was assumed. Results (**Table 2**) show that performance of broilers improved with increasing phytase addition. Supplementation of 500 or 1000 FTU/kg improved performance 1.5-2%. Results from Schutte and Kies (1995) were similar.

Similar responses were obtained with piglets. Calculations were made based on a review of 17 studies. In these studies, phytase was added to diets not limiting in digestible P, according to local standards or to Jongbloed et al. (1994). Results were subjected to linear regression analysis, using deviation of growth-corrected feed : gain ratios, compared to the control, as the

Table 2. Effect of microbial phytase on performance (1-38 days) of broilers fed a corn-soybean meal diet with adequate available phosphorus level (Kies and Schutte, 1997)

	Diet			
	1	2	3	4
Phytase addition, *FTU/kg*	0	250	500	1000
Total P, *g/kg*	7.8 / 6.7[1]	7.3 / 6.2	6.8 / 5.7	6.8 / 5.7
Available P, *g/kg*	4.5 / 3.5[1]	4.5 / 3.5	4.5 / 3.5	4.5 / 3.5
Gain, *g/bird*	2098[a,2]	2107[ab]	2145[c]	2137[bc]
Feed : gain ratio	1.581[a]	1.568[ab]	1.571[ab]	1.559[b]

[1] Starter and grower diet, respectively.
[2] Values in the same row without a common superscript differ significantly ($P < 0.05$).

dependent variable. A fixed effect for the trial number was included as the intercept (to correct for differences between trials): $Y = A_i + b \times X$, where Y = difference in growth-corrected feed conversion ratio (g/g), A_i = random intercept per trial (i = 1-17), b = regression coefficient and X = added phytase (FTU/kg). Details are provided by Kies et al. (1997). The resulting regression line ($Y = -0.000094 \times X$; $R^2 = 0.94$) and observations ($- A_i$) are presented in **Figure 2**.

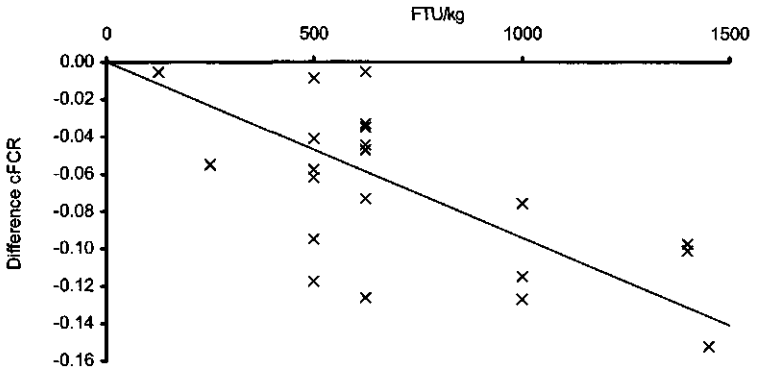

Figure 2. Effect of addition of microbial phytase on corrected feed conversion ratio of piglets (difference from controls) fed on diets not limiting in digestible phosphorus.

The effect of microbial phytase supplementation on performance in diets not limiting in digestible (available) P could be explained in several ways:

(a) P requirement is underestimated. Defined requirement is not necessarily the level to obtain maximal performance. This effect is unlikely to be a major factor in this case. When dietary digestible P level exceeds the animal's requirement, there is only a small effect on pig performance (Jongbloed, 1987).

(b) Phytase liberates cations from phytate and increases their availability. These cations (e.g. Ca, Mg, Mn, Zn, Cu and Fe) are, however, usually not limiting in practical diets for pigs and poultry. It is, therefore, unlikely that increase of their availability has a major impact on performance.

(c) Phytate can also bind with starch and protein, as shown in **Figure 3**. The action of phytase will liberate (a part of) these compounds, thereby increasing the energy and protein value of the diet.

Effect on protein and amino acid digestibility A number of trials have been conducted with poultry and pigs, to study the effect of microbial phytase on protein and amino acid digestibility. In general, dietary microbial phytase supplementation improved the digestibility values of amino acids and crude protein by 1 to 3%. There were, however, large variations between trials, probably caused by differences in composition of the diets and in methods for determining digestibility.

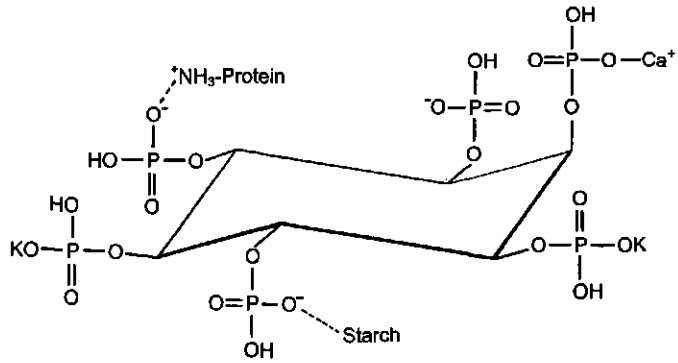

Figure 3. Possible interactions with a phytate molecule.

An illustration of the effect of phytase on performance of broilers, protein and amino acid digestibility and AME is presented in **Table 3** (Ravindran et al., 2001). In this experiment, different levels of phytase (up to 1000 FTU/kg) were added to a basal diet. Diets met the available P requirements of the birds (4.5 g/kg feed). Absence of a treatment effect on toe ash weight confirmed that P was not limiting. The basal diet was deficient in lysine (80% of requirement) in order to estimate the protein value directly (main objective of the experiment). Also, sensitivity for observing a response in broiler performance is higher. It was assumed that the sub-optimal lysine level did not affect amino acid digestibility. Main ingredients of the diet were wheat (330 g/kg), sorghum (220 g/kg) and soybean meal (220 g/kg). In addition, canola meal, corngluten meal and rice pollards were included, and an endoxylanase. Each diet was offered to 60 male birds (10 birds per cage). The experimental period was from 7 to 28 days of age. Excreta were collected from day 24 to day 27. At 28 days of age broilers were sacrificed and their ileal digesta were collected. Addition of phytase significantly ($P < 0.001$) improved weight gain, feed : gain ratio, AME and protein and amino acid digestibilities (Table 3). A number of quadratic relationships were significant (Ravindran et al., 2001).

Table 3. Effect of microbial phytase on performance, ileal digestibility of some amino acids and apparent metabolizable energy (AME) values in broilers (7-28 days) fed on a wheat/sorghum-based diet deficient in lysine (Ravindran et al., 2001)

	Phytase addition, *FTU/kg*							LSD
	0	125	250	375	500	750	1000	($P = 0.05$)
Weight gain, *g/bird*	823	832	847	857	864	867	861	13.1
Feed : gain ratio	1.79	1.79	1.79	1.76	1.73	1.72	1.74	0.031
Digestibility, *%*								
Crude protein	78.1	78.7	78.9	79.8	81.2	81.0	82.2	1.47
Arginine	82.1	82.4	82.8	84.9	85.6	85.2	86.2	1.30
Lysine	79.4	81.2	81.6	82.5	83.0	83.4	84.1	1.33
Threonine	74.9	75.6	76.8	76.3	78.5	77.9	79.6	1.90
Tryptophan	76.2	76.6	75.8	78.0	79.4	79.2	79.7	1.36
AME, *MJ/kg*	13.06	13.07	13.16	13.25	13.35	13.51	13.38	0.114

To take advantage of these results, feed producers could follow different strategies in feed formulation. Amino acid specifications used in feed formulation could be lowered, or higher digestible amino acid values could be assigned to feedstuffs of plant origin. These options may look confusing, but they could work if a fixed amount of phytase is always added to the diet. To take full advantage of phytase, however, it should be considered as a feed ingredient in its own right, thus assigning it with its own nutrient specifications (matrix values). The rate of inclusion of phytase need not be fixed, though a maximum inclusion needs to be defined. Optimal (nutritional and economic) dietary inclusion will result from linear programming.

A problem with considering phytase as an ingredient with its own nutrient values is the assignment of these values. It means that results, as those presented in Table 3, need some "translation". In 1997, we calculated the first matrix values for phytase (Kies et al., 1997). Different problems had to be overcome when making these calculations. For example, values need to be assigned to a certain phytase inclusion level, because the response on amino acid digestibility is probably not linear (as for P digestion: Figure 1). Phytase inclusion of 500 FTU/kg diet was chosen, because this amount is typically added to diets in practice (replacement of 1 g MCP-P). Results of the different experiments were re-calculated to this inclusion level by linear regression, if the maximum dose applied was larger than 500 FTU/kg. If the maximum dose in an experiment was less than 500 FTU/kg diet, it was assumed the result was obtained with 500 FTU/kg. Assuming that the effect of phytase on amino acid digestibility follows a similar exponential course as on P digestibility, both methods of estimation likely give an underestimation of this effect. In the dataset used, underestimation varies from 0 to more than 40%.

An example of applying this calculation is the estimation of the effect of phytase on lysine digestibility, using the results of Ravindran et al. (2001; Table 3). Lysine digestibility was 79.4% and 83.0% for dietary inclusion levels of 0 and 500 FTU/kg, respectively. Diets contained 9.7 g lysine per kg, thus digestible lysine levels were 7.7 and 8.1 g/kg, respectively. Phytase (500 FTU/kg) generated 0.35 g digestible lysine/kg feed. Calculating the effect using linear regression (over all treatments), the additional amount of digestible lysine is 0.21 g/kg (at 500 FTU/kg), a difference of 40%. Using non-linear regression analysis, an amount of 0.31 g digested lysine per kg feed was calculated. Because the linear regression method was used in our calculations, this allows for a (first) safety margin. The same approach was followed in all experiments for crude protein, the other amino acids and for AME.

Mean calculated quantities of extra digestible crude protein and amino acids and upper and lower limits of the 95% confidence interval are presented in **Table 4**. Two pig trials, showing extremely positive results for phytase, were excluded from the data. The table shows that the mean effect of phytase supplementation on protein and amino acid digestion is always positive, although sometimes small. Although the effects can be small, and the variation within experiments is in some cases large, for most amino acids the lower limit of the 95% confidence interval is positive, indicating statistical significance.

Another approach to test whether phytase supplementation has a positive effect on amino acid digestibility is to use a qualitative statistical method, like the Sign test. This test determines

Table 4. Digestible crude protein and amino acids generated by dietary phytase (500 FTU/kg) for poultry[1] and pigs[2] and the lower and upper limits of their 95% confidence interval.

	Poultry				Pigs			
	n	Lower limit	Mean	Upper limit	*n*	Lower limit	Mean	Upper limit
			g/kg diet				*g/kg diet*	
Crude protein	24	1.334	2.247	3.159	11	0.812	1.953	3.094
Arginine	25	0.080	0.137	0.194	13	0.043	0.088	0.133
Cystine[3]	13	0.019	0.036	0.053	11	0.001	0.024	0.048
Histidine	24	0.034	0.059	0.084	13	-0.003	0.015	0.033
Isoleucine	25	0.074	0.128	0.182	13	-0.004	0.040	0.084
Leucine	25	0.109	0.208	0.308	13	0.013	0.114	0.215
Lysine	25	0.072	0.121	0.169	13	0.023	0.075	0.127
Methionine[3]	14	-0.009	0.011	0.031	11	0.009	0.026	0.043
Phenylalanine	24	0.079	0.137	0.194	13	0.019	0.059	0.098
Threonine	25	0.076	0.137	0.197	13	0.002	0.054	0.106
Tryptophan	6	0.002	0.038	0.073	3	-0.008	0.030	0.067
Valine	25	0.087	0.158	0.230	13	-0.019	0.037	0.093
Glycine	19	0.028	0.074	0.120	-	-	-	-
Serine	19	0.037	0.110	0.183	-	-	-	-

[1] Results from 11 broiler trials, two turkey trials and one duck trial. Data from: Kornegay, 1996; Kornegay et al., 1999; Ledoux and Firman, 1998, 1999; Martin et al., 1998; Namkung and Leeson, 1999; Ravindran et al., 1999b, 2000, 2001; Schutte et al., 1997; Sebastian et al., 1997; Yi et al., 1996b; Zhang et al., 1999.

[2] Results from 8 growing / finishing pig trials and one sow trial. Data from: Gagné and Pomar, 1999; Johnston et al, 2004; Kemme et al., 1999; Kornegay et al., 1998; Mroz et al., 1994, 1998; Officer and Batterham, 1992a,b; Radcliffe and Kornegay, 2000.

[3] Only results obtained from experiments, in which the analyses were performed using an oxidation step prior to acid hydrolysis, are included.

the probability of the number of positive and negative results being equal; only non-zero effects are included in the test (Conover, 1980). The results are presented in **Table 5**. Not every diet containing phytase was included as an observation. In experiments with more than one non-zero phytase observation, this would mean that the control diet is counted more often than once, resulting in dependency of the variables. In those cases, a correlation coefficient was calculated (over all treatments in that experiment), and the sign of that coefficient was used as the observation. The advantage is that this method permits the inclusion of a number of observations that were not included in previous calculations (Table 4), e.g. those of Ravindran et al. (1999a). For most amino acids, a significant positive effect of phytase on amino acid digestibility is observed, for both poultry and pigs.

Mode of action Phytate can form complexes with protein, as shown in Figure 3. Proteolytic enzymes degrade complexed proteins more slowly (or not at all) relative to solubilized proteins. A lower or slower protein digestibility may be the result. Four different types of protein complexing have been identified. These are:

- Phytate-protein complexes naturally present in feedstuffs.
- *De novo* formation of phytate-dietary protein complexes in the digestive tract.
- Formation of complexes of phytate and free amino acids in the digestive tract.
- Formation of complexes of phytate and proteolytic enzymes in the digestive tract.

Table 5. Number of non-zero (n <> 0) and positive observations (positive effect of phytase; $n > 0$), and probability (P) for protein and amino acid digestibility studies in poultry and pigs[1].

	Poultry			Pigs		
	$n <> 0$	$n > 0$	P	$n <> 0$	$n > 0$	P
Crude protein	40	36	0.000	11	11	0.000
Lysine	38	33	0.000	14	11	0.029
Methionine	16	10	0.227	12	11	0.003
Cystine	15	12	0.018	12	7	0.387
Threonine	42	35	0.000	14	11	0.029
Tryptophan	6	5	0.109	3	3	0.125
Isoleucine	41	35	0.000	13	8	0.291
Arginine	41	37	0.000	13	11	0.011
Histidine	40	34	0.000	14	10	0.090
Leucine	42	35	0.000	13	12	0.002
Phenylalanine	40	33	0.000	14	12	0.006
Valine	41	35	0.000	14	8	0.395

[1] From the same references as used in Table 4 and additionally Ravindran 1999a (poultry).

It is generally assumed that natural phytate-protein complexes are present in feedstuffs (Ravindran et al., 1995). Quantification of these complexes is difficult, however, because such complexes cannot be distinguished from those formed during extraction procedures applied during analyses. *De novo* complexing of protein with phytate is likely to occur in the stomach.

Jongbloed et al. (1997) showed that a very strong complex is formed between soluble proteins from different feedstuffs and phytate, at pH values between 2 and 3. Such pH values may occur in the stomach. Pre-treatment of phytate with phytase prevented the formation of such complexes. After formation of a protein-phytate complex, hydrolysis of protein from this complex by pepsin was considerably accelerated by addition of phytase (**Figure 4**).

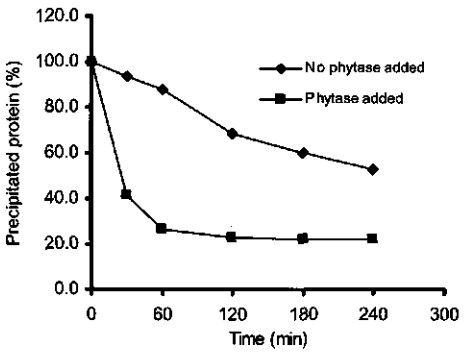

Figure 4. Soybean protein precipitated (%) at pH 2 after addition of pepsin or pepsin plus phytase.

Rutherfurd et al. (1997) showed that free lysine forms a complex with phytate. They incubated rice pollards (a feedstuff rich in phytate) with lysine hydrochloride. Approximately 20% of lysine was bound, but half of this was liberated after the addition of phytase. Protein digestion may also be inhibited indirectly because proteolytic enzymes in the digestive tract form complexes with phytate. Singh and Krikorian (1982) showed that trypsin activity could be inhibited by phytate, which may result in a decrease in protein digestibility.

In conclusion, phytase supplementation improves the digestibility of protein and amino acids, in addition to having a positive effect on P digestibility. Mechanisms to explain this improvement have been identified, but the exact mechanism is presently unclear.

EFFECT OF MICROBIAL PHYTASE ON ENERGY METABOLIZABILITY

As discussed, inclusion of phytase in diets not limiting in available P may result in improvement of weight gain and feed : gain ratio. Improved protein and amino acid digestibility may explain only a part of this effect, as calculated by Kies (1997). Increased mineral digestibility may also provide only a partial explanation for this performance-effect. It was hypothesized that phytase might improve utilization of energy, probably by increased digestibility or metabolizability.

The effect of microbial phytase on AME was studied in 24 broiler and 11 duck trials, and one turkey trial (Farrell and Martin, 1998; Farrell et al., 1993; Ledoux and Firman, 1998, 1999; Namkung and Leeson, 1999; Ravindran et al., 1999b, 2000, 2001; Schutte et al., 1997; Selle et al., 1999). Following the same procedures as outlined for amino acid digestibility, a mean increase in AME of 222 kJ/kg diet (95% confidence interval 155 to 289) was calculated (500 FTU/kg supplementation). In pigs, few experiments studying the effect of phytase on energy utilization have been performed and these do not permit calculations following a similar procedure to that used for poultry. It is proposed, therefore, to use the value for extra energy that would be derived from additional digestible protein. This is equivalent to 21.6 kJ NE/kg diet (32.5 kJ DE or 30.5 kJ ME/kg diet), when the diet is supplemented with 500 FTU/kg.

MATRIX VALUES AND ECONOMIC VALUE OF MICROBIAL PHYTASE

From the previously presented results, values for digestible amino acids and energy per kg phytase enzyme product can be calculated. These 'matrix-values' are summarized in **Table 6**, both for poultry and pigs. Calcium and P values are also presented, but not those of other cations. In different studies (e.g., Zhang et al., 1999) it was shown that the performance of animals relative to these extra values for protein and energy agrees with the expectation. Thus they can be used in practical diet formulation.

For the feed industry an interesting question is how much money can be saved by inclusion of phytase in animal feeds. There is no direct answer to this question because it depends on a number of factors such as the country (e.g. the requirements used), availability and utilization of feedstuffs, market prices and whether maximum P levels are used in the diets. Under Dutch conditions, feed costs can be reduced between 0 and more than EUR 3.50/mt by the addition

Table 6. Proposed matrix values for Natuphos® 5000 G for poultry and pigs. One kg of product is equivalent to the values shown[1].

	Poultry	Pigs
	g/kg	
P from MCP[2]	10000	10000
P from DCP[3]	11500	11500
Calcium	10000	10000
Crude protein	22500	20000
Arginine	1300	800
Cystine	300	300
Histidine	500	200
Isoleucine	1200	500
Leucine	2000	1200
Lysine	1200	800
Methionine	100	250
Phenylalanine	1300	600
Threonine	1300	500
Tryptophan	300	300
Valine	1500	400
Glycine	700	-
Serine	1100	-
AME, *MJ/kg*	2215	305
DE / NE, *MJ/kg*	-	325 / 215

[1] These values can be used from 0 to 500 FTU/kg diet (layers 0 to 300 FTU/kg). Using these values at higher inclusion rates may lead to overestimation of the nutritional value of the feed.

[2] A large variation exists in the expression of P values for animals. The most exact expression of the value of phytase is: for poultry and pigs, 500 FTU/kg diet (laying hens 300) is equivalent to 1 g P from MCP.

[3] Assumes that 1.15 g P from DCP (dicalcium phosphate) is equivalent to 1 g P from MCP.

of phytase. This is without a maximum limit on the total amount of P in the diet. When the total P concentration in the feed is lowered, as is the case in the Netherlands, then the phytase value increases substantially. In some instances effective diets cannot be produced without including phytase because of the contradictory requirements (a maximum limit on the total dietary P, and a minimum limit on the digestible dietary P). Because the use of animal by-products in feed is banned since December 2000, the advantage to be gained from inclusion of phytase has increased further. For illustrative purposes, examples of a broiler and layer diet formulated with or without phytase are presented in **Table 7**. The calculations are made applying the requirements and nutrient compositions as used in the Netherlands and the prices of feedstuffs prevailing during December 2000.

Table 7. Examples of composition (%) of a typical Dutch broiler and layer feed, formulated with or without phytase, using matrix values presented in Table 6.

		Broiler finisher		Layer	
Phytase:	Cost[1], *EUR/t*	No	Yes	No	Yes
Corn	143	10.0	8.7	25.0	25.0
Wheat[2]	128	50.0	50.0	27.7	28.2
Tapioca	93	2.4	-	-	-
Full fat soybeans	266	20.0	20.0	20.0	20.0
Corngluten feed	125	-	-	10.0	10.0
Soybean meal, 47% CP	275	8.6	13.1	1.7	1.1
Sunflower meal	162	-	-	3.0	3.8
Fat (animal, soy oil)	325 - 377	5.4	5.4	1.7	1.3
MCP	359	0.8	0.3	0.8	0.4
Other minerals, AA, premix	-	2.8	2.5	10.1	10.2
Natuphos 5000 G	11[2]	-	0.01	-	0.06
Costs (EUR/t)	-	207.36	201.32	160.50	158.14

[1] Prices as of December 2000 (feedstuffs, excluding premix).
[2] With addition of an endoxylanase product.
[3] EUR/kg; price varies due to differences in exchange rates, import duties, taxes and amount purchased.

PRACTICAL IMPLICATIONS

The effect of microbial phytase on P digestibility and the resulting decrease (by approximately 30%) of P output into the environment are well known. It has been shown that this enzyme also liberates extra protein (amino acids) and energy to the animal. Based on digestibility trials with poultry and pigs, matrix values have been calculated. It is concluded that inclusion of 500 FTU/kg in broiler and pig feed liberates approximately 2 g additional digestible crude protein/kg feed. Values are also available for the individual amino acids. For broilers it was shown that the AME value of the diet increases by 220 kJ, by inclusion of 500 FTU/kg. Using these values in formulations permits the feed producer to take full advantage of the nutritional benefits resulting from inclusion of phytase in diets. Feed costs could decrease by up to EUR 3.50/mt (more in diets with a maximal total P restriction).

Chapter 4

Effect of microbial phytase in diets for young piglets on energy metabolism; a preliminary study

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ABSTRACT

Positive effects of dietary phytase supplementation on pig performance are observed under both limiting and non-limiting conditions for phosphorus (P). An experiment was performed to study the impact of microbial phytase on energy metabolism of *ad libitum* fed, weaned piglets. Indirect calorimetry was used. Four groups of three barrows each were used per treatment. A complete diet was supplemented with phytase (1000 FTU/kg) and compared with a non-supplemented control diet, fed over a three-week period. Piglets in the phytase group consumed 12% more feed, grew faster and retained more energy, but these effects were not significant. In this group, more heat production was related to activity ($P = 0.07$). Generally, a large effect of phytase supplementation on parameters of energy metabolism was observed in the first two weeks of the experiment. In the third week no further difference was observed. Although the interaction week × treatment was not significant, this could indicate that phytase aids piglets in adapting to the post-weaning state. An adapted experiment was designed for follow-up work.

INTRODUCTION

Most phosphorus (P) of vegetable feedstuffs is present in the form of phytate. Phytate is degraded only to a limited extent in the gastro-intestinal tract of monogastric animals, which limits the availability of P for these animals. This results in a high P excretion in feces, which may result in environmental pollution in areas with intensive animal husbandry.

Microbial phytase was developed as a feed additive to improve the availability of P of vegetable origin, in the diets of pigs and poultry. Phytase hydrolyses phosphate groups from phytate and increases P-availability. An additional advantage is that phytase may also improve feed efficiency. This benefit, which may be as high as 3%, was indicated by the results of experiments performed with piglets fed diets not limiting in digestible P (Kies et al., 2001). There are different explanations for this effect. Firstly, phosphorus availability may be increased. The assumed P requirement does not necessarily give maximal performance. A digestible phosphorus level below requirement will reduce animal performance, but a slightly higher level will have little effect (Jongbloed, 1987). Secondly, solubility of cationic minerals (e.g. Ca, Zn and Fe) and proteins that are complexed to phytate may be increased by phytase. These nutrients are also released when phytase hydrolyzes phosphate groups from phytate (Simons et al., 1990; Pallauf et al., 1992a; Sandberg et al., 1996; Yi et al., 1996a; Oberleas and Chan, 1997; Selle et al., 2000). Since (micro-) minerals, except Ca, P and Na, are usually added to feeds in excess of the requirement of animals, it is unlikely that their improved bioavailability enhances animal performance. Amino acid digestibility increases with phytase supplementation (Kies et al., 2001), but it was calculated that this could explain only 10 to 25% of the performance effect (Kies, 1998). Thirdly, energy utilization may be increased. Improved digestion of starch or fat (Thompson and Yoon, 1984; Knuckles, 1988) may increase energy digestibility, but this was often not observed (Eeckhout and De Paepe, 1992a; O'Quinn et al., 1997).

Very little information is available about the impact of phytase on post-absorptive energy metabolism and protein gain. It can be hypothesized that a change in energy metabolism through phytase inclusion may result in improved pig performance. More specifically:

(a) Less energy is excreted with urine and feces, as the result of increased digestion (e.g. of fat and starch), or of reduced excretion of endogenous protein (Cowieson et al., 2004).
(b) Maintenance energy is reduced. Energy expenditure may be lower for nutrient transport (Summers et al., 1986) or for gastro-intestinal tissues and absorptive processes. Phytate can bind digestive enzymes, which could result in an increase in the need for enzyme production via a negative feedback mechanism (Singh and Krikorian, 1982; Selle et al., 2000). Phytate degradation would result in the production of less digestive enzymes. At a similar feed intake level, reduced maintenance energy requirement may affect performance or change the composition of retained energy. A shift in available energy or protein may change the rates of protein and fat deposition (Bikker et al., 1995).
(c) Faster adaptation of piglets to new feed and environmental conditions, especially shortly after weaning. Increased availability of nutrients may aid the animal to overcome its limited digestive capacity at a young age.

The objective of the present experiment was to investigate whether dietary microbial phytase supplementation affects the utilization and partitioning of energy in young piglets fed a diet not limiting in digestible P, and also their post-weaning adaptation.

MATERIALS AND METHODS

Animals, housing and diets The experiment consisted of four subtrials of three weeks. In each subtrial, three pairs of weanling barrows (littermates; Large White × [English Landrace × Duroc] or Dutch Landrace × Great Yorkshire) were used. Piglets weaned at about 21 days of age and 7.1 ± 0.1 kg of body weight were chosen. It was assumed that these young piglets are most sensitive to variation in energy partitioning as a consequence of differences in metabolic processes. Upon arrival (d 0), each littermate was allotted randomly to one of two dietary treatments (control vs. phytase supplementation). Each group of three piglets was housed in one of two identical climatic-respiration chambers (Verstegen et al., 1987). Inner dimensions of these chambers are 1.0 × 0.8 × 0.97 m (l × w × h). Temperature was kept within the thermoneutral zone (28°C on d 0, decreasing gradually to 23°C on d 20). Relative humidity was maintained at 65% (range 60 - 70%). Air velocity was < 0.20 m/s. A 12-h lighting scheme was adopted.

During the experimental period of three weeks, piglets had *ad libitum* access to water and feed. Two diets were used (control and phytase). They were of identical composition (**Table 1**), except for phytase addition (1000 FTU/kg feed), and were formulated to cover requirements for all nutrients (Jongbloed et al., 1994; CVB, 2002; NRC, 1998). Phytase was a 3-phytase (EC 3.1.3.8), obtained from *Aspergillus niger* (Natuphos®). Diets were pelleted at a temperature below 70°C, to prevent possible enzyme loss.

Measurements Individual body weights and feed intake were measured weekly. Each balance period, complete energy and nitrogen balances were measured per group of pigs. To calculate energy and nitrogen balances, feces with urine (mixed) were collected quantitatively per group over 6 (period 1) or 7 days (periods 2 and 3), and sampled. In feed and feces + urine samples, gross energy (GE) and nitrogen were measured. Intake of metabolizable energy (ME) per group was calculated from the energy content of feed, feces + urine and methane production.

Total heat production (H_{tot}) was measured in 9-minute intervals by measuring exchange of oxygen, carbon dioxide and methane (Verstegen et al., 1987) using the formula of Brouwer (1965). H_{tot} was measured during the last 5, 6 and 6 days of the three respective periods. Total energy retention (ER) was calculated by subtracting H_{tot} from ME intake. N-retention was estimated from N in feed, in feces + urine, in aerial NH_3 and in NH_4^+ of water that condensed on the heat exchanger. Energy retention as protein (ER_p) was derived from N retention, and energy retention as fat (ER_f) was calculated by subtracting ER_p from ER. ME required for maintenance (ME_m) was estimated based on ARC (1981), assuming efficiencies of 54% for protein and 74% for fat retention.

Table 1. Composition of the basal diet (as fed basis)

	Contents, *g/kg*		Contents, *g/kg*
Ingredient		Nutrient contents[3]	
Corn	400.0	Dry matter	880 (904)[4]
Barley	183.2	Crude protein	194 (196)
Toasted soybeans	160.0	Crude fat	62
Soybean meal	80.0	Ash	64
Sunflowerseed meal	80.0	Gross energy, *MJ/kg*	16.8 (17.4)
Skimmed milk powder	40.0	Digestible energy, *MJ/kg*	14.7
Vegetable oil	10.0	Net energy, *MJ/kg*	9.9
Monocalciumphosphate	12.5	Calcium	8.9
Sodium chloride	3.0	Total phosphorus	7.3
Limestone	14.0	Digestible phosphorus	3.8
Sodium bicarbonate	2.0	Phytate-phosphorus	3.0
L-Lysine.HCl	3.0	Ileal digestible lysine	10.1
DL-Methionine	1.0	Ileal digestible met + cys	6.2
L-Threonine	1.1	Ileal digestible threonine	6.5
L-Tryptophan	0.2		
Vitamin and mineral mixture[1]	10.0		
Microbial phytase, *FTU/kg*	0 / 1000[2]		

[1] Supplied per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 1,800 IU; vitamin E, 40 mg; vitamin K, 3 mg; vitamin C, 50 mg; riboflavin, 5 mg; d-pantothenic acid, 12 mg; niacinamide, 30 mg; folic acid, 1 mg; vitamin B_{12}, 40 µg; biotin, 0.1 mg; choline choride, 350 mg; Fe, 80 mg ($FeSO_4.7H_20$); Cu, 168 mg ($CuSO_4.5H_20$); Zn, 73 mg ($ZnSO_4.H_20$); Mn, 44 mg (MnO_2); Co, 0,53 mg ($CoSO_4.7H_20$); I, 0.38 mg (KI); Se 0.06 mg ($Na_2SeO_3.5H_2O$).

[2] Only added to the phytase diet. Analyzed 1160 FTU/kg (control: < 50 FTU/kg).

[3] Calculated values (g/kg; CVB, 2000). Ca and P in corn, barley, soybeans, soybean meal, sunflowerseed meal and rice bran were analyzed prior to formulating the diets.

[4] Between parentheses: analyzed values.

Physical activity was recorded in the same time intervals as H_{tot}. Physical activity per group of piglets was monitored with a radar device according to the method of Wenk and Van Es (1976). The calculation of activity-related heat production (H_{act}) and heat production corrected for activity (H_{rest}) were carried out as described by Gentry et al. (1997).

Analytical procedures Kjeldahl nitrogen was analyzed (ISO, 1979) in feed and in mixed feces + urine (fresh). Nitrogen was also analyzed in condensed water collected from the respiration chambers and in acidified liquid samples through which outflowing air from the chambers was led, to trap gaseous ammonia. Energy was analyzed in feed and feces + urine, using adiabatic bomb calorimetry (IKA-C700, Janke & Kunkel GmbH & CoKG, Staufen, Germany). Feed samples were analyzed for phytase activity (Engelen et al., 2001).

Statistical analyses For all traits, group was the experimental unit. Energy data were expressed in kJ/($kg^{0.75}$.d). Effects of phytase supplementation on weekly mean values of the measured parameters were tested with the GLM procedure of SAS (Version 6.12; SAS Inst. Inc., Cary, NC, USA) by means of *F*-tests using a split-plot model, with balance period values within groups taken as repeated measurements:

$$Y_{ijkl} = \mu + T_i + F_j + e_{1,ijk} + W_l + W \times F_{jl} + e_{2,ijkl},$$

where Y_{ijkl} = trait; μ = overall mean; T_i = fixed effect of sub-trial i (i = 1,..,4); F_j = fixed effect of experimental diet j (j = control, phytase); $e_{1,ijk}$ = error term 1, which represents the random effect of group k (k =1,..,4) nested within trial i and feeding treatment j; W_l = fixed effect of balance period l (l = 1, 2, 3); $e_{2,ijkl}$ = error term 2. The effects of trial and experimental diet were tested against error term 1, other effects against error term 2. Results are presented as Least Square Means with their standard error. Statistical calculations were corrected for different group sizes, due to the removal of some animals from the respiration chambers, using the weight statement of the GLM procedure of SAS.

RESULTS

Three animals (out of 24) were removed from respiration chambers during the experiment. One animal (control) did not eat, the other animals (littermates; one control and one phytase) showed dermatitis. The other animals appeared healthy.

The performance level of the animals was good (**Table 2**). Feed intake and growth rate improved numerically with phytase supplementation. The differences between treatments were large in the first and second week, but disappeared in the third week.

As a result of a higher feed intake, gross energy intake was higher in the phytase group (Table 2). Over the first two weeks, this difference was nearly 20%, but it disappeared in the third week. Energy metabolizability (ME/GE) was higher in the phytase groups during the first week, but somewhat lower the third week (interaction: $P < 0.10$). Over the three weeks, the relative value was nearly 1% higher for the phytase than for the control treatment ($P = 0.20$).

Total heat production was 5% higher with phytase, again with a large difference in the first two weeks, which disappeared in the third week (**Table 3**). Heat production related to activity tended to be higher in the phytase group than in the control ($P = 0.07$), but no other differences in heat production characteristics appeared. Energy retention was higher with phytase (17% over the entire period), but this was not significant. Calculated ME_m was similar in both treatments. For most energy-related parameters, there was a difference between the treatments during the first two weeks, but this disappeared by week three. Exceptions were activity related heat production and ME_m, which were similar in the first week, but higher in the phytase animals during the last two weeks. The week × feed interaction was, however, not significant for any of these parameters.

DISCUSSION

The hypothesis, that the positive effect of phytase on the performance of young piglets is partly due to post-absorptive effects on energy metabolism, could not be confirmed in the present study. A number of numerically important differences were observed between the two treatments, however. These were related to feed intake, heat production parameters, energy retention and early adaptation. Indications were that growth rate and feed conversion efficien-

Table 2. Effect of dietary phytase on performance, energy intake and metabolizability and methane production of weanling piglets during the three-week experiment[1].

Trait	Control diet	Phytase diet	SEM[1]	*P*-value		
				feed	week	feed × week
Initial weight, *kg*	7.0	7.2	0.03	< 0.001		
Number of piglets						
Week 1	12	12				
Week 2	11	11				
Week 3	10	11				
Growth rate, *g/d*						
Week 1	90	132				
Week 2	229	284	31	0.14	< 0.001	0.96
Week 3	383	441				
Feed intake, *g/d*						
Week 1	125	154				
Week 2	295	364	24	0.22	< 0.001	0.48
Week 3	546	557				
GE intake, $kJ/(kg^{0.75}.d)$						
Week 1	487	583				
Week 2	1023	1212	77	0.25	< 0.001	0.41
Week 3	1567	1543				
ME intake, $kJ/(kg^{0.75}.d)$						
Week 1	385	476				
Week 2	811	965	68	0.27	< 0.001	0.38
Week 3	1265	1224				
ME/GE ratio, %						
Week 1	79.1	82.1				
Week 2	78.9	79.2	0.95	0.20	0.30	0.09
Week 3	80.7	79.3				
Methane production, $kJ/(kg^{0.75}.d)$						
Week 1	4.2	4.8				
Week 2	6.8	7.4	0.85	0.49	0.02	1.00
Week 3	6.9	7.5				

[1] n= 4 per treatment per week.

cy were improved by dietary phytase supplementation, which agrees with earlier findings (Kies et al., 2001).

A large week effect on parameters measuring energy metabolism was observed ($P < 0.001$ for all parameters studied). This confirms the observation that young piglets, shortly after weaning, are not yet in a steady state regarding their energy metabolism (Schrama, 1997).

Piglets consumed small amounts of feed during the first week, resulting in a negative energy balance. Comparison of the results with other experiments performed in our laboratory (Van Diemen et al., 1995; Gentry et al., 1997; Moon et al., 1998; Sijben et al., 1998), also using young weaned piglets, showed similar findings. Apparently, piglets weaned at 21 days are for some time in negative energy balance (**Figure 1A**). In particular, the ME_m during the first week of the present experiment was high. Because in the other experiments piglets were weaned at about 28 days of age, comparison by age results in comparable values (**Figure 1B**).

Table 3. Heat production characteristics, energy retention and energy requirements for maintenance in weanling barrows during the three-week experiment[1].

Trait	Control diet	Phytase diet	SEM	*P*-value		
				feed	week	feed × week
Total heat production (H_{tot}, *kJ/(kg$^{0.75}$.d)*)						
Week 1	510	541				
Week 2	579	647	19	0.16	< 0.001	0.20
Week 3	733	728				
Activity related heat production (H_{act}, *kJ/(kg$^{0.75}$.d)*)						
Week 1	123	123				
Week 2	103	129	9	0.07	0.23	0.39
Week 3	128	138				
Non-activity related heat production (H_{rest}, *kJ/(kg$^{0.75}$.d)*)						
Week 1	387	418				
Week 2	476	518	16	0.38	< 0.001	0.22
Week 3	605	590				
Total energy retention (ER, *kJ/(kg$^{0.75}$.d)*)						
Week 1	-125	-66				
Week 2	231	318	55	0.37	< 0.001	0.53
Week 3	532	496				
Energy retention in protein (ER_p, *kJ/(kg$^{0.75}$.d)*)						
Week 1	54	74				
Week 2	146	182	17	0.21	< 0.001	0.42
Week 3	243	234				
Energy retention in fat (ER_f, *kJ/(kg$^{0.75}$.d)*)						
Week 1	-178	-139				
Week 2	86	136	40	0.46	< 0.001	0.60
Week 3	288	262				
Maintenance energy requirements (ME_m, *kJ/(kg$^{0.75}$.d)*)						
Week 1	527	528				
Week 2	425	445	20	0.25	< 0.001	0.89
Week 3	424	436				

[1] n= 4 per treatment per week.

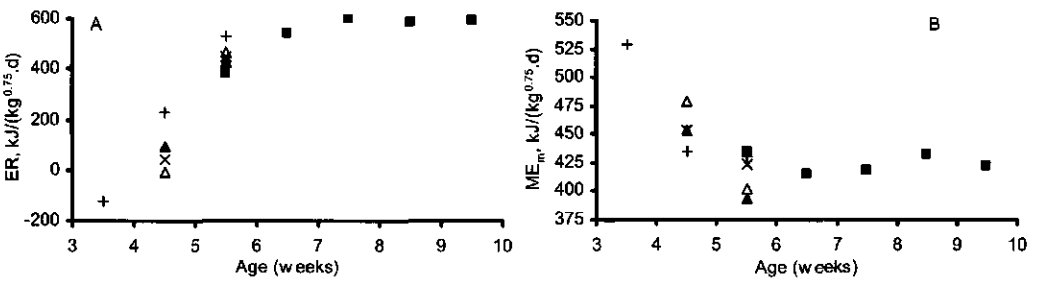

Figure 1. Comparison of mean observed energy retention (ER; pane A) and energy requirements for maintenance (ME_m; pane B) in young weaned piglets in different studies.

Data from: ×: Gentry et al. (1997); ■: Van Diemen et al. (1995); ▲: Moon et al. (1998); △: Sijben et al. (1998); +: this study (mean of treatments). In current study piglets were weaned at 3 weeks of age, in the other studies at 4 weeks.

Numerically important effects of phytase supplementation were observed. Because they were not significant, their interpretation needs some caution. Feed intake was higher for the phytase treatment. During the first two weeks this effect was nearly 20%. Consequently phytase increased the GE and ME intake, the latter also because the ME/GE ratio was higher. A higher feed intake can maintain gut integrity, which is beneficial for young piglets (Makkink, 1993; Spreeuwenberg, 2002). Phytase supplementation improved growth rate. Methane production, H_{tot}, H_{act}, H_{rest}, ER, ER_p, ER_f and ME_m were higher with the phytase treatment. Most of these effects were large during the first week, but disappeared by the third week of the experiment. Interestingly, this was not the case for H_{act} and ME_m. No difference was observed for these parameters during the first week, but they were higher for the phytase treatment in the second and third weeks. These observations suggest that animals receiving the phytase diet adapt faster to the change in environment and diet, probably due to increased digestion of minerals and other nutrients, which helps the animals to overcome their limited digestive capacity.

As indicated, metabolizability of feed was higher for the phytase treatment. The average difference over the three-week period was 0.6%-units, or about 104 kJ/kg feed. In experiments with (fast growing) poultry, phytase increased dietary energy metabolizability as calculated in a meta-analysis (Kies et al., 2001). Dietary supplementation with 500 FTU/kg feed resulted in a 220 kJ/kg feed higher ME. This is about 1.8% of the ME-content of a typical broiler diet. In the present experiment, phytase increased the ME content of the diet for piglets by about half of this value. Possibly, the digestive capacity in poultry is affected more by phytase, or young growing poultry are more comparable with piglets directly after weaning.

The piglets receiving dietary phytase retained more energy, both in protein and in fat. Also this advantage disappeared in the third week of the experiment. Even this short period of higher growth may, however, be of advantage for the later life of the animal. Early energy stores may prove useful when conditions are less favorable for the piglets.

In conclusion, dietary phytase supplementation stimulated feed intake and performance of *ad libitum* fed, newly weaned piglets. The higher consumption means that more energy was available for production. This resulted in higher protein and fat retention. Animals were also more active on the phytase diet. Many results showed a clear effect during some weeks of the experiment, but few effects were significant. It was decided, therefore, to run a follow-up experiment using a different design. To reduce variation, piglets in the experiment were fed restrictedly. This is difficult to realize, shortly after weaning. Therefore, the experiment started at three weeks after weaning of the piglets. With such a set-up it is possible to distinguish activity-related heat production during feeding and during the rest of the day. Results are reported elsewhere (Kies et al., 2005).

Chapter 5

Mineral absorption and excretion as affected by microbial phytase, and their effect on energy metabolism in young piglets

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ABSTRACT

Positive effects of dietary phytase supplementation on pig performance are not only observed when phosphorus is limiting. Improved energy utilization might be one explanation. Using indirect calorimetry, phytase-induced changes in energy metabolism were evaluated in young piglets with adequate phosphorus intake. Eight replicates each of eight, group-housed, barrows were assigned to either a control or a phytase-supplemented diet [1500 phytase units (FTU)/kg feed]. Piglets were fed a restricted amount of the control or phytase diet. The diets were made limiting in energy content, by formulating them to a high digestible lysine : DE ratio. Fecal nutrient digestibility, portal blood variables, organ weights and apparent absorption and the urinary excretion of ash, Ca, P, Na, K, Mg, Cu and Fe, were also measured. A model was developed to estimate the energy required for absorption and excretion, which are partly active processes. Phytase tended to improve energy digestibility ($P = 0.10$), but not its metabolizability. Energy retention and heat production were not affected. At the end of the 3-week period, pancreas weight ($P < 0.05$) and blood pH ($P < 0.01$) were lower, and CO_2-pressure was higher ($P < 0.01$) due to phytase. This suggests that phytase reduced energy expenditure by the digestive tract, and increased metabolic activity in visceral organs. The potential increases in energy retention due to phytase were counterbalanced by increased energy expenditure for processes such as the higher absorption of minerals (for most, $P < 0.05$), and their subsequent urinary excretion. The energy cost of increased absorption of nutrients and of deposition and excretion of minerals was estimated as 4.6 kJ/($kg^{0.75}$.d), which is 1% of the energy required for maintenance. The simultaneous existence of both increases and decreases in heat production resulted in the absence of a net effect on energy retention.

INTRODUCTION

Most of the phosphorus (P) in vegetable feedstuffs is in phytate. Phytate is degraded only to a limited extent in the gastrointestinal tract of monogastric animals, restricting the availability of P for these animals. This results in a high P excretion in feces, which may cause pollution of the environment in areas with intensive animal husbandry. The negatively charged phytate ions can form strong complexes with cationic minerals and other positively charged compounds in the gastrointestinal tract (Champagne, 1988; Torre et al., 1991; Oberleas and Chan, 1997; Bebot-Brigaud et al., 1999; Frossard et al., 2000; Weaver and Kannan, 2002).

To improve the availability of P, of vegetable origin, in pig and poultry diets, microbial phytase is widely used as a feed additive. Phytase hydrolyses phosphate groups from phytate and increases the availability of P. An additional advantage is that phytase may also improve feed efficiency, as was calculated from experiments performed with piglets fed diets not limiting in digestible P (Kies et al., 2001). This effect, which may be as high as 3%, has several possible explanations. Firstly, phosphorus availability may be increased. The P requirement assumed does not necessarily give maximum performance. A digestible phosphorus level slightly above the requirement has, however, little effect on performance (Jongbloed, 1987). Secondly, the solubility of cationic minerals (e.g., Ca, Zn and Fe) and proteins that are complexed to phytate may be increased. These nutrients are released when phytase hydrolyzes phosphate groups from phytate (Simons et al., 1990; Pallauf et al., 1992a; Sandberg et al., 1996; Yi et al., 1996a; Oberleas and Chan, 1997; Selle et al., 2000). Because (micro-) minerals, except Ca, P and Na, are usually added to feeds in excess of the requirement of animals, it is unlikely that their improved bioavailability enhances animal performance. Amino acid digestibility is increased by phytase supplementation (Kies et al., 2001). It was estimated (Kies, 1998) that this could explain only 10 to 25% of the performance effects. The improvement of amino acid digestibility, however, is often small and not significant (Adeola and Sands, 2003). Thirdly, energy utilization may be increased, e.g. due to greater digestion of starch or fat (Thompson and Yoon, 1984; Knuckles, 1988). In several studies, however, no positive effect of phytase addition on energy digestibility was observed (Eeckhout and De Paepe, 1992a; O'Quinn, 1997).

In earlier studies, the effects of phytase on post-absorptive energy metabolism and on protein gain were not investigated. It was hypothesized that phytase might improve performance by a change in energy metabolism. More specifically: firstly, less energy is excreted with urine and feces as a result of either increased digestion (e.g. of fat and starch) or of reduced excretion of endogenous protein (Cowieson et al., 2004). Secondly, maintenance energy is reduced. Energy expenditure may be lower for nutrient transport (Summers et al., 1986) or for gastrointestinal tissues and absorptive processes. Phytate can bind digestive enzymes, which could result in an increased need for enzyme production via a negative feedback mechanism (Selle et al., 2000; Singh and Krikorian, 1982). Thus phytate degradation would result in the production of a smaller amount of digestive enzymes. Thirdly, increased mineral absorption may result in energy expenditure for the piglet. Minerals absorbed in excess of their requirement will be largely excreted in urine. Absorption and excretion are both (partly) ATP-requiring processes

and can, therefore, affect energy metabolism (Balaban and Mandel, 1980; Mandel and Balaban, 1981; Summers et al., 1986). A difference in energy metabolism may affect performance, or in alteration of the composition of retained energy. A shift in available energy or protein may change the rates of protein and fat deposition (Bikker et al., 1995).

The objective of the present experiment was to investigate whether dietary microbial phytase supplementation affects utilization and partitioning of energy in young piglets. Increased absorption and excretion of ash, Ca, P, Na, K, Mg, Cu, Fe and Cl were quantified, and the energy costs required for these processes were estimated using a mathematical model.

MATERIALS AND METHODS

Animals, housing and diets Four subtrials of 3 weeks were performed. In each subtrial, 8 pairs of barrows (Yorkshire × [Finnish Landrace × Yorkshire]; littermates) were used. The experiment started at about day 46 of age (average liveweight 11.3 ± 0.25 kg), which was about 3 weeks post-weaning. The ethics committee of Wageningen University and Research Center approved the experiment described.

At the start of the experiment, piglets were allotted to 1 of 2 dietary treatments (control vs. phytase supplementation) based on weight and litter. Each group of 8 piglets was housed in 1 of 2 identical climate respiration chambers (2.5 x 1.5m), comparable in design to those described previously (Verstegen et al., 1987). Ambient temperature was maintained at 25, 24 and 23°C during weeks 1, 2 and 3, respectively. Relative humidity was maintained at 65% (range 60-70%). Air velocity was < 0.20 m/s. A 12-h light : dark cycle was used.

At the start of the experiment, piglets were switched immediately to the experimental diets, offered at 2.3 times the energy requirement for maintenance (ME_m). The 2 diets were identical in composition (**Table 1**), except for the addition of phytase (1500 phytase units (FTU)/kg feed; phytase from Natuphos®) to one of the diets. A FTU is defined as the amount of enzyme that liberates 1 µmol inorganic orthophosphate/min from a 5.1 mM sodium phytate at pH 5.5 and 37°C. It was shown (Düngelhoef and Rodehutscord, 1995; Kornegay, 2001) that the maximal effect of phytase on P digestibility is reached at 1000-1500 FTU/kg. To study the direct effect of phytase on energy metabolism, diets were formulated to be limiting in energy: the ileal digestible lysine/digestible energy (DE) ratio was 0.74. This is 10% higher than the recommended dietary level of lysine in relation to energy content, for piglets of 10-20 kg body weight (Bikker, 1994; CVB, 2002). Diets were not limiting in other nutrients (CVB, 2002; NRC 1998) and were adequate in digestible P (Jongbloed et al., 1994). Both diets were high in phytate P (3.8 g/kg) to ensure substrate availability, and thereby experimental contrast. Because feeding Ca and P slightly above requirement does not affect performance (Jongbloed, 1987), it was assumed that Ca and P released by phytase would not affect energy metabolism. Mineral levels (Table 1) varied from the recommended level for Na to about 8 times that for Fe, relative to NRC (1998) recommendations. The Cu level was far in excess of the require-ment of 5 mg/kg feed, as is common in practice. Diets were pelleted at a temperature

TABLE 1. Composition of the basal diet (as-fed basis)

Ingredient		Nutrient contents[4]	
	g/kg		*g/kg*
Corn	435.0	Dry matter	880 (898)[5]
Soybean meal	318.4	Crude protein	212 (220)
Rice bran (extracted)	184.0	Crude fat	59 (69)
Vegetable oil	15.0	Ash	75 (81)
Monocalcium phosphate	14.0	Gross energy, *MJ/kg*	16.6 (17.3)
Sodium chloride	3.5	Digestible energy, *MJ/kg*	14.7
Limestone	7.0	Net energy, *MJ/kg*	9.9
L-Lysine.HCl	1.8	Calcium	10.5 (11.0)
DL-Methionine	0.9	Total phosphorus	9.1 (9.0)
L-Threonine	0.2	Digestible phosphorus	3.9
L-Tryptophan	0.2	Phytate-phosphorus	4.7 (3.8)
Diamol[1]	10.0	Base excess[6], *mEq/kg*	251
Vitamin and mineral mixture[2]	10.0	Apparent ileal digestible lysine	10.8
Microbial phytase, *FTU/kg*	0 / 1500[3]		

[1] Diatomaceous shell powder, SiO_2, added as an indigestible marker.
[2] Supplied per kilogram of diet: 3.1 mg retinyl acetate; 45 µg cholecalciferol; 40 mg dl-a-tocopherol; 3 mg menadione; 50 mg ascorbic acid; 5 mg riboflavin; 12 mg D-pantothenic acid; 30 mg niacinamide; 1 mg folic acid; 40 µg cobalamin; 0.1 mg biotin; 350 mg choline choride; 80 mg Fe ($FeSO_4.7H_20$); 168 mg Cu ($CuSO_4.5H_20$); 73 mg Zn ($ZnSO_4.H_20$); 44 mg Mn (MnO_2); 0.53 mg Co ($CoSO_4.7H_20$); 0.38 mg I (KI); 0.06 mg Se ($Na_2SeO_3.5H_2O$).
[3] Added to the phytase diet only. Analyzed in feed pellets: 1520 FTU/kg (control: <50 FTU/kg).
[4] Calculated values (CVB, 2000). Ca and P in corn, barley, soybeans, soybean meal, sunflowerseed meal and rice bran were analyzed before diets were formulated.
[5] Analyzed values are in parentheses. Analyzed mineral levels (/kg feed) were 9.6 g P, 11.7 g Ca, 1.5 g Na, 10.4 g K, 3.3 g Cl, 3.5 g Mg, 187 mg Cu and 638 mg Fe.
[6] $Na^+ + K^+ - Cl^-$.

< 70°C to prevent loss of enzyme activity. Piglets had free access to water. On the day of slaughter, piglets consumed their respective diet *ad libitum* for 4 h before slaughter.

Measurement of energy and nitrogen balance and physical activity Individual body weights were determined weekly. Feed refusals were recorded daily for each treatment group. For each balance period, complete energy and nitrogen balances were measured per group of pigs. To this end, feces + urine were collected quantitatively per group, over 6 (period 1) or 7 days (periods 2 and 3), and sampled. In the feed and feces + urine samples, gross energy (GE) and nitrogen were measured. Intake of metabolizable energy (ME) per group was calculated from the energy content of feed, of feces + urine, and of methane produced.

Total heat production (H_{tot}) was measured in 9-minute intervals by measuring the exchange of oxygen, carbon dioxide and methane (Verstegen et al., 1987), using the formula of Brouwer (1965). H_{tot} was measured during the last 5, 6 and 6 days of the 3 periods, respectively. The respiration chamber had to be opened twice daily to feed the piglets. Heat measurements are, therefore, based on 22 hours/day. Energy retention was calculated by subtracting H_{tot} from ME intake. ME_m was estimated based on ARC (1981) data, assuming efficiencies of 54% for protein and 74% for fat retention.

Physical activity was recorded over the same time intervals as H_{tot}. Physical activity per group of piglets was monitored with a radar device according to the method of Wenk and Van Es (1976). Calculation of activity-related heat production (H_{act}) and heat production corrected for activity (H_{rest}) was done as described by Gentry et al. (1997).

Apparent fecal digestibility and urinary mineral excretion For measurement of apparent fecal digestibility, fresh "grab" samples were taken daily from each group of pigs directly after the morning feeding, taking care to collect feces from the different piglets. These samples were taken for 5, 6 and 6 days in periods 1 to 3, respectively. The feces (100 g/d) were pooled for each period and stored at -20°C before analyses. Apparent fecal digestibilities were calculated using acid-insoluble ash as a marker (Schrama et al., 1998). Mineral excretion in urine was calculated from the difference of the quantities excreted within feces + urine and feces alone.

Measurements at slaughter One day after the last balance period, the pigs consumed their feed *ad libitum* for 4 h. They were then weighed, anesthetized, the abdominal cavity was opened and directly 2 blood samples were taken from the portal vein. Within 3 min, 1 sample was analyzed for pH, partial oxygen pressure (pO_2) and partial carbon dioxide pressure (pCO_2) using i-STAT cartridges. The other blood sample was collected into a heparinized tube (Li-Heparin) and stored on ice. Within 4 h, this sample was centrifuged at 1100 × *g* for 12 min, and plasma was stored at –20°C for analysis for mineral contents. After the blood samples were taken, pigs were killed by injection of 1 mL of T61 directly into the portal vein. Weights of the emptied gastrointestinal tract (separated for stomach, small intestine, cecum and colon + rectum), pancreas, liver, kidneys (without fat) and heart were recorded.

Analytical procedures Kjeldahl nitrogen was analyzed (ISO, 1979) in feed, feces (fresh), mixed feces + urine (fresh), in condensated water collected from the respiration chambers, and in acidified liquid samples through which outflowing air from the chambers was led to trap gaseous ammonia. Crude fat, crude ash, acid-insoluble ash and energy were analyzed in feed, freeze-dried feces and freeze-dried feces + urine. Crude fat was analyzed after acid hydrolysis by extraction with petroleum ether [boiling range 40-60°C; (ISO, 1996)]. Crude ash and acid-insoluble ash were analyzed according to International Organization for Standardization methods (ISO, 1978a,b). Energy content was analyzed using adiabatic bomb calorimetry (IKA-C700, Janke & Kunkel). Feed samples were analyzed for phytic acid (Bos et al., 1993) and phytase activity (Engelen et al., 2001).

Minerals and chloride were determined in feed and freeze-dried samples of feces and feces + urine. Minerals were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) using yttrium as an internal standard (NEN-ISO, 1998). Chloride was measured titrimetrically after water extraction from the samples (EG, 1971). Heparinized blood plasma samples were analyzed electrochemically for Na, K, Cl, Ca (ionized), lactate and glucose, using an EML 105 (Radiometer). Blood P was determined in plasma samples after deproteinization with trichloroacetic acid by ICP-AES.

Model calculations Potential energy costs associated with the increased absorption of amino acids, fatty acids and minerals from the gastrointestinal tract, increased retention of Ca and P in bone tissues, and increased tubular reabsorption of minerals from primary urine due to phytase supplementation, were estimated using a model. A complete model description is provided in Appendix 1. Calculations were performed using Excel® 2000 (Microsoft). The "Goal Seek" tool of Excel® was used to calibrate renal reabsorption of the different minerals to the mineral excretion rates, observed in the present experiment.

Statistical analyses For all traits, group was the experimental unit. Data on GE, ME, ER, ER_p, ER_f, H_{tot}, H_{act} and H_{rest} were expressed in kJ/($kg^{0.75}$.d). The effect of phytase supplementation was tested with the GLM procedure of SAS (Version 6.12; SAS Institute) by means of *F*-tests using a split-plot model, with balance period values within groups taken as repeated measurements:

$$Y_{ijkl} = \mu + T_i + F_j + e_{1,ijk} + W_l + W \times F_{jl} + e_{2,ijkl}$$

where Y_{ijkl} = trait; μ = overall mean; T_i = fixed effect of subtrial i (i = 1,..,4); F_j = fixed effect of experimental diet j (j = control, phytase); $e_{1,ijk}$ = error term 1, representing the random effect of group k (k =1,..,4) nested within trial i and feeding treatment j; W_l = fixed effect of balance period l (l = 1, 2, 3); $e_{2,ijkl}$ = error term 2. The effects of trial and experimental diet were tested against error term 1, other effects against error term 2. Results are presented as Least Square Means with their SE. Per treatment per trait, n was thus 12.

For traits measured at the end of each subtrial, at slaughter (blood variables and weights of organs and intestines; n= 4 per treatment per trait), the model was used without period effect:

$$Y_{ij} = \mu + T_i + F_j + e_{ij}.$$

RESULTS

General observations and animal performance In the experiment, 1 piglet died (during the first balance period of subtrial 1; phytase treatment). No feed-refusals were recorded. For most parameters the week-effect was significant. Because no significant week × dietary treatment interactions were observed (unless indicated), results are presented as means over the 3-week period.

Weight gain of piglets was slightly higher in those administered phytase than for control piglets (329 vs. 319 ± 4 g/d). Because feed intakes did not differ (484 g/d), the gain : feed ratio tended to be greater in the phytase group (681 vs. 661 ± 7 g/kg, $P = 0.13$).

Digestibility Phytase tended ($P \leq 0.10$) to increase apparent fecal digestibility of dry matter, nitrogen, fat and energy by 2.0, 1.9, 1.2 and 1.3%, respectively (**Table 2**). Phytase increased ash digestibility by almost 10% ($P < 0.05$). For all nutrients, apparent fecal digestibility increased with time ($P < 0.01$). There was no week × feed interaction, except for ash ($P < 0.01$). Ash digestibility was higher in pigs fed phytase than in those fed the control diet, but the difference became smaller over time.

TABLE 2. Effect of dietary phytase supplementation on apparent fecal digestibility in piglets during the 3-week experiment[1]

Trait	Control diet	Phytase diet	SEM	*P*-value
	%			
Dry matter	82.6	84.6	0.45	0.05
Nitrogen	80.2	82.0	0.52	0.09
Crude fat	81.9	83.1	0.26	0.05
Crude ash	45.0	54.8	1.40	0.02
Energy	84.7	86.0	0.41	0.10

[1] Values are least-square means, n =12.

Energy metabolizability, heat production and energy retention GE intakes did not differ between diets (**Table 3**), but due to the increased apparent fecal energy digestibility, DE intake tended to be higher ($P = 0.19$) in piglets fed phytase. There was no difference in ME intake. Methane production ($P < 0.05$) and urinary energy excretion were higher ($P = 0.17$) in piglets administered phytase [1.2 and 12 kJ/(kg$^{0.75}$.d), respectively]. Energy retention and ME_m did not differ between piglets fed the 2 diets.

Measurements at slaughter After this 3-wk experiment, weight of the small intestine was numerically slightly lower ($P = 0.19$) in phytase-treated piglets, than in the control piglets (4.48 vs. 4.69 ± 0.09% of empty body weight; n=4). Weight of the pancreas was almost 6% lower in piglets receiving phytase (0.24 vs. 0.26 ± 0.003 % of empty body weight; $P < 0.05$). Weights of the entire gastro-intestinal tract, heart and liver were not different.

In the portal blood of piglets fed phytase (4 h after *ad libitum* consumption of feed), the pH was lower ($P < 0.01$), and partial carbon dioxide pressure was higher ($P < 0.01$; **Table 4**). Plasma glucose, K and P concentrations were 20% ($P < 0.05$), 31% ($P < 0.01$) and 18% ($P <$

TABLE 3. Effect of dietary phytase supplementation on energy intake, energy loss with urine and methane, and heat production in piglets during the 3-week experiment[1]

Trait	Control diet	Phytase diet	SEM	*P*-value
	kJ/(kg$^{0.75}$.d)			
GE intake	1162	1162	2.8	0.84
DE intake	983	1000	6.8	0.19
ME intake	958	961	3.1	0.65
Urinary Energy	19	32	5.0	0.17
Methane production	5.5	6.7	0.16	0.02
Total heat production (H_{tot})	638	640	3.2	0.66
Activity related heat production (H_{act})	123	122	1.5	0.55
Activity corrected heat production (H_{rest})	515	519	3.8	0.55
Total energy retention (ER)	213	249	24	0.37
Energy retention as protein (ER_p)	148	163	7	0.21
Energy retention as fat (ER_f)	65	86	17	0.46
ME for maintenance (ME_m)	459	469	5	0.25

[1] Values are least-square means, n =12.

TABLE 4. Effect of dietary phytase supplementation on the pH, partial oxygen pressure (pO_2), and partial carbon dioxide pressure (pCO_2) in portal blood, and on concentrations of glucose, lactate, Na, K, Cl, Ca and P in portal blood plasma of young piglets[1,2]

Trait	Control diet	Phytase diet	SEM	*P*-value
pH	7.06	6.98	0.008	<0.01
PO_2, *kPa*	5.0	4.2	0.38	0.23
PCO_2, *kPa*	12.1	13.5	0.16	<0.01
Glucose, *mmol/L*	8.4	10.2	0.36	0.04
Lactate, *mmol/L*	5.7	7.0	0.50	0.17
Sodium, *mmol/L*	138	137	0.3	0.11
Potassium, *mmol/L*	6.1	8.0	0.16	<0.01
Chloride, *mmol/L*	95	98	2.0	0.36
Ionized calcium, *mmol/L*	1.29	1.31	0.018	0.55
Phosphorus, *mmol/L*	3.58	4.22	0.162	0.07

[1] Samples taken after slaughtering the piglets were, about 4 h after initiating *ad libitum* consumption of the diets.
[2] Values are least-square means, $n = 4$.

0.07) higher, respectively, in phytase-fed piglets than in control piglets. Plasma concentrations of Na, Cl and Ca were unaffected by treatment.

Mineral flows The apparent absorption of ash and all minerals measured, was higher in piglets administered phytase (**Table 5**). For ash, Ca, P, Na and K, this effect was significant ($P < 0.05$). Dietary chloride absorption was unaffected by treatment. Phosphorus and calcium comprised most (75%) of the (measured) minerals absorbed in greater quantities due to phytase. Increased absorption was followed by increased urinary excretion, i.e. nearly 70% of the extra ash absorbed was recovered in urine. For most minerals, this represented >80%, but for Ca it was only 28%. Phytase addition increased fecal P-digestibility from 52% to 70%, elevating P absorption with 1.7 g/kg feed.

TABLE 5. Effect of dietary phytase supplementation on apparent absorption and urinary excretion of ingested minerals in piglets during the 3-week experiment[1]

Trait	Intake[2]	Apparent absorption				Urinary excretion			
		Control	Phytase	SEM	*P*-value	Control	Phytase	SEM	*P*-value
Ash, $g/(kg^{0.75}.d)$	5.4	2.4	3.0	0.09	0.02	1.3	1.6	0.06	0.03
					$mg/(kg^{0.75}.d)$				
Calcium	726	364	439	10.5	0.01	41	62	8.9	0.20
Phosphorus	598	313	416	8.7	0.01	46	131	7.9	0.01
Sodium	94	78	83	0.6	0.01	30	33	1.7	0.34
Potassium	645	495	533	6.0	0.02	427	461	9.3	0.08
Chloride	202	186	184	1.2	0.53	130	120	7.4	0.38
Magnesium	215	68	80	3.9	0.13	26	38	3.4	0.09
Copper	11.6	1.4	2.5	0.31	0.09	1.4	2.5	0.23	0.04
Iron	39.6	7.7	9.1	1.03	0.42	3.5	4.6	0.77	0.39

[1] Values are least-square means, $n = 12$.
[2] Intake means of the treatment groups; feed intakes were almost equal due to restricted feeding of piglets.

Energy costs of absorption and excretion Using a mathematical model (Appendix 1) it was estimated that the energy expenditure, associated with the phytase-induced increased absorption of amino acids, fat and minerals from the gastro-intestinal tract, mineral reabsorption from the kidney, and retention of Ca and P in bone tissues, was 4.6 kJ/($kg^{0.75}$.d) higher (**Table 6**). This is just over 1.0% of ME_m. Most of this energy (74%) was required for the reabsorption of minerals from primary urine in the kidney. The calculated, fractional reabsorption rates ($kRA_{M,pu,pl}$; Appendix 1) were 0.976, 0.913, 0.964, 0.749 and 0.715 per cardiac cycle (defined in Appendix 1) for Ca, P, Na, K and Mg + Fe + Cu, respectively.

TABLE 6. Predicted energy costs for the phytase-induced absorption of protein, fatty acids and minerals from the gastrointestinal tract, deposition of Ca and P in bone tissues, and reab-sorption of minerals from primary urine in the kidney of piglets during the 3-week experiment[1]

	Calculated energy costs
	kJ/($kg^{0.75}$.d)
Absorption of amino acids, fatty acids	0.28
Absorption of minerals	0.56
Deposition of Ca and P in bone tissue	0.38
Reabsorption of minerals in the kidney	3.41
Total	4.63

[1] Predictions according to the model described in Appendix 1.

DISCUSSION

Because heat production parameters and energy retention did not differ between treatments, the hypothesis that the positive effect of phytase on performance of young piglets is due in part to post-absorptive effects on energy metabolism could not be confirmed in the present study. A number of numerically important differences were observed between the two treatments, however. For example, growth rate and feed conversion efficiency were slightly higher in phytase-supplemented piglets.

Effect of phytase on energy utilization Apparent fecal digestibility of energy was higher in phytase-supplemented piglets, which could be attributed mainly to higher protein and fat digestibilities. The positive effect of dietary phytase on fecal protein digestibility was reported previously (Eeckhout and De Paepe, 1992a; Mroz et al., 1994), but the magnitude of the effect was often small (O'Quinn et al., 1997; Johnston et al., 2004). The effect of phytase on fecal fat digestibility was measured in few experiments, mainly with a positive effect (Fandrejewski et al., 1997). In general, no improvement of energy digestibility was reported (Eeckhout and De Paepe, 1992a; O'Quinn et al., 1997; Johnston et al., 2004).

Energy metabolizability did not differ for the 2 treatments (82.7 vs. 82.5% for phytase and control, respectively). Consequently, energy losses with urine and gases were higher in phytase-supplemented piglets (13 and 1.2 kJ/($kg^{0.75}$.d), respectively; Table 3). Diets were desig-

ned to be limiting in energy. It could, therefore, be expected that more nitrogenous components would be excreted in the urine of piglets administered phytase, given the higher protein digestibility (Table 2). Piglets excreted 0.67 (phytase) or 0.64 (control) g N/($kg^{0.75}$.d) in urine, which explains only about 1 kJ($kg^{0.75}$.d) of the difference in urinary energy. The remaining difference, about 12 kJ/($kg^{0.75}$.d) (1.3% of ME intake) cannot be attributed to urinary loss of nitrogenous compounds. Explaining this deficit requires more extensive urinary measurements than could be performed in the current experiment, because urine was not collected separately. It is speculated that because dietary phytase increased the mineral absorption rate (discussed later), this could increase blood plasma volume, and consequently blood pressure. As a result, more plasma compounds will be filtered into the primary urine, and have to be reabsorbed. Possibly, with the increase of renal ultra filtration, more (energy-rich) organic compounds, which would normally be reabsorbed, are lost in urine. For instance, reabsorption of glucose is almost, but not totally, complete (Bonnardeaux and Bichet, 2004). Increased renal filtration may increase urinary losses of glucose, or similar energy-rich compounds. This requires further investigation.

In fast-growing poultry, dietary phytase clearly enhances energy metabolizability. A mean increase of ME content with 220 kJ/kg feed was calculated, when 500 FTU/kg feed was supplied (Kies et al., 2001). This is about 1.8% of the ME-content of a typical broiler diet. In the present experiment, phytase supplementation increased the ME content of the diet for piglets by only about 0.3%.

No difference in energy partitioning was observed between treatments. In a preliminary, unpublished experiment [Chapter 4] with newly weaned piglets consuming feed *ad libitum*, there were indications that retention might be increased with phytase supplementation. Energy retention increased by 17%, but this was largely the result of a higher feed intake. In an experiment with growing pigs (26-52 kg) fed restricted diets (2.9 × ME_m), phytase supplementation to diets not limiting in P increased energy retention by 6% compared to control pigs, as measured by total-body electrical conductivity on carcasses (Shelton et al., 2003). In both experiments the effects were, however, not significant.

Organ weight and blood variables After the experimental period of 21 days, weights of the pancreas and small intestine were slightly lower in phytase-fed than in control piglets. Lower pancreas weight might be due to a lower production of pancreatic digestive enzymes because less of these enzymes are bound to phytate (Singh and Krikorian, 1982; Selle et al., 2000; Cowieson et al., 2004). Lower organ weight might indicate that work required for digestive processes is slightly less (Koong et al., 1982; Bikker, 1994; Kerr et al., 2003). Corresponding with the increased digestibility, it could mean that the digestion process is facilitated by phytase.

One sample of portal blood was taken from each piglet 4 h after initiation of feeding. Because kinetic information is lacking, no firm conclusions on metabolic activity in visceral tissues can be drawn. Some interesting differences occurred between treatments, however. The pH, typically about 7.4 (Dersjant-Li et al., 2002b), was very low in both treatments, i.e. about 7.

Lower pH and pO2, and higher pCO_2 were observed in phytase-treated piglets. This could indicate increased oxidative metabolism in portal-drained viscera at the time of sampling, due to a higher absorption rate of nutrients. Increased metabolic activity of the viscera was not reflected, however, in higher heat production at 4 h post-feeding (data not shown). Higher absorption of Na and K coincides with increased bicarbonate absorption, which may also explain the higher pCO_2 of portal blood (Dersjant-Li et al., 2002b). Absorption of sodium requires oxygen, due to the activity of Na, K-ATPase (Mandel and Balaban, 1981; Summers et al., 1986), which could explain the slightly lower pO_2.

Portal plasma glucose concentration was 21% higher in phytase-treated piglets than in control piglets. Blood glucose level depends on many factors, including insulin, which we did not measure. It may reflect a higher rate of absorption from the viscera, a lower utilization by visceral tissues, a lower portal blood flow, or a combination of these factors. A higher absorption rate would conform to the higher energy digestibility. For both treatments, starch digestibility was almost complete at the end of the ileum (data not shown); thus the extent of starch digestion cannot explain the higher plasma glucose concentration. The result for glucose is in agreement with those of Johnston et al. (2004), who found a higher plasma glucose level (5%) in pigs administered phytase. They took a blood sample from the vena cava, which is an important difference compared to the current experiment (portal vein). This experiment compared pH, pO_2 and pCO_2 in portal and in jugular blood of 6 piglets. The mean values (phytase and control) for these 3 variables were 7.07, 5.0 and 11.5 for portal blood, and 7.27, 5.6 and 6.6 for jugular blood, respectively. These values are not indicative for glucose concentration, but indicate that caution is required when comparing results of blood sampled from different veins.

Together, these blood variables suggest that gut metabolism is more intensive in phytase-treated piglets 4 h after the start of feeding. But this did not result in changes in heat production. Dietary phytase may simultaneously both increase and decrease heat production, resulting in no net effect. A higher mineral load might increase heat production. Minerals are absorbed and when they are in excess of the piglet's needs, they must be excreted. Both absorption and excretion are partially active processes, thus requiring energy.

Effect of phytase on mineral metabolism Dietary phytase supplementation increased apparent fecal ash digestibility by almost 10%. This is the result of increased absorption of all minerals studied and is in agreement with earlier findings (Jongbloed et al., 1992, 2000; Pallauf et al., 1992a; Yi et al., 1996d; Gebert et al., 1999a). In those experiments, the effect of phytase on the absorption of P and divalent cations was studied. In the current experiment, phytase addition also increased fecal digestibility of the monovalent cations Na and K (6%, for both; $P < 0.02$). Na- and K-phytate complexes are highly soluble. For instance, Na-phytate is >96% soluble over the pH-range 0.3-11.2 (Scheuermann et al., 1988). Therefore, it is likely that digestibility of these minerals is not inhibited by phytate; their absorption was not studied *in vivo* previously in this context.

Most of the extra-absorbed minerals were excreted in the urine, indicating that their availability was in excess of the piglets' requirements. This corresponds with the findings of O'Quinn et al. (1997). In finishing swine, they recovered 79% of the extra-absorbed P in urine when phytase supplementation increased from 300 to 500 FTU/kg; at that level the P requirement of swine was approached. In our experiment, using diets not limiting in P, the relative urinary loss of phytase-induced P absorption was 83%.

Effect of minerals on energy metabolism Absorption of many minerals is in part an active process that requires energy. Most minerals are excreted passively, but there is an extensive, partly active, reabsorption of minerals in the kidneys (Martens et al., 1991; Randall et al., 1997). Many active processes are driven by Na-K-ATPase. This enzyme generates a Na and K concentration gradient and electric potential difference, which drives the absorption of other ions and molecules (Mandel and Balaban, 1981; McBride and Kelly, 1990). Na-K-ATPase-dependent respiration has been estimated as high as 70% of kidney oxygen consumption (Balaban and Mandel, 1980; Summers et al., 1986). The work of the kidneys requires 14% of maintenance oxygen consumption (Balaban and Mandel, 1980; Summers et al., 1986); thus about 10% of ME_m would be related to mineral reabsorption in the kidneys. Using our model, energy costs for mineral reabsorption from the kidney were estimated at 30 kJ/($kg^{0.75}$.d) (mean of treatments; data not shown), or 7% of ME_m. Our estimate seems low, indicating that the energy costs of urinary reabsorption may be underestimated.

When constructing the model, several assumptions were made, some of which potentially underestimate energy costs. These assumptions include: *1*) Excretion of minerals by some organs, and possible subsequent reabsorption, is neglected. With saliva and other gastrointestinal juices, a large quantity of minerals is excreted into the gastrointestinal tract (Partrigde, 1978a). Much of this is reabsorbed from the gut. The potential importance of mineral excretion in the gastrointestinal tract is illustrated by the relatively low ileal ash digestibility (Partrigde, 1978b). *2*) The potential energetic effect of the acid-base balance is not included in the calculations. This balance affects energy metabolism of piglets (Dersjant-Li et al., 2002a) and is probably altered by the extra amounts of minerals absorbed. On the other hand, some assumptions may lead to overestimation of the energy expenditure of mineral reabsorption. In particular, it is not always clear what proportion of the minerals is actively (re-) absorbed. We estimated these values on the basis of a number of references (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000), but there are conflicting views. For instance, the energy costs involved in the absorption of calcium from the gastrointestinal tract in humans are subject to debate (McCormick, 2002, 2003; Bronner et al., 2003).

Using the model, extra energy expenditure for membrane transport and for Ca and P deposition in bones was estimated at 4.6 kJ/($kg^{0.75}$.d) for piglets administered phytase. This is equivalent to 1% of the estimated ME_m. A sensitivity analysis of the model for energy costs of renal reabsorption of minerals showed especially the importance of measured urinary mineral excretion, which determines the calculated reabsorption coefficient of the minerals. For example, to equalize urinary sodium excretion to 3 mg/($kg^{0.75}$.d), as measured in the experiment, a reabsorption coefficient of 0.964 was calculated. If this coefficient were fixed at 0.99, a value

often mentioned in literature (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000), calculated urinary excretion would be 1.1 mg/($kg^{0.75}$.d). The higher reabsorption coefficient increases the estimated energy costs of renal sodium reabsorption by 39%.

Dietary treatment did not affect H_{tot}, but the kinetics of heat production within the day showed some differences. For 3 of the hours between 7 and 12 h after afternoon feeding, H_{tot} was higher in phytase-fed piglets ($P < 0.05$). Mean heat production during these 6 h was 8 (range 2-14) kJ/($kg^{0.75}$.d) higher in phytase-fed than in control piglets. It is speculated that this increased heat production was related to increased mineral excretion. A diurnal variation of mineral excretion has been shown for P, Na, K and Cl (Toor et al., 1965; Mudge et al., 1973). In a study of human beings in a hot environment, the peak in urinary Na excretion shifted due to heat stress or work (Toor et al., 1965). Diurnal shifts in mineral excretion could not be measured in the present experiment, nor could it be measured whether a similar effect on heat production exists after morning feeding, because it coincided with the heat production peak after the afternoon meal.

In conclusion, phytase addition improved nutrient digestibility. Because the extra digested energy was lost post-absorption, energy metabolizability was not affected. Furthermore, protein and fat deposition rates were unaffected by dietary phytase. Mineral absorption and subsequent urinary excretion were increased by phytase supplementation. Phytase-induced effects on organ weights and blood variables suggested that the energy expenditure of the digestive tract was reduced, and metabolic activity in visceral organs increased. The possible energy-saving benefits of supplemental phytase at the level of digestion might be counterbalanced by the increased energy costs for other processes, such as the increased absorption and urinary excretion of minerals. These costs were subsequently estimated, using a simulation model. Phytase-induced energy expenditure associated with increased (re-) absorption of protein, fatty acids and minerals, and deposition of Ca and P in bone tissues, was estimated to be just over 1% of ME_m. The simultaneous existence of both increases and decreases in heat production processes resulted in the absence of a net effect on energy retention.

APPENDIX 1: Description of a model to estimate the energy costs associated with phytase-induced increase of nutrient absorption and excretion.

Definition:

- One cardiac cycle is defined as the time required for a quantity of blood equal to the total blood volume of the animal, to be ejected by the heart into the systemic circulation.

General assumptions:

- The increase of active transport of ions is assumed to depend totally on Na-K-ATPase at the cost of one ATP per (net) transported ion, mono- or divalent (Mandel and Balaban, 1981; Berg et al., 2002).
- Possible saturation of transport carrier molecules is ignored, as well as possible further metabolism of nutrients, e.g. urea formation or synthesis of proteins from amino acids (Van Milgen, 2002).
- A dietary energy equivalent for ATP of 99 kJ/mol was taken, an average value calculated on the basis of complete oxidation of protein, glucose and fat (Gerrits et al., 1997) with an efficiency according to the non-integral values of Van Milgen (2002).
- Calculations were made on the differences between the dietary treatments (i.e., phytase-induced), not on total nutrient absorption and excretion rates.
- Energy costs for pumping minerals from extracellular fluid into intracellular fluid, or the reverse, are ignored.

Absorption from the gastro-intestinal tract:

- Intestinal absorption for mineral M = (intake – fecal excretion)$_M$ mg/(kg$^{0.75}$.d). Na and K were assumed to be absorbed actively (energy dependent) for 100%, Ca, Mg, Fe, and Cu for 80%, and P for 75% (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000). Chloride was ignored in the calculations; it is absorbed passively.
- Phytase increased fecal protein absorption [239 mg/(kg$^{0.75}$.d)]. The molecular weight of an amino acid was assumed to be 100 g/mol. The energy cost for intestinal absorption is 1 ATP per amino acid (Berg et al., 2002).
- Phytase increased fecal fat absorption [104 mg/(kg$^{0.75}$.d)]. Fat is absorbed as monoacylglycerol and two fatty acids. The molecular weight of a fatty acid was assumed to be 280 g/mol. Re-esterification to triglyceride (2 ATP/fatty acid) requires 1.33 ATP/ fatty acid (Berg et al., 2002).

Deposition of Ca and P into bone tissues:

- Compared to the control, phytase treatment increased calcium and phosphorus retention by 54 and 18 mg/(kg$^{0.75}$.d), respectively. Retained Ca and P were deposited into bone tissues only. Costs for deposition of other minerals are ignored. Energy costs for the deposition of Ca and P into bone were assumed to be 2 mol ATP/mol Ca or P (analogous to Gerrits et al., 1997).

Reabsorption from primary urine:

A part of the blood passes through the kidneys, where a proportion is filtrated in the glomeruli into primary urine. Minerals are reabsorbed from primary urine into plasma (recycling), or excreted into urine. The mathematical representation of this system is as follows:

Pool sizes of mineral M (expressed in $mg/kg^{0.75}$):

$Q_{M,pl}$: blood plasma
$Q_{M,if}$: interstitial fluid
$Q_{M,cb}$: intracellular fluid and bones
$Q_{M,TBF}$: total body fluids ($= Q_{M,pl} + Q_{M,if} + Q_{M,cb}$)
$Q_{M,pu}$: primary urine

Mineral fluxes:

Mineral flows from one pool to the other are represented by mass-action kinetics:

$$F_{M,pl,pu} = k_{M,pl,pu} \times Q_{M,pl} \text{ [mg/(kg}^{0.75}\text{.cardiac cycle)]} \quad (1.1)$$
$$F_{M,pu,pl} = k_{M,pu,pl} \times Q_{M,pu} \text{ [mg/(kg}^{0.75}\text{.cardiac cycle)]} \quad (1.2)$$
$$F_{M,pu,ur} = k_{M,pu,ur} \times Q_{M,pu} \text{ [mg/(kg}^{0.75}\text{.cardiac cycle)]} \quad (1.3)$$

in which $k_{M,pl,pu}$, $k_{M,pu,pl}$, and $k_{M,pu,ur}$ are the fractional transport constants (/cardiac cycle) from plasma to primary urine, from primary urine to plasma, and from primary urine to urine, respectively.

Pools of minerals in plasma, interstitial and intracellular fluids, and in bones were considered together ($Q_{M,TBF}$), with a constant distribution over the three pools at the start of each cardiac cycle.

The following differential equations were used to calculate the change in pool sizes with time:

$$dQ_{M,TBF}/dt = F_{M,pu,pl} - F_{M,pl,pu} \quad (1.4)$$
$$dQ_{pu}/dt = F_{M,pl,pu} - F_{M,pu,pl} - F_{M,pu,ur} \quad (1.5)$$

The total body pool size of each mineral ($Q_{M,TBF}$) at $t = 0$ was assumed to be the quantity of mineral M (extra-absorbed due to phytase) from the gastro-intestinal tract (appearing at t=0). The pool size of minerals in plasma, relative to the total body fluids, was recalculated as:

$$Q_{M,pl} = D(Q_{M,pl} / Q_{M,TBF}) \times Q_{M,TBF} \quad (1.6)$$

The distribution constants ("$D(Q_{M,pl} / Q_{M,TBF})$") are indicated in **Table 7**.

From equation 1.6, from similar equations for the mineral pools of interstitial fluid and intracellular fluid and bones, and based on an assumption of the distribution between the different body fluids, net flow rates between the three body compartments could be calculated.

Calculations:

For present calculations, constants were parameterized as follows:

- One cardiac cycle takes 35 s, based on a piglet weighing 15 kg (Melbin and Detweiler, 1993)
- Renal blood flow (RBF) is 0.22 [fraction of cardiac output; (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000)]
- Glomerular filtration rate (GFR) is 0.20 [fraction of RBF; (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000)]
- The fractions of minerals re-absorbed actively from primary urine are given in Table 7.

The flux $F_{M,pu,pl}$ (eq. 1.2) was calculated by calibrating the rate of reabsorption $kRA_{M,pu,pl}$ (eq. 1.7) to fit the measured, cumulative, daily phytase-induced excretion of mineral M (Table 1; eq. 1.8). This was done using the "Goal Seek" tool of Excel®.

$$k_{M,pu,pl} = D(Q_{M,pl} / Q_{M,TBF}) \times RBF \times GFR \times kRA_{M,pu,pl} \quad (1.7)$$

$$S\ (t = 0, \ldots, 2469)\ F_{M,pu,ur} = UR_M\ [mg/(kg^{0.75}.d)] \quad (1.8)$$

where 2469 is the number of cardiac cycles per day and UR_M the measured urinary excretion of mineral M.

Flows of the minerals ($F_{M,pu,pl}$) were integrated over a day and cumulative energy expenditure was calculated, using the assumptions described previously. The calculated reabsorption rates ($kRA_{M,pu,pl}$) and energy costs are presented.

Table 7. Assumed partitioning of minerals in plasma, relative to total body fluids, and assumed fraction of minerals actively reabsorbed from primary urine[1,2]. Parameters used in the model to estimate energy costs of mineral reabsorption in piglets.

Trait	Ca	P	Na	K	Mg + Fe + Cu[3]
$D(Q_{M,pl} / Q_{M,TBF})$[4]	0.13	0.19	0.24	0.08	0.13
Active reabsorption[5]	0.25	0.80	0.95	0.90	0.25

[1] Chloride was not included in the calculations, since it is absorbed passively

[2] Assumptions mainly based on human data (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000; Groff and Gropper, 2000; Berl and Verbalis, 2004; Yu, 2004).

[3] Mg + Fe + Cu were grouped together; most parameters were assumed to be equal to those for Ca

[4] "Distribution constants": estimated proportion of extra-absorbed mineral M (in ionic form) in plasma, relative to TBF (Randall et al., 1997; Halperin and Goldstein, 1999; De Jong et al., 2000). These values are not (necessarily) equal to the distribution in different compartments of the body.

[5] Fraction of reabsorption (kidney) assumed to be energy dependent.

Chapter 6

Effect of graded doses of microbial phytase on the digestibility of various minerals in piglets

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ABSTRACT

An experiment with 224 piglets (initial BW 7.8 kg) was conducted to determine the dose effect of dietary phytase supplementation on apparent fecal digestibility of minerals (P, Ca, Mg, Na, K and Cu) and on performance. Four blocks, each with eight pens of seven piglets, were formed. Eight dietary treatments were applied in the 43 d experiment: supplementation of 0 (basal diet), 100, 250, 500, 750, 1,500, or 15,000 phytase units (FTU) or of 1.5 g digestible P (positive control) per kg feed. The basal diet, with corn, barley, soybean meal and sunflowerseed meal as the main components, contained per kg 1.2 g digestible P (dP). Fresh fecal grab samples were collected in weeks four and five of the experiment. Average daily feed intake, average daily gain (ADG) and gain : feed ratio all increased with increasing phytase dose ($P < 0.001$). Digestibility of all investigated minerals increased ($P < 0.001$) with increasing phytase dose. Supplementation with 15,000 FTU increased P digestibility from 34 to 84%, generating 1.76 g dP per kg feed. At this level, 85% of the phytate phosphorus was digested, compared to 15% in the basal diet. Digestibility of the monovalent minerals increased from 81 to 92% (Na), and from 76 to 86% (K). In conclusion, phytase supplementation to a dP deficient diet improved performance of piglets and digestibility of minerals, including monovalent minerals, up to a level of 15,000 FTU/kg. Phytate-P could be almost completely digested. Thus, dietary phytase supplementation beyond present day industrial standards (500 FTU/kg) could further improve mineral utilization, and consequently reduce detrimental mineral output to the environment.

INTRODUCTION

Microbial phytase is added to animal feeds throughout the world. The enzyme improves phosphorus (P) digestibility of feeds for monogastric animals, like pigs. Subsequently, excretion of P with manure can be decreased. Most P in plants is stored as phytate, the salt of phytic acid (*myo*-inositol hexakisphosphate). Pigs digest phytate-P poorly.

Under normal physiological conditions, phytate is a negatively charged ion that is able to bind cations such as Ca, Mg and Zn, and also proteins (Ravindran et al., 1995; Bebot-Brigaud et al., 1999). Phytase hydrolyses the ortho-phosphate groups from phytate. Phytate-bound nutrients are liberated as well. The result is not only a higher digestibility of P, but also of protein (Kies et al., 2001) and of minerals. Dietary microbial phytase was reported to increase digestibility of Ca, Mg, Mn, Zn, Cu and Fe in pigs at a dose level of 500 to 1500 phytase units (FTU)/kg of feed (Pallauf et al., 1992a; Adeola, 1995; Jongbloed et al., 1995).

There is hardly any information available about the effect of phytase doses higher than 1,500 FTU/kg on mineral digestibility. Düngelhoef and Rodehutscord (1995) and Kornegay (2001) estimated that only a small additional effect on P digestibility in pigs would be obtained at dose levels exceeding 1,500 FTU/kg. The effect of high phytase doses on P and Ca digestibility was studied in broiler chicks (Augspurger and Baker, 2003; Shirley and Edwards, 2003), and in pigs (Harper et al., 1999). The authors concluded that phytase continued to improve performance, bone characteristics, and P and Ca digestibility up to a dose of 10,000 (Harper et al., 1999; Augspurger and Baker, 2003) or 12,000 FTU/kg (Shirley and Edwards, 2003).

The present experiment was performed to obtain more information about the effect of graded phytase doses up to a high level (100, 250, 500, 750, 1,500 and 15,000 FTU/kg feed), on the digestibility of P, Ca, Mg, Na, K and Cu in piglets.

MATERIALS AND METHODS

Animals and Housing Crossbred ([Yorkshire × Dutch Landrace] × Yorkshire) female and castrated male piglets (224 in total) were used from weaning (at about 28 d of age). Mean initial weight was 7.8 kg. Each of the eight treatment groups consisted of four replicates (pens) with seven piglets each. The seven piglets in each pen (four females and three castrates, or the inverse) were selected from seven pairs of sows and were randomly chosen. No littermates were used in any one pen. Selection criteria of the piglets were health status and the combination of weight and sex. Piglets remained in the farrowing house during the 43 d experiment. Four farrowing houses were used, each one being considered a block and each block containing eight pens. The eight treatments were randomly assigned to pens in each of the four blocks. Pen size was 1.6 × 1.8 m. Temperature at weaning was kept at 25°C and was lowered by <1°C every wk thereafter. Ventilation was thermostatically controlled. The ethics committee of DLO-Institute for Animal Science and Health approved the experiment

Treatments, diets and feeding Eight treatments were investigated in the experiment. To a basal diet, 0 ("basal diet"), 100, 250, 500, 750, 1,500, or 15,000 FTU was added per kg. As

the positive control, a diet supplemented with 1.5 g digestible P (dP)/kg was used. One FTU is defined as the phytase activity that liberates 1 µmol orthophosphate from 5.1 mM sodium phytate per minute at 37°C and at pH 5.5 (Engelen et al., 1994). Natuphos® Granulate (obtained from DSM Food Specialties, Delft, the Netherlands) was the phytase source. It was added based on analyzed activity. The 1.5 g dP/kg added to the positive control was in the form of monocalcium phosphate monohydrate (MCP), with an assumed P digestibility of 83% (CVB, 2000).

Composition of the basal diet and proximate analyses are presented in **Table 1**. A mix of the ingredients (without mineral mix, microbial phytase and MCP) was produced. This mix was split into eight equal parts, to which a starch-based premix, containing mineral mix, microbial phytase and (or) MCP were added. After mixing, feeds were pelleted (diameter 3.2 mm) without addition of steam to prevent possible inactivation of phytase. Temperature during the pelleting process was below 60 °C. The basal diet contained nutrients at or above the levels recommended by the CVB (2002), except for Ca and P. Digestible P content of the basal diet (0 FTU/kg) was estimated at 1.25 g/kg. It was assumed that 100, 250, 500, 750, 1,500 and 15,000 FTU generated 0.15, 0.4, 0.8, 1.0, 1.1 and 1.1 g dP/kg of feed, respectively. To obtain a fixed Ca : dP ratio of 2.8, Ca content of the diets was increased by addition of limestone. Analyzed Ca and P contents and phytase activity, and calculated and measured dP content are presented in **Table 2**. Analyzed and calculated values agreed well. Piglets had *ad libitum* access to feed and water. Water was normal tap water. Contents of Ca, P, Mg, Na and K were about 64, 0.01, 4.5, 19.8 and 1.0 mg/L, respectively. The Cu level was negligible. The impact of minerals in the drinking water is ignored in digestibility calculations, because there is little difference between treatments.

Observations, collection and analytical procedures The experiment lasted 43 days. Pig weight and feed intake were recorded on day 0, 8, 29 and 43. Health status of the pigs was monitored twice daily throughout the experiment. Fresh fecal grab samples were collected in weeks four and five taking care to collect feces from the different piglets. Sampling was on Tuesdays and Thursdays at 0800 to 0830, 1000 to 1030, 1300 to 1330 and 1500 to 1530. The samples were pooled per pen per wk and frozen at -18°C pending analysis. Feed was sampled during production.

All analyses were performed on freeze-dried feed and feces samples. Feeds were analyzed for DM, ash, Ca, P, Mg, Na, K, Cu, Cr and phytase activity. Phytate-P and nitrogen were analyzed in the basal diet only. Feces were analyzed for DM, ash, Ca, P, Mg, Na, K, Cu and Cr. Dry matter, ash and nitrogen (Kjeldahl) were assayed using AOAC procedures (1984). Mineral levels (except Cr) were determined using inductively coupled plasma atomic emission spectrometry, according to NEN-ISO (1998). The method by Williams et al. (1962) was used to assess chromium. Phytate-phosphorus was measured by the enzymatic method (Bos et al., 1993), and phytase activity according to Engelen et al. (1994). Digestibility coefficients of DM, ash and the minerals under investigation, were calculated using Cr as an indigestible marker.

Table 1. Feed composition and nutrient contents of the basal diet[1] (as-fed)

Ingredient		Analyzed composition[4]	
	g/kg		*g/kg*
Corn	321	DM	868
Barley	300	CP	202
Soybean meal	108	Ash	38
Sunflower seed meal	65	Ileal digestible Lys[5]	9.9
Soybeans, toasted	40	NE, *MJ/kg*[5]	9.6
Potato protein	38	Ca	3.5
Acid casein	33	P	3.7
Cane molasses	35	Digestible P[5]	1.25
Soy oil	13.5	Phytate P	2.6
Fumaric acid	10		
Salt	2.5		
L-Lysine.HCl	0.95		
DL-methionine	0.25		
Cr-premix[2]	1.0		
Choline chloride (40%)	0.30		
Premix[3]	2.0		
Limestone	6.1		
Cornstarch	23.4		

[1] Limestone was added to obtain a Ca : dP ratio of 2.8. To the positive control diet, 7.96 g MCP was added per kg feed. Cornstarch was used to balance to 1000 g/kg.
[2] Contained 0.25 g chromium oxide per kg feed.
[3] The vitamin-mineral premix provided per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,000 IU; vitamin E, 20 IU; vitamin K_3, 1.5 mg; thiamine, 1 mg; riboflavin, 4 mg; D-panthothenic acid, 15 mg; niacin, 25 mg; vitamin B_{12}, 20 µg; folic acid, 0.2 mg; pyridoxine, 1.5 mg; Fe ($FeSO_4.7H_2O$), 150 mg; Cu ($CuSO_4.5H_2O$), 125 mg; Zn ($ZnSO_4.7H_2O$), 80 mg; Mn (MnO), 30 mg; Co ($CoSO_4.7H_2O$), 0.15 mg; I (KI), 0.5 mg; and Se ($Na_2SeO_3.5H_2O$), 0.3 mg.
[4] Variation between diets was small. Mg, Na and K contents of the basal diet were 1.6, 1.1 and 8.1 g/kg, respectively. Cu content was 113 mg/kg.
[5] Calculated values (CVB, 2000).

Table 2. Analyzed Ca, P and phytase activity of the diets, and calculated and measured digestible P (dP) levels

FTU added/kg	Ca	P[1]	Phytase	Calculated dP	Measured dP
	g/kg	*g/kg*	*FTU/kg*	*g/kg*	*g/kg*
0	3.5	3.7	350	1.25	1.22
100	3.9	3.7	470	1.40	1.46
250	4.6	3.6	600	1.65	1.69
500	5.8	3.6	810	2.05	2.01
750	6.3	3.6	1190	2.25	2.19
1,500	6.5	3.6	1740	2.35	2.60
15,000	6.7	3.6	13900	2.35	3.04
Positive control	7.6	5.4	340	2.75	2.58

[1] All calculations on phytase effect are based on the mean P level of these diets: 3.63 g/kg.

Statistical Analysis Data were analyzed by ANOVA as a randomized complete block design with pens as the experimental units using Genstat 5, version 3.1 (Genstat Committee, Rothamsted, UK, 1993). Because no treatment × week interaction was observed for the digestibility coefficients, results for the two-week collection period were averaged and analyzed as such. Significant differences ($P < 0.05$) among treatments were tested by Student's t-test. Finally, exponential dose-response equations were fitted for performance and P-digestibility for all treatments excluding the positive control.

RESULTS

General observations and performance Few health problems occurred in this experiment. A limited number of animals were treated for lameness. Eight piglets died, mainly due to edema disease. Mortality was not related to treatment. Mean final weight of the piglets was 27.5 kg.

Performance of piglets fed the positive control diet was significantly better than for those fed the basal diet (**Table 3**). These results indicate that the basal diet was clearly deficient in dP. Dietary phytase supplementation affected average daily feed intake (ADFI), ADG and gain : feed ratio exponentially. The calculated relationships were:

$$\text{ADFI (g/d)} = 751 - 167 \times e^{-0.0021 \times FTU} \quad (R^2 = 0.98;\ \text{S.E.: } 10.8;\ P < 0.001);$$
$$\text{ADG (g/d)} = 512 - 141 \times e^{-0.0018 \times FTU} \quad (R^2 = 0.98;\ \text{S.E.: } 9.4;\ P < 0.001);$$
$$\text{Gain : feed} = 687 - 49 \times e^{-0.0008 \times FTU} \quad (R^2 = 0.91;\ \text{S.E.: } 6.9;\ P < 0.02),$$

in which FTU is the added microbial phytase activity (FTU/kg).

Digestibility Values Treatment affected the digestibility of all measured parameters ($P < 0.01$; **Table 4**). DM digestibility was slightly but significantly lower in the positive control diet compared to the other diets, with the exception of the basal diet and the diet with 750

Table 3. Average daily feed intake (ADFI; g/d), average daily gain (ADG; g/d) and gain : feed ratio (G : F; g/kg) of piglets receiving diets with different levels of phytase or a diet containing 1.5 g dP from monocalcium phosphate monohydrate (positive control)[1,2]

FTU added/kg	ADFI	ADG	G : F
0 (basal diet)	588 ^{a}	370 ^{a}	629 ^{a}
100	603 ^{a}	388 ^{a}	645 ^{ab}
250	666 ^{b}	436 ^{b}	654 ^{bc}
500	697 ^{bc}	458 ^{b}	658 ^{bc}
750	708 ^{bcd}	467 ^{bc}	659 ^{bc}
1,500	747 ^{cd}	499 ^{cd}	667 ^{c}
15,000	752 ^{d}	517 ^{d}	689 ^{d}
Positive control	699 ^{bcd}	468 ^{bc}	671 ^{cd}
SEM	18.4	11.4	6.5
P-value	< 0.001	< 0.001	< 0.001

[1] Data are means of four replications of seven piglets over a 43-d feeding period; mean initial BW was 7.8 kg.
[2] Values within a column with no common superscript differ significantly ($P < 0.05$).

Table 4. Effect of dietary supplementation with different levels of phytase on apparent fecal digestibility of DM, ash and some minerals in piglets[1,2]

FTU/kg	DM	Ash	Ca	P	Mg	Na	K	Cu
0 (basal diet)	84.4 ^{ab}	52.3 ^{a}	57.2 ^{a}	33.5 ^{a}	19.8 ^{a}	81.1 ^{a}	76.1 ^{abc}	-5.8 ^{a}
100	84.7 ^{bc}	55.2 ^{b}	64.0 ^{b}	40.1 ^{b}	24.6 ^{cd}	83.4 ^{ab}	73.5 ^{a}	-2.3 ^{bc}
250	85.2 ^{c}	58.5 ^{c}	66.9 ^{c}	46.6 ^{c}	24.2 ^{bc}	81.0 ^{a}	76.0 ^{abc}	-3.0 ^{ab}
500	84.5 ^{b}	60.4 ^{d}	70.6 ^{d}	55.3 ^{d}	25.4 ^{cd}	83.0 ^{ab}	77.2 ^{bc}	-1.5 ^{bc}
750	84.4 ^{ab}	61.6 ^{d}	69.2 ^{cd}	60.4 ^{e}	24.4 ^{cd}	86.0 ^{bc}	78.5 ^{c}	0.4 ^{cd}
1,500	84.6 ^{b}	66.2 ^{e}	71.8 ^{d}	71.5 ^{f}	27.7 ^{d}	88.8 ^{c}	81.5 ^{d}	2.8 ^{d}
15,000	85.3 ^{c}	70.2 ^{f}	75.8 ^{e}	83.8 ^{g}	33.8 ^{e}	92.1 ^{d}	85.7 ^{e}	6.0 ^{e}
Pos. control	83.9 ^{a}	55.7 ^{b}	58.1 ^{a}	47.8 ^{c}	20.8 ^{ab}	84.4 ^{b}	75.3 ^{ab}	-2.2 ^{bc}
SEM	0.22	0.61	0.95	1.06	1.17	1.05	0.90	1.05
P value	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Data are means of four replications of seven piglets. Digestibility was measured in the fourth and fifth weeks of the experiment; these data were averaged per treatment.
[2] Values within a column with no common superscript differ significantly ($P < 0.05$).

FTU/kg. Between the other diets, differences were small. Ash and phosphorus digestibility increased significantly with each increase of phytase dose, up to a level of 15,000 FTU/kg ($P < 0.001$). Also calcium digestibility increased in a dose-dependent manner, but this effect may be biased due to different limestone levels in the diets. Magnesium, sodium, potassium and copper digestibility increased with phytase dose ($P < 0.001$), but differences per subsequent level of supplementation were not always significant. Copper digestibility was negative at phytase doses below 750 FTU/kg. An exponential model was fitted to the dP levels (excluding the positive control diet), resulting in the following equation:

$$\text{Digestible P (g/kg)} = 3.02 - 1.76 \times e^{-0.00102 \times FTU} \quad (R^2 = 0.997;\ \text{S.E.: } 0.045;\ P < 0.001).$$

The estimated dP level of the basal diet according to this model was 1.26 g/kg (measured was 1.22 g/kg; Table 2). The plateau level was 3.02 g dP/kg, which was reached at about 5,000 FTU/kg (3.04 g measured at 15,000 FTU/kg). Consequently, the maximal amount of dP generated by phytase in this diet was 1.76 g/kg.

Similar exponential models were fitted to the other minerals. Digestible DM gave no good fit, and digestible ash and Ca are biased, due to the inclusion of limestone to keep a constant Ca : dP ratio. These equations are, therefore, not presented. The equations for Mg, Na, K and Cu are as follows:

$$\text{digestible Mg (mg/kg)} = 54.7 - 18.8 \times e^{-0.00045 \times FTU} \quad (R^2 = 0.89;\ \text{S.E.: } 2.80;\ P < 0.03);$$
$$\text{digestible Na (mg/kg)} = 960 - 117 \times e^{-0.00067 \times FTU} \quad (R^2 = 0.922;\ \text{S.E.: } 14.7;\ P < 0.02);$$
$$\text{digestible K (g/kg)} = 6.8 - 0.89 \times e^{-0.00059 \times FTU} \quad (R^2 = 0.945;\ \text{S.E.: } 0.09;\ P < 0.01);$$
$$\text{digestible Cu (mg/kg)} = 7.22 - 12.96 \times e^{-0.00083 \times FTU} \quad (R^2 = 0.958;\ \text{S.E.: } 1.20;\ P < 0.01).$$

The amounts of these minerals digested due to phytase can easily be calculated from these equations.

DISCUSSION

Addition of microbial phytase to the basal diet, with a measured dP content of 1.2 g/kg, improved piglet performance. The recommended dP level for weaned piglets is 3.7 g/kg (CVB, 2002), thus all diets had dP contents below requirement. Piglets will, therefore, not show their maximal performance (Jongbloed, 1987). Phytase liberates P from phytate, which increases dP supply to the piglets. Consequently, performance improves, as observed in previous studies (Beers and Jongbloed, 1992; Cromwell et al., 1993; Kornegay, 2001). In the present experi-ment, performance improved up to a phytase inclusion level of 15,000 FTU/kg. The maximal effect of phytase supplementation on performance has been estimated to be in the range of 500 to 1,500 FTU/kg, in basal diets with similar dP contents to the one in the present experi-ment (Beers and Jongbloed, 1992; Gentile et al., 2003). In a review, Kornegay (2001) came to a similar conclusion. At a phytase dose of 1,500 FTU/kg, dP level was similar to that of the positive control diet (2.6 g/kg). At this phytase level, feed intake and ADG were improved by 6.9 and 6.6%, respectively, as compared to the positive control diet. Although not significant, this may indicate some effect of phytase on performance beyond the dP-related effect (Kies et al., 2001).

The exponential models used showed an excellent fit to the data, as shown for dP (**Figure 1**). In this figure, the curve calculated from Kornegay (2001; equation '1,500') is also depicted (after correction for the difference at 0 FTU/kg). The curves are similar up to about 750 FTU/kg. After that, the curve calculated from Kornegay's equation plateaus rapidly, at a level about 0.8 g dP/kg feed lower than the plateau of the curve from the current experiment.

Supplementation of microbial phytase to low-P diets improved P digestibility. This confirms previous results (Jongbloed et al., 1992; Adeola et al., 1995; Kornegay and Qian, 1996). Generation of digestible P by phytase was, however, much higher than expected, especially at the highest dose (Table 2). The assumed dP generation with the addition of 15,000 FTU/kg

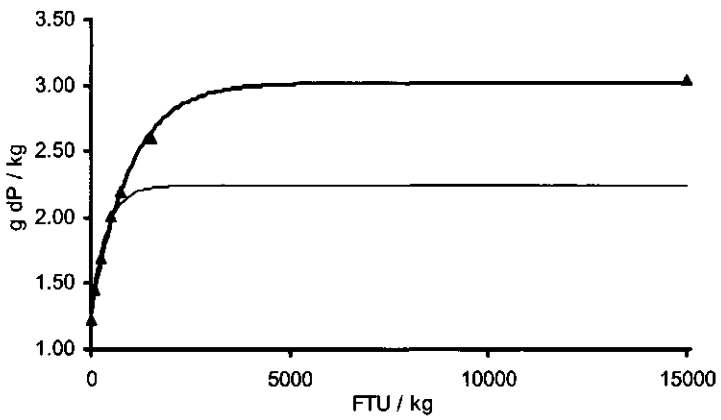

Figure 1. Observed (▲) and predicted (—) digestible phosphorus (dP) content of a piglet diet containing 3.63 g P/kg with different levels of phytase supplementation. Also depicted is the estimated curve according to Kornegay (2001; '1,500'-equation), after correction for the difference in the level at 0 FTU/kg (—).

was 1.1 g/kg, but the measured value was 1.83 g/kg. In earlier dose-response experiments, maximum dP generation was found at much lower phytase doses. Beers and Jongbloed (1992) found a maximum at around 1,000 FTU/kg, both for corn-soybean meal and for by-product based diets. Also the curves calculated from the experiments of Kornegay and Qian (1996) and Yi et al. (1996d) showed no further increase in P digestibility at doses higher than 1,000 to 1,500 FTU/kg feed. Based on a literature review, Düngelhoef and Rodehutscord (1995) calculated a dose-response curve and concluded that little improvement in P digestibility at doses higher than 750 FTU/kg could be expected.

For P digestibility, results similar to those in current experiment were obtained in earlier experiments investigating very high doses of microbial phytase. Harper et al. (1999) concluded that a dose of 10,000 FTU/kg continued to improve performance, bone mineralization, and mineral digestibility of grower pigs. Augspurger and Baker (2004) reported improvements of performance and bone characteristics in broilers, with phytase inclusion up to 10,000 FTU/kg, although they observed some differences between phytase sources. Shirley and Edwards (2003) performed a broiler trial with phytase doses up to 12,000 FTU/kg. They observed that performance, bone characteristics and P-retention improved with increasing phytase dose. In their study, phytate-P disappearance increased up to 85 and 95% at supplementations of 6,000 and 12,000 FTU/kg, respectively. These findings are comparable to our results, in piglets. Assuming a digestibility of 80% for non-phytate P (Jongbloed, 1987), phytate-P digestibility was 85% at 15,000 FTU/kg, compared to 15% in the basal diet.

The continuing improvement of digestibility up to very high phytase doses is intriguing, in light of earlier assumptions that the maximum effect would be realized at a dose of 1,000 to 1,500 FTU/kg. The following explanation can be hypothesized. The main site of phytase activity is the stomach (Jongbloed et al., 1992; Yi and Kornegay, 1996). This is due to several factors: the residence time of feed and phytase, pH value, grade of phytate accessibility, and grade of phytase degradation in the stomach, and the phytase and phytate characteristics. In the case of a 'mega-dose' of phytase (15,000 FTU/kg), soluble phytate is the limiting factor in the biochemical reaction (Kemme, 1998). Possibly, therefore, phytate is degraded faster or to a greater extent than is the case with 'normal' doses of about 500 FTU/kg, where soluble phytate is available in excess. This could result in a faster or better accessibility of phytate for phytase. When more phytate is hydrolyzed, there is a chance that additional phytate molecules become accessible for the enzyme to degrade. Phytase creates space for additional phytate hydrolysis. Phytate molecules that would normally not be accessible can be degraded.

Another speculation regarding the large effect at the high phytase dose is that much active phytase escapes the stomach to the small intestine. Phytase from *Aspergillus niger* has its optimal pH at 5.5. At pH 6.0 to 6.5, which is the pH in the upper half of the small intestine at 4.5 h after feeding (Van der Meulen and Bakker, 1991), the relative phytase activity at 37 °C is 35 to 80% of its maximum activity (Engelen et al., 1994). Thus phytase can still be active in the small intestine. When phytase is included at 500 FTU/kg feed, the activity in the small intestine is of limited magnitude. But in the case of a mega-dose, the time that phytase can degrade phytate may be extended because quantitatively less phytase is degraded by proteo-

lytic enzymes. This may result in the high level of phytate hydrolysis, as observed. An argument against this hypothesis could be that at the higher pH phytate can precipitate. Cheryan (1980) reviewed that phytate solubility decreases rapidly at higher pH values, because it precipitates with cations, e.g. Ca or Mg. This decrease of solubility depends on the ratio of mineral : phytate. The solubility of calcium phytate decreases rapidly at a pH above about 6. The magnesium salt precipitates at a higher pH. This indicates that in the upper small intestine, phytate is probably still available in a soluble form, and that it is available for hydrolysis by phytase. De Rham and Jost (1979) showed that phytate might be soluble at pH from 5.5 to 11. Thus the hypothesized explanation for the large effect at the high phytase dose seems feasible. It needs to be tested in experiments in which phytate degradation and phytase activity in the duodenum of piglets fed diets with a high phytase activity are measured.

Ca digestibility increased with increasing levels of phytase addition, which is in agreement with earlier reports. O'Quinn et al. (1997) found a linear increasing Ca digestibility with increasing phytase supplementation up to 1000 FTU/kg. The effect is, however, not always significant (Murry et al., 1997). In the current experiment, a constant Ca:dP ratio was realized in the diets, by adding limestone to diets that contained microbial phytase. The observed increase in Ca digestibility was, therefore, confounded with limestone level. Jongbloed et al. (1995) showed increased Ca digestibility with phytase addition to the diet of grower pigs, but the increase was smaller at a high level of dietary Ca than at a low level. Digestibility of Mg and Cu increased with phytase supplementation. This is in agreement with earlier observations in piglets (Pallauf et al., 1992a; Adeola, 1995; Kies et al., 2005), in grower pigs (Jongbloed et al., 1995) and in sows (Jongbloed et al., 2004).

The improved digestibility of Na and K with phytase supplementation is surprising. Phytase addition increased fecal digestibility of these monovalent cations up to 10%-units ($P < 0.001$). This confirms recent findings in sows (Jongbloed et al., 2004) and in piglets (Kies et al., 2005). The effect of phytase on digestibility of monovalent cations was not reported prior to those experiments. Sodium and potassium phytates are highly soluble: sodium-phytate dissolves more than 96% over the pH-range of 0.3 to 11.2 (Scheuermann et al., 1988). Probably, for this reason inhibition of Na and K-absorption by phytate has not been studied *in vivo*. Kies et al. (2005) calculated that increased absorption of minerals, and their subsequent excretion with urine, might cost energy. Increased Na and K digestibility alters the anion-cation difference, which might affect energy utilization by pigs (Dersjant-Li et al., 2002a). These processes affect energy utilization to a relatively small extent, but the effect of increased mineral digestibility needs to be considered when utilizing microbial phytase to obtain its maximal effect on animal performance.

IMPLICATIONS

Supplementation of piglet diets with microbial phytase at levels higher than 500 FTU/kg can permit degradation of phytate almost completely. In addition the digestibilities of other minerals increase further with high phytase supplementation levels. Dietary mineral inclusion levels can be reduced and the excretion of P and other minerals into manure can be decreased

further than realized presently. To take full advantage of phytase, the effect on mineral digestibility, including Na and K, should be taken into consideration. Whether it is economically advantageous to increase the phytase dose beyond 500 FTU/kg depends on the balance of these advantages and of costs and characteristics of the phytase product, and needs to be evaluated per case. It is likely that economic optimal phytase dose is higher than the present industry standard of 500 FTU/kg.

Chapter 7

Effect of phytase supplementation to a high- and a low phytate diet on the recovery of mucin in ileal digesta from growing pigs

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Submitted.

ABSTRACT

Secretion of mucin in the gastro-intestinal tract makes up a substantial part of the maintenance amino acid (AA) requirements of pigs. AA digestibility is slightly increased by phytase. It was investigated whether the recovery of mucin in ileal digesta from growing pigs (40-70 kg) was affected by dietary phytate level and by phytase supplementation. Because endogenous protein synthesis is energy demanding, the energy required for mucin production was also estimated. Eight barrows, fitted with simple T-cannulas at the distal ileum, were fed one of two basal diets with a low or a high phytate level (2.2 or 4.8 g phytate-P/kg). Both basal diets were tested with or without phytase supplementation (0 or 2,000 FTU/kg). The experiment was conducted following a repeated Latin Square design. Feeds were offered at 2.4 times requirement for ME. Ileal digesta were collected over a 36 h period, on the last three days of each of the 14-day experimental periods. Ileal mucin recovery was calculated from the glucosamine/galactosamine ratio, and from the daily ileal galactosamine output. Ileal mucin recovery was higher at the high than at the low-phytate inclusion level but this was not significant. On a mean basis, on the low phytate diet a larger proportion (69.5%) of the mucin was of gastric origin than on the high phytate diet (64%; $P < 0.01$). Without phytase, mean ileal mucin recovery was higher than with phytase administration (10.2 vs. 8.7 g/d). Gastric mucin recovery was higher without than with phytase (7.3 and 5.5 g/d), but intestinal mucin recovery was not affected. About 3.3% of the ileal AA were of mucus origin. This value was higher for serine (Ser; 8.0%) and for threonine (Thr; 12.1%). Mucin protein decreased apparent ileal AA digestibility by 0.8%-units. For Ser and Thr this was 1.9 and 3.9%-units, respectively. Of the differences in apparent ileal AA digestibility due to phytase, in most cases mucin could explain less than 10%, but for Thr this was 31%. These values were small but in all cases significant ($P < 0.01$). The mean energy costs required for mucin secretion was estimated at 180 kJ/d, or about 2% of maintenance energy requirement. No difference was observed between basal diets, but phytase supplementation resulted in a 29 kJ/d lower value ($P < 0.01$) than with the basal diets. In conclusion, phytase supplementation decreased mucin secretion. Recovery of AA with mucin and energy required for mucin secretion were both lower. These effects were significant, but of small magnitude. Mucin secretion was hardly affected by basal diet composition.

INTRODUCTION

Phytase products are used worldwide to increase phosphorus (P) digestibility of feeds for pigs and other monogastric animals. Phytase hydrolyzes phosphate groups from phytate, which is the main P store in vegetable feedstuffs. Phytate can bind cationic minerals and proteins. As a consequence, their digestibility may be hampered. In several studies, both in pigs and poultry, ileal protein digestibility improved with dietary phytase supplementation (Kies et al., 2001). The effect is often quite small, but is of practical relevance. Proteins in ileal contents of pigs are mainly of endogenous origin. They consist of undigested mucin, enzymes and desquamated cells (Auclair, 1986; Nyachoti et al., 1997). Improved protein digestibility by phytase is possibly, therefore, a result of a decreased endogenous protein recovery. Compared to feeding glucose only, feeding phytic acid to precision-fed broilers increased endogenous protein loss (Cowieson et al., 2004). Addition of phytase reduced these protein losses. Phytate increased the excretion of sialic acid, an acidic sugar found in mucin (Mantle and Allen, 1981). It was concluded, therefore, that a large part of the endogenous proteins lost was of mucus origin (Cowieson et al., 2004). Mucin can make up a substantial proportion of endogenous protein, though it varies with feed characteristics (Lien et al., 1997, 2001).

The objective of this study was to determine the recovery of mucin in ileal digesta from growing pigs fed high and low phytate diets, with or without phytase supplementation. It was also an objective to estimate the impact of a possible difference in mucin production on ileal protein digestibility, and the energy costs related to mucin production.

MATERIALS AND METHODS

The animals used in this study were cared for in accordance with the guidelines established by the CCAC (1993), and the Faculty of Agriculture, Forestry and Home Economics Animal Care Committee of the University of Alberta approved the experimental proposal.

Animals, diets and experimental procedures Eight Genex F2 (Large White × Landrace) barrows with an initial BW of 25.3 ± 0.4 kg (mean ± SEM) were obtained from the University of Alberta Swine Research Unit. The pigs were housed individually in stainless steel metabolic crates in a temperature-controlled (20-22°C) room. Following a 14 d adjustment period they were fitted with a simple T-cannula at the distal ileum, about 5 cm from the ileo-cecal sphincter. The surgical procedures were adapted from Sauer et al. (1983). Preparation of the cannula was according to De Lange et al. (1989). Pre- and post-operative care was as described by Li et al. (1994).

Following a 7 d recuperation period after surgery, barrows were fed four experimental diets according to a repeated 4 × 4 Latin Square design (n = 8). The dietary treatments were basal diet (low or high phytate level; **Table 1**), either supplemented with phytase (2,000 FTU/kg), or not (0 FTU/kg). One FTU is defined as the phytase activity that liberates 1 µmol orthophosphate from 5.1 mM sodium phytate per minute at 37°C and pH 5.5 (Engelen et al., 1994). Natuphos® Granulate (obtained from DSM Food Specialties, Delft, The Netherlands) was used as the phytase source. Chromic oxide was used as a digestibility marker. Pigs were fed

Table 1. Formulation (g/kg) and nutrient contents of the experimental diets

	Phytate level	
	Low	High
Ingredient		
Corn	321	295
Barley	160	150
Wheat	50	50
Corn starch	150	0
Soybean meal	250	190
Canola meal	20	50
Rice bran	20	200
Canola oil	4.0	42
Mono-dicalcium phosphate[1]	6.0	3.3
Limestone	10.2	11.0
Salt	3.6	3.5
Lysine-HCl	0.0	0.4
Choline chloride[2]	0.5	0.5
Vitamin premix[3]	1.0	1.0
Mineral premix[4]	1.0	1.0
Chromic oxide	2.5	2.5
Microbial phytase (FTU/kg)	0 / 2,000[5]	0 / 2,000[6]
Nutrient contents[7]		
Dry matter (DM)	883	896
Crude protein (CP)	180	188
Neutral detergent fiber (NDF)	195	263
Metabolizable energy (ME[h]; MJ/kg)	13.7	13.7
Calcium (Ca)	8.5	13.1
Total P	5.1	7.7
Available P[8]	2.3	2.3
Phytate-P	2.2	4.8
Lysine	9.1	9.7

[1] Contained P, 21.0%; Ca, 15.0%; F, 2.1 g/kg and Fe, 9.0 g/kg.
[2] Contained 60% choline chloride.
[3] The vitamin premix provided (per kg of diet): vitamin A, 12,000 IU; vitamin D_3, 1,500 IU; vitamin E, 50 IU; vitamin K_3, 1.5 mg; riboflavin, 5.5 mg; niacin, 25 mg; pantothenic acid, 15 mg and vitamin B_{12}, 0.02 mg.
[4] The mineral premix provided (per kilogram of diet): Fe, 135 mg; Zn, 135 mg; Mn, 40 mg; Cu, 20 mg; I, 0.5 mg; Co, 0.5 mg and Se, 0.3 mg, as ferrous sulfate, zinc carbonate, manganese sulfate, copper sulfate, potassium iodate, cobalt sulfate and sodium selenite, respectively.
[5] Analyzed: 120 and 1,910 FTU/kg.
[6] Analyzed: 140 and 1,740 FTU/kg.
[7] Analyzed (g/kg, unless mentioned otherwise).
[8] Calculated (NRC, 1998).

the diets at a rate of 2.4 × maintenance requirements for metabolizable energy (i.e., 418 kJ/$kg^{0.75}$), based on their mean BW at initiation of each 14 d experimental period. Pigs were weighed then and at the conclusion of the experiment. The total daily allowances were offered in two meals of equal amounts at 0800 and 2000 h. Water was mixed with the feed (as mash) at a rate of 2.5 to 1.

Ileal digesta were collected according to the procedure of Li et al. (1994) for 36 h, from 0800 to 2000 h on days 12, 13 and 14. Digesta were frozen at –28°C immediately following collection. Samples were pooled and then freeze-dried.

Analytical procedures Diets, ingredients and ileal digesta were ground through a 0.5-mm screen. Analytical procedures were as described by Liao et al. (2005b). Crude mucin was isolated by ethanol precipitation of soluble ileum contents, as described by Lien et al. (1997). Freeze-dried crude mucin was analyzed for glucosamine and galactosamine. In crude mucin and in freeze-dried ileal digesta, these carbohydrates were analyzed as their alditol acetates and measured by gas-liquid chromatography (Lien et al., 1997). Analyses were performed in duplicate.

Calculations and statistical analyses The daily ileal DM flow was calculated from DM intake and apparent ileal DM digestibility, assuming that DM digestibility during digesta collection time (0800-2000 h) is equal to that during the period that digesta were not collected. Apparent ileal digestibility of DM in the experimental diets was determined using the equation:

$$D_D = 100\% - [(I_D \times A_F) / (A_D \times I_F)] \times 100\%$$

where D_D is the apparent ileal DM digestibility in the test diet (%), I_D and I_F are the chromic oxide concentrations (g/kg) in the diet and in ileal digesta, respectively, and A_F and A_D are the DM concentrations (g/kg) in ileal digesta and in the diet, respectively.

The ratios of N-acetylglucosamine (GlcNAc) to N-acetylgalactosamine (GalNAc) are different in gastric than in intestinal mucin (Scawen and Allen, 1977; Mantle and Allen, 1981; Mantle et al., 1981). This was used to calculate the relative part of mucin, from these different origins, in the ileal mucin. Regression equations were derived from GlcNAc : GalNAc ratios in purified gastric (Scawen and Allen, 1977) and intestinal (Mantle and Allen, 1981; Mantle et al., 1981) mucins (Lien et al., 1997). Ileal mucin recovery and the gastric mucin part of it were calculated assuming that the mucins were not proteolytically degraded. Daily ileal mucin output was calculated according to Lien et al. (1997) as:

$$\text{mucin output (g/d)} = \text{GalNAc} / \%\text{GalNAc}$$

with GalNAc = ileal GalNAc output in g/d, and

$$\%\text{GalNAc} = 32.30 - 22.74x + 8.83x^2 - 1.37x^3$$

where %GalNAc = GalNAc content of mucin mixtures, and x = the GlcNAc/GalNAc ratio in crude mucin.

The contribution of gastric mucin in the ileal mucin mixture was calculated as:

$$\%\text{ gastric mucin} = -80.23 + 183.26x - 71.19x^2 + 11.05x^3$$

where x = the GlcNAc/GalNAc ratio in crude mucin, also according to Lien et al. (1997).

Data were subjected to statistical analysis for a repeated Latin Square design using the General Linear Model Procedure of SAS (Version 6.03; SAS Inst. Inc., Cary, NC, USA). The main effects of diets ($n = 4$), pigs ($n = 8$) and periods ($n = 4$) were included in the model. Effects of diet and phytase supplementation were tested by orthogonal contrasts.

RESULTS AND DISCUSSION

All pigs remained healthy and usually consumed their meal allowances within 30 min after feeding. The experiment covered the BW period of 40.6 to 69.8 kg. Postmortem examinations conducted at the conclusion of the experiment revealed no intestinal adhesions or any other abnormalities. Feed intake (as is) was 1,175, 1,256, 1,436 and 1,590 g/d, in the periods 1 through 4, respectively.

For calculation of the AA and carbohydrate recovery, the composition of gastric mucin, as reported by Scawen and Allen (1977), was used and of intestinal mucin as reported by Mantle and Allen (1981). These calculations are based on the following assumptions: constant mucin composition (production vs. ileal passage); mucins are in their native form (thus no degradation); and the relative proportion of gastric and intestinal mucins is similar in soluble and insoluble fractions (Lien et al., 1997).

Dietary treatment significantly affected ileal recoveries of crude mucin, gastric mucin and intestinal mucin (g/d), and the percentage of gastric mucin (**Table 2**). On the low phytate diet, recovery of crude mucin was numerically lower than at the high phytate diet. The relative part of gastric mucin was higher at the low phytate than at the high phytate level ($P < 0.01$). Gastric mucin recovery (g/d) was comparable for the two diets, but intestinal mucin recovery was higher at the high phytate level ($P < 0.001$).

Phytase decreased mucin production significantly (Table 2). This seems contradictory to the absence of an effect between the low vs. high phytate diets. An explanation may be found in the difference in feed composition between the two basal diets, thus in a difference in dietary content of components other than phytate. These compounds may also affect mucin production. Decreasing mucin production with phytase supplementation would suggest an increase by phytate. Dietary phytate level of the high phytate diet was mainly increased by inclusion of rice bran. Thus, a compound of rice bran would decrease mucin production. Rice bran contains a high level of fiber. Fiber, however, is related to a higher mucin production (Lien et al., 2001). Lectins or tannins may increase mucus production (Lien et al., 2001), but rice bran is not a known source of these components (Farrell, 1994). Thus, it cannot be explained that there is no difference in mucin production between basal diets.

Table 2. Recovery of mucin (total, and of gastric and intestinal origin) in ileal digesta of pigs (40-70 kg) fed low or high phytate diets, supplemented or not with microbial phytase

Diets	Low phytate		High phytate		SEM[1]	*P*-value	P_D[2]	P_P[3]
Phytase, *FTU/kg*	0	2,000	0	2,000				
Crude mucin, *g/d*	9.61	8.59	10.71	8.72	0.45	< 0.02	0.19	< 0.01
Gastric mucin, *%*	74.1	64.9	67.0	60.7	1.9	0.001	< 0.01	< 0.001
Gastric mucin, *g/d*	7.34	5.60	7.17	5.36	0.52	< 0.03	0.69	< 0.01
Intestinal mucin, *g/d*	2.27	2.98	3.54	3.36	0.20	< 0.01	< 0.001	0.20

[1] Standard error of the means (n= 8).
[2] Orthogonal contrast *P*-values: effect of diet.
[3] Orthogonal contrast *P*-values: effect of phytase supplementation.

The lower recovery of crude mucin with phytase supplementation was the result of a lower recovery of gastric mucin ($P < 0.01$); no difference appeared in the recovery of intestinal mucin. Because gastric mucin is virtually indigestible (Lien et al., 2001), gastric mucin production was lower in the pigs receiving phytase. Both phytate and mucin are negatively charged under gastro-intestinal conditions (Bebot-Brigaud et al., 1999; Dekker, 1990). Therefore, it is not likely that a large quantity of mucin binds directly to phytate. Also, mucin contains little of the basic AA that can form binary complexes with phytate (Cheryan, 1980). But phytate can bind pepsin (Camus and Laporte, 1976). Glycoprotein output of the stomach is positively correlated to the pepsin concentration (Allen et al., 1980; Munster et al., 1987). When more pepsin is bound to phytate a negative feedback mechanism may stimulate pepsin secretion. Subsequently, mucin production may be stimulated. Phytase hydrolyses phytate to inositol phosphates with less than six phosphate groups, which have a lower ability to bind pepsin (Knuckels et al., 1989). This may explain the lower production of gastric mucin with phytase.

In the current experiment, ileal protein digestibility was also measured (reported by Liao et al., 2005b). Intestinal glycoprotein secretion may make up a substantial part of maintenance AA needs (Van der Schoot et al., 2002). Differences in ileal digestibility could, therefore, be explained by mucin production. Based on the AA composition of gastric and intestinal mucins (Scawen and Allen, 1977; Mantle and Allen, 1981) it was calculated that about 3.3% of the ileal digesta AA are of mucus origin. This value is higher for Ser (8.0%) and for Thr (12.1%), both major AA in mucin (Scawen and Allen, 1977; Mantle and Allen, 1981; Lien et al., 1997). This indicates that a large part of ileal digesta protein originates from mucin, which agrees with Van der Schoot et al. (2002). Pro, another major AA of mucin, was not analyzed in ileal digesta in the present experiment. In **Table 3** an example is given of the calculated effect of mucin on the ileal digestibility of threonine.

On the low phytate diet, digestibility of CP and of all AA was higher than on the high phytate diet. The mean difference was 3.1%-units (in most cases $P < 0.05$; Liao et al., 2005b). By correcting the ileal digesta protein quantity for mucin-protein, the "mucin-free" CP digestibility can be calculated, and subsequently the dietary effect on this corrected digestibility. On average, mucin affected apparent ileal AA digestibility negatively by 0.8%-units, but it was higher for Ser (1.9%) and for Thr (3.9%). Only very small differences were observed between the low- and high phytate diets (maximum 0.3% for Thr).

Phytase supplementation improved the digestibility of CP and of AA by 1.0%-units (mean value), which was, for most individual AA, not statistically significant (Liao et al., 2005b). To obtain an indication whether the lower mucin production with the phytase treatment explains part of this effect, we performed the same calculations as for the basal diets. Lower mucin-protein output resulted in a slightly higher apparent digestibility (mean difference 0.1%-unit) on the phytase treatment. The maximum difference was observed for Thr (+ 0.4%) and the smallest effect for Leu (+ 0.04%-unit). An example of the calculation for Thr is given in Table 3. Despite the small, calculated differences due to phytase supplementation, they were significant for CP and for all individual AA ($P < 0.01$). With phytase, ileal Thr digestibility was (not-significantly) higher than without phytase (68.3 vs. 67.0; Liao et al., 2005b). Lower

Table 3. An example of the calculation of the effect of mucin on threonine (Thr) passage and digestibility[1]

Parameter	Measured value	Calculated value
Feed intake, *g/d*	1436	
Thr content feed, *%*	0.66	
Thr intake, *g/d*		9.48
Ileal Thr passage, *g/d*		2.96
Ileal Thr digestibility, *%*		68.77
Ileal mucin		
Gastric, *g/d*	8.27	
Intestinal, *g/d*	2.57	
Mucin Thr[2]		
Gastric, *mg/d*		233.1
Intestinal, *mg/d*		142.8
Total, *mg/d*		375.9
Ileal Thr_{mucin}/Thr_{total}, *%*		12.7[3]
Ileal Thr - Thr_{mucus}, *g/d*	2.58	
"Mucus-corrected" Thr digestibility, *%*		72.78
Difference ileal Thr digestibility -"Mucus-corrected" Thr digestibility, *%*		4.01[4,5]

[1] The pig received the high-phytate diet without microbial phytase; experimental period 3.
[2] Assumptions: gastric mucin contains 15.4% protein; content of Thr in protein is 18.3%. Intestinal mucin con-tains 21.2% protein; content of Thr in protein is 26.2%.
[3] Mean value for all treatments: 12.1%.
[4] Mean value for all treatments: 3.9%.
[5] Mean values for treatments without and with phytase: 4.10 and 3.68%. The difference is 0.42%-units, or 31% of the difference in digestibility between these treatments.

mucin production explained 31% of this difference. A similar calculation was done for the other AA. In most cases, AA recovery with mucin explained less than 10% of the difference in ileal digestibility. The rest may be partly explained by the dissolution of complexes of phytate with other proteins, including endogenous proteins. It has been shown that phytate can bind amylase (Knuckels and Betschart, 1987), pepsin (Camus and Laporte, 1976), trypsin (Singh and Krikorian, 1982) and lipase (Knuckels, 1988). In the present experiment, production of these endogenous proteins was not measured.

Endogenous protein production is a highly energy-demanding process, as described by Nyachoti et al. (1997). These authors calculated that energy required for gut protein synthesis increased from 174 to 414 kJ/d, when piglets (13 kg BW) were fed a diet containing no or a high level of trypsin inhibitors, respectively. This was the equivalent of 6 and 13% of the energy required for maintenance, respectively. Using the approach of Nyachoti et al. (1997), the energy involved in mucin production in the current experiment can be estimated. Based on the composition of gastric and intestinal mucins (Scawen and Allen, 1977; Mantle and Allen, 1981), their energy content was estimated. Including the energy required for synthesis of the sugar derivates sialic acid, glucosamine and galactosamine, which are present in relatively

high levels in mucin (Berg et al., 2002), but excluding energy required for synthesis of protein, the energy content is 18.4 kJ/g. Energy content of gastric and intestinal mucin differed little, thus the mean value was taken. Protein contents of mucin were assumed to be 15.4 and 21.2% for gastric and intestinal mucin, respectively. The energy cost for protein synthesis was estimated to be 4.5 kJ/g (Webster, 1981). The estimated energy content of mucin and requirement for synthesis of mucin protein were 184, 165, 205 and 167 kJ/d for the low phytate diets (without and with phytase) and high phytate diets (without and with phytase), respectively. On average, the energy required for ileally-recovered mucin is 2% of the energy requirement for maintenance, for a 55-kg pig. The difference between the low phytate (174 kJ/d) and high phytate diets (186 kJ/d) was not significant. Without phytase supplementation, the energy cost related to mucin production (195 kJ/d) was higher ($P < 0.01$) than when phytase was supplemented (166 kJ/d). This difference (29 kJ/d) is about 0.3% of the energy required for maintenance. There was no phytate × phytase interaction.

In conclusion, dietary phytase supplementation decreased the amount of mucin synthesized, and recovered in ileal digesta. Differences in apparent ileal AA digestibility were significantly related to mucin protein, but their role was in most cases small. For Thr, 31% of the observed increased ileal digestibility by phytase could be attributed to a lower recovery of mucin. With phytase supplementation, energy required for mucin production was 29 kJ/d lower than for the control. This is about 0.3% of maintenance energy requirement. Basal diet composition had no effect on these parameters. In the current experiment, only the recovery of mucin was measured. None of other endogenous proteins were investigated. A high dietary phytate level might enhance the secretion of endogenous proteins other than mucin. To determine the effect of phytate and of phytase on endogenous production of protein, more research is needed.

IMPLICATIONS

Phytase supplementation reduced mucin secretion. The difference could explain a small, though significant part of ileal AA recovery. Estimated energy required for mucin secretion was significantly reduced by phytase. By reducing mucin secretion, phytase may improve apparent ileal AA digestibility and reduce energy requirement. The magnitude of these effects was, however, small. Of the frequently observed improvement in ileal AA digestibilities, the main part is probably due to a lower secretion of other endogenous proteins. This effect requires further investigation.

General discussion

INTRODUCTION

Recently, the positive effect of phytase on amino acid digestibility has been disputed in a number of papers (Peter et al., 2000; Peter and Baker, 2001; Adeola and Sands, 2003). In this final chapter, the results presented in Chapters 2 and 3 will be evaluated with regard to these authors' criticisms. Also, the physiological basis for the phytase effect on protein digestibility will be further discussed, based on the *in vitro* experiments of Chapter 1. In Chapters 4 and 5, indications of some impact of phytase on energy metabolism were obtained. In this general discussion, the physiological basis for such an effect will be addressed. Conclusions from Chapters 6 and 7 are also part of this discussion. The effect of phytate, and thus of phytase, must be explainable by the chemical interactions between phytate on one side, and nutrients and endogenous compounds on the other side. Therefore, a short link will be made to physical chemistry, before ending this chapter with conclusions and ideas for future research.

PHYTASE AND PROTEIN DIGESTIBILITY – QUANTITATIVE ASPECTS

The impact of phytase on ileal amino acid digestibility shows large variation. In particular Officer and Batterham (1992a) measured a large positive effect. These authors investigated, however, a rather extreme diet: they studied amino acid digestibility in growing pigs fed a sugar-based diet containing 40% Linola™ meal. Linola meal is the solvent-extracted meal from a linseed cultivar with a low linolenic acid content, having a rather poor feeding value (Batterham et al., 1991). The large improvement of ileal protein digestibility they observed with phytase (12%-units for both crude protein and lysine) indicated that phytase could improve protein digestibility *in vivo*. Effects of similar magnitude were not observed in subsequent experiments, when practical diets were used in the experiments. But in most of the studies, phytase increased digestibility (Chapters 2 and 3).

Peter et al. (2000) and Adeola and Sands (2003) dispute this positive effect. The conclusion of Peter et al. (2000), and other reports from the same laboratory, is based on Protein Efficiency Ratio (PER) studies. Adeola and Sands (2003) discussed ileal amino acid digestibility studies. First we will look at the PER studies.

Protein Efficiency Ratio (PER) studies In the PER studies, protein utilization was measured in young chicks. PER is the ratio of body weight gain to protein consumption. Diets must be limiting in protein content. In the studies of Peter et al. (2000), Peter and Baker (2001), Boling-Frankenbach et al. (2001) and Augspurger and Baker (2004), a feedstuff was added to a starch-sugar based diet, without or with supplementation of phytase. Most studies were performed using 4 pens containing 4 chicks each, from 8 to 17-21 days of age. Usually, the diets contained 10% crude protein. In most experiments, consequence of the low protein content was a low dietary phytate level. Phytate-P content was below 1.5 g/kg feed, with the exception of some of the treatments of Boling-Frankenbach et al. (2001). In Chapter 1, it was shown that protein complexes with phytate at low pH, if the ratio of protein to phytate is about 10-20 : 1, maximally. This is much lower than the ratio in most of the PER experiments. For piglets, it has been shown that phytase does not affect performance of piglets, when the

diet contains a phytate level below about 1.6 g/kg (**Figure 1**). The diets with higher phytate content tested by Boling-Frankenbach et al. (2001), were rather extreme: they contained, e.g., 23% cottonseed meal, 70% wheat bran, or 75% rice bran. The authors did not discuss the impact of such extreme ingredient composition on growth and protein utilization by young chicks, but such an effect may not be excluded. The methodology applied in these PER experiments probably prohibits finding a positive effect of phytase on protein utilization.

Interestingly, phytase supplementation improved the PER in a number of diets without phytate (casein and meat and bone meal diets). In **Figure 2**, the results of the PER studies are given relative to the phytate level. Peter et al. (2000) suggested that the positive effect of phytase is independent of the phytate moiety. This is extremely unlikely, however, because phytase is a highly specific enzyme for the hydrolysis of phosphates, especially from phytate (Misset, 2003). It does not degrade proteins (Chapter 1 of this thesis). Also, casein is highly digestible: measured apparent and true lysine digestibilities in piglets were 98 and 100%, respectively (Kies et al., 1986). There is no room for improvement with phytase supplementation.

Peter et al. (2000) observed a decreased PER with phytase in a corn gluten meal (CGM) diet. They suggested that phytase liberated amino acids from CGM, but none or little of the lysine, the most limiting one. This would result in an even more imbalanced digestible amino acid profile, with negative consequences for growth and protein utilization. The increase of amino acid (AA) digestibility by phytase seems, however, quite general and not AA-specific (Ravindran et al., 1999a; Chapters 2 and 3 of this thesis). Also CGM has little room for improvement in protein digestibility; apparent amino acid digestibility in poultry is about 95% (CVB, 2000). Furthermore, it is unlikely that a 1-2% improvement in protein digestibility will show a clear improvement in growth of chickens fed a diet containing about 10% protein. The PER method appears unsuitable to test the effect of phytase on protein utilization.

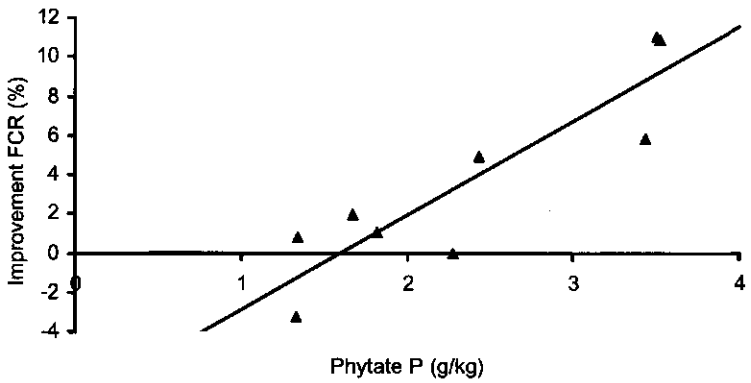

Figure 1. Improvement of feed conversion ratio of piglets (% change compared to control diets) by dietary phytase supplementation, dependent on the dietary phytate level (Kies et al., 1997).

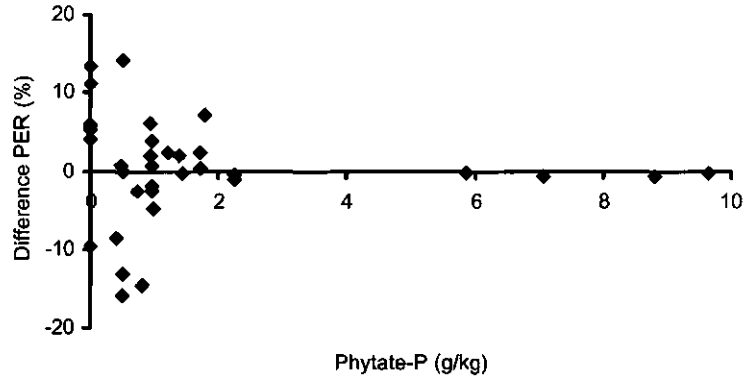

Figure 2. Relative difference (%) in Protein Efficiency Ratio (PER) between diets with and without phytase supplementation, dependent on the dietary phytate level (Peter et al., 2000; Peter and Baker, 2001; Boling-Frankenbach et al., 2001; Augspurger and Baker, 2004). Phytate levels are calculated from CVB (2000). Phytase was supplemented at 1200 FTU/kg, except by Augspurger and Baker (2004); their 500 FTU/kg-results are included in the figure.

Amino acid digestibility studies Based on ileal amino acid digestibility studies, Adeola and Sands (2003) concluded that phytase does not improve protein utilization in pigs and poultry. Since 2000 (when the calculations for Chapter 3 were made), a limited number of new papers appeared describing the impact of phytase on ileal amino acid digestibility. In poultry, new information from Rutherfurd (2002, 2004a) and Dilger et al. (2004) became available. Rutherfurd et al. (2002) measured a large increase in true ileal amino acid digestibilities. In the experiments of Dilger et al. (2004) and Rutherfurd (2004a), phytase improved apparent ileal amino acid digestibility to a similar extent to that found in earlier experiments. The mean apparent ileal lysine digestibility in broilers (taken as an example) increased from 80.4 to 82.6% with phytase supplementation, including the new studies, and from 80.3 to 82.4% without them. The interpretation of the phytase effect seems not influenced by more recent results, compared to the findings in Chapter 3.

Also in pigs new results have become available: Traylor et al. (2001), Sands (2002), Rice (2002), Omogbenigun et al. (2003), Johnston et al. (2004) and Liao et al. (2005a,b). Without the results given in these reports (only using the references given in Chapter 3), the apparent ileal lysine digestibility increased from 79.4 to 81.4% with phytase supplementation. Including these new reports, it increased from 79.2 to 80.6%. The mean effect of phytase was thus somewhat lower than when based only on the earlier experiments.

In most experiments, both in broilers and pigs, dietary phytate levels were higher than 1.6 g/kg. An exception is the experiment of Valaja et al. (1998), in which a diet containing no phytate was used. Their findings were therefore excluded from the calculations in Chapter 3.

There are three possible explanations for the discrepancy in the conclusions of Adeola and Sands (2003) when compared with this report. Firstly, they used a limited number of experiments to derive their conclusions. The calculations in Chapter 3 were based on more results,

which were selected only on a few criteria (e.g. full report available). Secondly, Sands (2002) measured a mean effect of phytase of -0.5 and -1.2%-units in low and high-phytate diets, respectively, on apparent ileal lysine digestibility in pigs. The 1.2% decrease with the high-phytate diet is not according to expectations, and might give reason for a lack of confidence in a positive action of phytase. Also Liao et al. (2005b) measured a smaller (positive) effect in pigs with a high than at a low-phytate diet. In poultry, however, Ravindran et al. (2000) measured a larger increased amino acid digestibility with phytase in broilers at a high rather than at a low phytate level. This aspect is thus somewhat confused. Thirdly, in most experiments the improvement of digestibility did not reach statistical significance, and was therefore interpreted by Adeola and Sands (2003) to have no effect. However, with the inclusion of all results in a meta-analysis, including numerical effects, significant improvements were observed for the digestibility of most amino acids, as shown in Chapter 3.

PHYTASE AND PROTEIN DIGESTIBILITY – PHYSIOLOGICAL ASPECTS

Four theories have been postulated to explain an effect of phytase on protein digestibility (Chapter 3):

1. Phytate-protein complexes being naturally present in feedstuffs.
2. *De novo* formation of phytate – dietary protein complexes in the digestive tract.
3. Formation of complexes of phytate and free amino acids in the digestive tract.
4. Formation of complexes of phytate and endogenous protein (especially digestive (proteolytic) enzymes) in the digestive tract.

Natural phytate-protein complexes From Chapter 1 it appears unlikely that, in the feedstuffs tested, naturally occurring protein-phytate complexes are a major issue, at least for the soluble protein and phytate parts. This is in agreement with the findings of Reddy (2002), who showed that globoid crystals, that contain most of the phytate in many feedstuffs, often contain only a small amount of protein.

De novo phytate-protein complexing That large protein-phytate complexes are (*de novo*) formed under acidic conditions, in the stomach of pigs and the proventriculus and gizzard of poultry (Chapter 1), is more likely. According to the results of our *in vitro* experiments, 1% dietary phytate could potentially complex with about all dietary protein.

Under acidic conditions, proteins can complex directly with phytate via the positively charged amino groups in the protein. These positively charged amino groups may be the α-NH_2-terminal groups of protein, or positively charged (amino) groups of lysine, arginine and histidine. Binding is in this order, according to Lásztity and Lásztity (1990). Consequently, it was expected that phytase would increase the digestibility of these basic amino acids to a larger extent than that of other amino acids (Zhang, 1999). From the lack of a larger increase in lysine digestibility compared to the other amino acids, Biehl and Baker (1997) concluded that the phytase effect may be unrelated to the release of protein-bound amino acids from the phytate complex. From Chapter 1 it appears that phytase does not hydrolyze protein. Protein

is released from the phytate complex, which may enable proteolytic enzymes to degrade the protein faster. Although the peptides that are bound to phytate probably contain a higher level of basic amino acids, the complex includes all amino acids, whose digestibility may be reduced. The lack of a clear effect of basic amino acid contents of feedstuffs compared to the effect of phytase on lysine digestibility is evident from **Figure 3**. A higher proportion of basic amino acids in the protein did not affect the response positively.

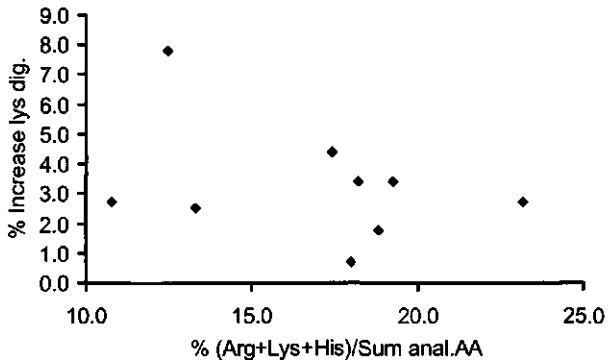

Figure 3. The increase in apparent ileal lysine digestibility due to phytase, dependent on the proportion of basic amino acids in the protein (sum of analyzed AA; Ravindran et al., 1999a).

Complexing of phytate and free amino acids The third possible explanation for the positive phytase effect is related to the binding of free amino acids by phytate, or phytate-rich feedstuffs (Rutherfurd et al., 1997, 1998, 2004b). These authors showed that solubility of free lysine, methionine, threonine and tryptophan decreased when they were mixed with different feedstuffs. The extent of binding varies between amino acids and between feedstuffs. Binding may be both to phytate and to other components of the feedstuffs. Partial release of amino acids after incubation with phytase indicates that they are in part bound to phytate. In digestibility studies the effect of phytase on amino acids that are supplemented in a free form to diets is not larger than the effect on other amino acids. So there seems to be no specificity of phytase for any amino acid. Prior to intestinal absorption, proteins may be degraded to free amino acids, mainly in the brush-border area of enterocytes (Argenzio, 1993c). It is unlikely that these free amino acids are bound to phytate at this level, and subsequently released due to hydrolysis by phytase. In practical diets, therefore, release of phytate-bound free amino acids by phytase action seems to be of a limited quantitative order.

Complexing of phytate and endogenous proteins The fourth mechanism that may explain an effect of phytase on protein digestibility is the complexing of endogenous proteins, especially digestive enzymes, to phytate. This could reduce protein digestibility in two ways. Firstly, protein is complexed to phytate, making it less accessible for proteolytic hydrolysis. Secondly, if the complexed protein itself is a proteolytic enzyme, the digestive capacity may be reduced. Endogenous proteins make up the largest part of protein recovered at the distal ileum of animals, thus it will affect ileal protein digestibility strongly. It consists of undigested

mucin, enzymes and desquamated cells (Auclair, 1986; Nyachoti et al., 1997). Complexing of desquamated cell proteins to phytate has not been specifically investigated. They behave similarly to dietary proteins, which complex to phytate under acidic conditions (Chapter 1). Moreover, most of the desquamated cells will originate from the small intestine, where pH is 6 to 7. At this pH, protein-phytate complexing is of little quantitative importance (Chapter 1).

Digestive enzymes that are able to complex to phytate include pepsin (Camus and Laporte, 1976), amylase (Knuckels and Betschart, 1987), trypsin (Singh and Krikorian, 1982) and lipase (Knuckels, 1988). When phytate is, in part, degraded, it loses the ability to complex to proteins, as shown with pepsin (Knuckels et al., 1989) and cationic minerals (Sandberg et al., 1989). The increase in protein digestibility with phytase may be the result of reduced complexing with endogenous proteins.

Cowieson et al. (2004) showed that excretion of endogenous protein increased by feeding phytic acid to broilers. This excretion was reduced almost to the basal level when phytase was added. The authors also measured a higher recovery of sialic acid when phytate was fed, which was reduced almost to the basal level by addition of phytase (Cowieson et al., 2004). Sialic acid is an acid sugar that is part of mucins (Mantle and Allen, 1981). These results show that phytase prevented an increase in the production of mucin. In the experiment described in Chapter 7, phytase reduced the ileal recovery of mucin also. It is not likely that phytate complexes with mucin, because both substances are negatively charged (Dekker, 1990; Bebot-Brigaud et al., 1999). Also, mucin contains a relatively small amount of basic amino acids (Scawen and Allen, 1977; Mantle and Allen, 1981). There might, however, be an indirect effect.

Mucin production in the stomach is positively correlated to the pepsin concentration (Allen, 1981; Munster et al., 1987). Munster et al. (1987) investigated the increased production of gastric mucin by pepsin quantitatively, in rats. Assuming that the rat and pig are comparable on this point, and that the difference in gastric mucin production is only due to a difference in the production of pepsin, then the effect of phytase on pepsin production can be estimated. The production of gastric mucin glycoproteins increased 600 µg with 4.2 mg pepsin (Munster et al., 1987). In the experiment described in Chapter 7, pigs produced 5.48 and 7.25 g gastric mucin/d with and without phytase, respectively. This would be equivalent to the production of 38.5 and 50.9 g pepsin/d (Munster et al., 1987). The effect this will have on ileal amino acid digestibility can now be estimated, e.g. for threonine. Pepsin contains 9.5% threonine (Blumenfeld and Perlmann, 1959). Assuming an ileal digestibility of endogenous proteins of 75% (Nyachoti et al., 1997), ileal threonine (of mucin + pepsin origin) recovery would be 1.24 and 1.57g/d, with and without phytase, respectively. This would be a relative reduction in endogenous threonine of 21%. Cowieson et al. (2004) measured a relative reduction in threonine of endogenous origin of about 8%.

The potential amount of ileal threonine recovery due to mucus and residual pepsin proteins could explain a difference (due to phytase) in ileal threonine digestibility of 2.5%-units. The measured difference (Chapter 7) was 1.3%. Thus, the reduction of pepsin and, consequently, of gastric mucin production with dietary phytase, can explain the measured difference in digestibility of threonine.

PHYTASE AND APPARENT METABOLIZABLE ENERGY IN POULTRY

Rojas and Scott (1969) were the first to show that microbial phytase could improve the metabolizable energy (ME) content of feedstuffs for broilers. They investigated cottonseed meal, which was pre-treated with a crude fermentation broth of *A. niger* (*ficuum*). Depending on the type of cottonseed meal, ME increased up to 32% after phytase incubation. Also, in soybean meal they measured an improvement, of up to 11%. In more practical diets, studied more recently (Chapters 2 and 3), the effects were not as large as those measured by Rojas and Scott (1969). In Chapter 3, an improvement in AME of 222 kJ/kg was calculated, for an application of 500 FTU phytase/kg of feed. This is about 1.5 to 2% of the energy content of a typical broiler diet. Energy losses in excreta are, thus, reduced by 1.5 to 2% (relative to the gross energy content of the feed).

Do these values agree with the results of digestibility studies? The effect of phytase supplementation on protein digestibility was measured in a number of studies, but results on the digestibility of fat and carbohydrates are scarce. Camden et al. (2001) measured ileal protein, fat and starch digestibility. Depending on source and dietary concentration, phytase supplementation increased ileal digestibility of protein, fat and starch by 1.5-3, 3-3.5 and 1-1.5%-units, respectively. Using ME-energy factors (De Groote, 1999), the mean energy level of the corn-soybean meal diet used in the experiment (Camden et al., 2001) increased with 231 kJ/kg. Ileal digestibility, however, does not take the energy value of post ileal fermentation products into account. Camden et al. (2001) measured ileal digestible energy and AME. These values increased with 260 and 170 kJ/kg (mean of phytase treatments), respectively. The improvement of the AME value by phytase could be explained by the increased digestibility.

Ileal protein, fat and fiber digestibility were also measured in an, as yet unreported, experiment (A. Kies, V. Ravindran and W. Hendriks; Massey University, Palmerston North, New Zealand). In this experiment, in broilers (7 to 28 d), corn-wheat-soybean meal based diets were fed. Also included was 5 or 15% rice bran. Diets were calculated to contain similar levels of AME and digestible amino acids, and were designed to be not limiting in digestible P (CVB, 2002). Broilers on the low rice bran diet performed better than those on the high rice bran diet. Without phytase, weight gain was 1214 g/bird and feed conversion ratio 1.55 on the low rice bran diet, and 924 and 1.70, respectively, on the high rice bran diet. Some of the effects with phytase (*A. niger*; Natuphos®) supplementation (500, 750, or 1000 FTU/kg) are summarized in **Table 1**.

Crude fiber digestibility was negative in nearly all cases, but positively affected by phytase. Dietary crude fiber levels were 2.5 and 3.1% for the low and high rice bran diets, respectively. AME was less improved by phytase than in earlier experiments (Chapters 2 and 3). Making a calculation to attribute the differences in protein and fat digestibility to the difference in AME, similar to the study of Camden et al. (2001), 49 and 290 kJ/kg can be explained by phytase in the low and high rice bran diets, respectively. It seems that differences in digestibility could explain a large part of the effect of phytase on AME.

Table 1. Improvement in weight gain, feed conversion, AME and ileal nutrient digestibility due to phytase addition (mean of 3 doses) to either a low (5%) or a high (15%) rice bran diet in broilers (7-28 d; Kies, Ravindran and Hendriks, unpublished)

Parameter	Low rice bran	High rice bran	P-value
Weight gain (g/bird)	+ 61	+ 44	< 0.05
Feed conversion ratio	- 0.02	- 0.03	N.S.
AME (kJ/kg)	+ 90	+ 160	N.S.
Ileal crude protein digestibility (%)	+ 1.2	+ 4.6	0.11
Ileal crude fat digestibility (%)	+ 0.1	+ 2.9	N.S.
Ileal crude fiber digestibility (%)	+ 7.2	+ 9.8	0.11

Dietary levels of calcium may affect the measured AME. In the experiment by Kies, Ravindran and Hendriks (unpublished), a treatment with decreased Ca level was also included (6.2 and 5.0 g/kg in the normal and low Ca diets, respectively). Low Ca resulted in a higher ileal protein and fat digestibility, although the effect was not significant. AME was higher at the low Ca level than on the control diet ($P = 0.07$). These results agree with earlier experiments of Ravindran et al. (2000). These authors hypothesized that at the higher Ca level insoluble phytate-Ca-fatty acid complexes are formed, resulting in lower fat digestibility, and thus a lower AME. This hypothesis has not yet been tested. That should be done by measurement of such complexes in intestinal contents. Phytase degrades phytate, preventing formation of such complexes, but also increases Ca digestibility (Chapter 3), permitting a reduction of Ca levels in the diet.

Composition of body weight gain may be affected by differences in digestibility of the diets, e.g. protein and fat, but may also be due to a difference in post-absorptive energy utilization. In broilers, Kies, Ravindran and Hendriks (unpublished) measured body composition. The energy content of gained body mass was calculated from the gain of protein and fat (energy contents 23.6 and 39.7 kJ/g, respectively). On the low rice bran diet, the mean energy content per gram of body mass gain increased 1.4% with phytase, from 8.26 to 8.38 kJ. On the high rice bran diet, the increase with phytase was 1.0%: from 8.53 to 8.60 kJ/g gain. The overall effect was significant (for rice bran, phytase inclusion and interaction: $P < 0.001$). This effect may be due to the higher availability of protein and fat, but the observation does not exclude that post-absorptive energy utilization is affected by phytase.

In conclusion, phytase improves the ileal protein and fat digestibility in poultry, thus the ileal energy digestibility and probably the AME. This effect may be due to prevention of the formation of phytate-protein and of phytate-Ca-fatty acid complexes. That phytase improves post-absorptive energy utilization cannot be excluded. This will be further discussed for pigs.

PHYTASE AND ENERGY UTILIZATION IN PIGS

In pigs, the effect of phytase on energy metabolism seems to be different from that in poultry. In broilers, phytase increases the (ileal) digestibility of protein and of fat, which can explain a large part of the effect of phytase on AME. In pigs, clear evidence for the improvement of

energy digestibility is lacking. Forty-eight measurements of the effect of phytase on energy digestibility resulted in a mean positive effect of only 0.06%-units. The experiments were performed with different diets, phytases and phytase doses, but these variables did not affect the results (Eeckhout and DePaepe, 1992a,b; Ketaren et al., 1993; Murry et al., 1997; O'Quinn et al., 1997; Gebert et al., 1999a; Radcliffe and Kornegay, 2000; Sands et al., 2001; Brady et al., 2002; Sands, 2002; Sauer et al., 2003; Adeola et al., 2004; Johnston et al., 2004; Kies et al., 2005; Liao et al, 2005a,b.).

The observed improvement in pig performance by phytase (Chapter 3) could not be fully explained by the increased digestibility of amino acids (Kies, 1998). It was, therefore, hypothesized that phytase might affect post-absorptive energy utilization in pigs. This higher energy utilization relates to lower energy losses with urine, or with a smaller amount of energy lost as heat. Maximizing the utilization of protein might reduce the urinary energy losses. Only in a few experiments has the metabolizability of energy in pigs been measured. Walz and Pallauf (2002) did not observe a consistent phytase effect. Also, in the experiments of Liao et al. (2005b) and Chapters 4 and 5, no effect of phytase on energy metabolizability was observed.

In broilers, energy content of gained mass was increased by phytase supplementation (Kies, Ravindran and Hendriks, unpublished). In pigs such an effect is not consistent in the literature (Ketaren et al., 1993; Gebert et al., 1999b; Walz and Pallauf, 2002; Shelton et al.; 2003).

In Chapters 4 and 5, the effect of phytase on heat production was investigated. No effect was observed. There were, however, a number of interesting changes in the different variables measured. In Chapter 4, in newly weaned piglets during the first two weeks of the experiment, intake of feed containing phytase was 23% higher than that of the control diet. In weaned piglets, a low feed intake results in a lower amount of secreted digestive enzymes than at a higher feed intake (Makkink, 1993). Piglets have overcome this limited enzyme production by day 10 post-weaning Makkink (1993). Binding of a part of the digestive enzymes to phytate reduces the digestive capacity of the piglet further. In Chapter 7, it was shown that phytase reduces the production of gastric mucin. It was hypothesized that this is due to an indirect effect, via the reduction of pepsin production. Probably, phytase aids the piglet post weaning to adapt to solid feed, and to utilize their digestive potential better. Two weeks post-weaning piglets adapt to their new feed anyway (Makkink, 1993). This coincides with the vanishing of the advantage of phytase, as observed in Chapter 4. Spreeuwenberg (2002) concluded that limited post weaning energy intake decreases gut integrity. Thus also a positive effect of phytase on energy utilization might also be a factor in the better adaptation of piglets during the first weeks after weaning.

In Chapter 5, the energy metabolism of piglets three weeks post-weaning was investigated. Contrary to most findings, phytase increased energy digestibility. It did, however, not affect energy metabolization due to increased energy losses with methane and urine. Heat production variables were not affected by phytase in those piglets, even though a number of the variables showed differences that point towards possible differences in energy metabolism. The visceral organs seemed to be more active at 4 h after initiation of feeding on the phytase diet than on the control diet. Heat production was not affected by treatment, thus this could

mean that absorption kinetics were different. Because piglets were fed restrictedly, this was not reflected in a different extent of absorption or heat production. Kinetic studies of both digestibility and heat production at a higher feed intake are required, to clarify the potential of phytase in this regard.

In Chapter 5, we developed the hypothesis that phytase affects energy utilization simultaneously in a positive and in a negative way. A positive effect would be the reduction of energy required for production of endogenous protein, e.g. digestive enzymes. A negative effect could be the higher energy requirement for absorption and excretion processes, but also a higher blood pressure, as already speculated (Chapter 5). In particular the case of minerals is obvious: phytase increases the absorption of minerals, but when they are available above the animals' requirement, they need to be excreted, mainly into urine. Both these processes are, in part, active processes, which mean they require energy. A simple mathematical model indicated that energy required for absorption, retention and excretion for the extra-absorbed nutrients due to phytase, was about 1% of energy required for maintenance.

The increased mineral absorption also included the monovalent minerals sodium and potassium. This effect was observed in the experiments described in Chapters 5 and 6 and was recently also reported in sows (Jongbloed et al., 2004). It may be the result of a higher release of Na and K that is bound to phytate. Despite a high solubility of Na and K-phytate (Scheuermann et al., 1988), this would mean that not all Na and K is released from phytate within the gastro-intestinal tract, when no phytase is supplemented. The higher digestibility could also be the result of a decreased secretion of digestive fluids. Assuming that phytate binds (e.g.) trypsin, a negative feedback will result in an increased pancreatic exocrine secretion. Degradation of phytate by phytase prevents this binding, thus will result in lower production of pancreatic juice. Because pancreatic juice contains a high concentration of Na (Low, 1989; Argenzio, 1993b), this results in a lower secretion of Na into the small intestine. Cowieson et al. (2004) hypothesized similarly, after observing an increased secretion of endogenous Na when feeding phytic acid to chicks, which was reduced when phytase was also fed. With this hypothesis, however, the increased digestibility of K by phytase cannot be explained. The electrolyte concentration in digestive juices is typically similar to that in plasma, containing a high level of Na, but not of K (Argenzio, 1993b). The increased absorption of minerals, including these monovalent minerals, should be taken into consideration in feed formulation, to prevent the negative effect of the higher mineral load on energy requirement.

Possible energy saving by adding phytase can be explained from reduction of the production of endogenous proteins (digestive proteins). In Chapter 7, it was shown that phytase decreases the production of gastric mucin. It was calculated that phytase saved 29 kJ/d for the lower mucin production itself. Lower mucin production is probably initiated via reduced pepsin production, as discussed. Previously, it was calculated that the mucin produced would result from the production of 38.5 and 50.9 g pepsin/d for the phytase and control treatments, respectively. The energy consequences of the difference in pepsin production can be estimated. Pepsin is produced from pepsinogen. The pepsinogen of pigs contains 371 amino acids and is hydrolyzed into pepsin and a peptide containing 44 amino acids (Pelmont, 1989). Assuming an even

distribution of the weight of amino acids over both peptides formed, production of 38.5 and 50.9 g pepsin requires the production of 43.7 and 57.7 g pepsinogen/d, respectively. The energy content of protein is 23.6 kJ/g, and the energy cost for protein synthesis (peptic binding) was estimated to be 4.5 kJ/g (Webster, 1981). An ileal digestibility of 75% is assumed for endogenous protein, thus for both pepsin and the 44 amino acid-peptide (Nyachoti et al., 1997). Of the ileal undigested protein, 80% is assumed to be fermented. The energy value of fermented protein can be assumed at 14 kJ/g protein [23.6 – 3.6 (urea)] × 0.70 (efficiency factor for volatile fatty acids; Bakker, 1996). Overlooking energy costs for protein re-synthesis of absorbed amino acids and for the absorption processes, energy required for compensating these losses is 332 and 438 kJ/d for the phytase and control treatments, respectively. The difference, 106 kJ/d, is 1.3% of the ME_m for a 55 kg pig. Phytase may reduce energy requirement, via an effect on pepsin production and the related production of mucin, by 135 kJ/d, or 1.6% of ME_m for a 55-kg pig.

The magnitude of the effects of phytase on energy requirement: 1) an increased requirement for nutrient (re-) absorption and retention of minerals (1% of ME_m), and 2) a decreased requirement with a lower production of mucin and pepsin (1.6% of ME_m), do not differ much. So positive and negative effects on energy requirement both play their role, which resulted in an absence of a net effect of phytase in the pig experiments. All these effects need to be experimentally confirmed.

ABSORPTION OF INOSITOL PHOSPHATES

In Chapter 6, it was shown that phytase continues to degrade phytate at a much higher level of incorporation than thought previously (Düngelhoef and Rodehutscord, 1995; Kornegay, 2001). From the exponential model (Chapter 6), it was calculated that phytase could increase the digestible P level of the diet with 1.76 g/kg. Assuming that non-phytate P has a digestibility coefficient of 80% (Jongbloed, 1987), this means that digestibility of phytate P in this experiment was 85%. This large degree of phytate degradation could reduce the need for inorganic phosphate even more than realized with the present level of phytase supplementation.

The extent of phytate hydrolysis may also be related to the physiology of inositol phosphates, and be linked to energy utilization. This aspect was not measured, however, and is speculative. Phytase from *Aspergillus niger* has a relatively low affinity for inositol monophosphate (IP_1; Misset, 2003). The degradation of IP_6 was 85%. It could be concluded, therefore, that all phytate had been hydrolyzed to this metabolite. The remaining IP_1 (17% of phytate-P) would be excreted with manure. This is, however, not likely. Kemme et al. (1999) showed that levels of the "lower" inositol phosphates (i.e. IP_{1-3}) in ileal digesta of pigs were very low. Rapp et al. (2001) showed similar results in mini-pigs. Skoglund et al. (1998) found small amounts of IP_1 and IP_2 in the feces of pigs. In ileostomized humans a low level of IP_3 was also found (Sandberg and Andersson, 1988), but IP_1 and IP_2 were not measured in that experiment. All these findings suggest that "lower" inositolphosphates are quantitatively of minor importance at the ileal level in pigs. Moreover, there is no accumulation of these metabolites in the ileum. Thus, IP_1 seems not to appear at the terminal ileum in a large quantity. The last phosphate group

from IP_1 may be degraded by phosphatases from intestinal or microbial origin (Kemme et al., 1999). Possibly, under conditions of the gastro-intestinal tract, phytases might degrade the last phosphate group from IP_1.

Alternatively, IP_1 may be absorbed by the small intestine. In a number of studies, absorption of inositol phosphates has been investigated. Sakamoto et al. (1993) showed in rats that inositol phosphates are well absorbed. The authors suggested that IP_6 is absorbed, but they mainly measured IP_1 and inositol in blood plasma. They hypothesized that IP_6 is absorbed but that it very rapidly degrades to lower IP's. But they could not exclude that IP_{1-5} were absorbed, rather than IP_6. Also Grases et al. (2001) concluded from a kinetic study with humans that IP_6 is absorbed directly. They could measure IP_6 in plasma and in urine, but it can be calculated that less than 0.1% of the IP_6 dose is recovered in plasma as IP_6. These studies show that it is likely that IP-metabolites are absorbed. This is probably mainly in the form of IP_1, or other lower inositol phosphates, rather than as IP_6, contrary to the hypothesis of Sakamoto et al. (1993) and Grases et al. (2001). Storage in cells would be in the form of IP_6, because the cellular amount of this metabolite is higher than of IP_{1-5} (Szwergold et al., 1987; Efanov et al., 1997; Vucenik and Shamsuddin, 2003). The lower cellular concentrations of IP_{1-5} are logical due to their regulatory involvement (Ferris et al., 1989; Eckmann et al., 1997; Woodcock, 1997).

The interest in absorption of inositol phosphates results from a number of positive properties that have been attributed to phytate. A lower glycemic index due to the binding of starch, antioxidant properties, prevention of heart disease, prevention of renal calculi and prevention of colon cancer have been attributed to phytate (Thompson, 1986; Zhou and Erdman, 1995; Feil, 2001). Organisms certainly have a need for inositol phosphates, but they can synthesize them. An example is inositol 1,4,5-triphosphate, a secondary messenger, which acts in the cascade of many physiological processes. Shamsuddin and co-workers studied the anti-cancer effect of phytic acid (Sakamoto et al., 1993; Shamsuddin, 1999; Vucenik and Shamsuddin, 2003). According to Vucenik and Shamsuddin (2003), IP_6 reduces cell proliferation, and induces cell maturation and differentiation. They measured, however, transport of IP_1 to tumor cells (Vucenik and Shamsuddin, 2003). Assuming that in pigs and chickens lower IP's have similar functions, it could be that IP_1 has growth regulating properties. In pigs, absorption of inositol phosphates was not measured directly. The hypothesized positive effect on growth regulation is speculative, and is presently not investigated. It could, however, be one of the elements to explain part of the improvement in performance, observed in animals receiving phytase.

PHYSICAL AND PHYSIOLOGICAL ASPECTS

In the former part of this thesis, it is shown that reducing the anti-nutritional effect of phytate by phytase improves digestibility of minerals and protein and to some extent also energy utilization. The explanation of the anti-nutritional effect of phytate has to be found in the chemistry of phytic acid and its complexing with cationic materials. Acid-base properties of phytate are highly complex. Only some basal properties of this chemistry will, therefore, be discussed. The chemical properties have also to be seen in the context of the physiological circumstances of the animal.

A typical feed (without addition of acids) dispersed into water, has a pH of about 5.5-6. In the stomach, this value gradually decreases to a value of about 2-4. After entering the small intestine, the pH rises rapidly to about 6, to reach a value of about 6.5-7.5 in the distal ileum. Protein, Ca and P are absorbed throughout the small intestine, but especially in the duodenum and proximal jejunum (Partridge, 1978a; Laplace, 1982; Grimble and Silk, 1989; Van der Meulen and Bakker, 1991). In poultry, these values are similar. Feed, however, first enters the crop, with a pH of about 4.5-5.5, and subsequently the proventriculus and gizzard (Van der Klis, 1993). Throughout the digestive tract, an abundant quantity of Na is available in endogenous secretions, in normal, healthy animals (Partridge, 1978a; Van der Klis, 1993).

The complexing ability of the phytate ion depends on its valency, which is a result of its acid-base properties. In the literature, variable protonation constants have been reported, as discussed by Li et al. (1989), Bebot-Brigaud et al. (1999) and De Stefano et al. (2003). This is probably because different ionic media with different strengths have been used. Presence of sodium ions, and probably other ions too, affect properties of phytate. De Stefano et al. (2003) showed that protonation of phytate depends on both ionic medium and strength. These authors measured protonation constants of phytic acid in NaCl and tetraethyl ammonium iodide (Et_4NI) media. The latter is a 'non-interacting' medium. With Na, protonation constants were considerably lower. For example, at an ionic strength of about 0.5 M, pK_a values for the reaction H_5-phytate^{7-} → H_6-phytate^{6-} were 4.7 and 6.0 for NaCl and Et_4NI, respectively (at 25 °C). With Na, phytate is less protonated at a certain pH, thus there are more binding sites available for protein. De Rham and Jost (1979) showed that both Na and Ca affect protein-phytate complexing. Probably, the presence of different cationic species also affects the protonation constants and stability of the complexes formed, to an extent that depends on the cationic species and concentration (De Stafano et al., 2002). Therefore, it is hard to predict the binding capacity of phytate for cationic minerals and protein in complex media like digesta.

Bebot-Brigaud et al. (1999) showed that the conformation of the phytate ion depends on the availability of Na-ions (and possibly of other cations). In a tetraethyl ammonium perchlorate solution, the conformation of the phytate-phosphate groups was 5 axial/1 equatorial in the pH range 1-13. With Na, however, this switched to 5 equatorial/1 axial. The position of the phosphate groups may affect the biochemical activity of phytate, and the bioavailability of complexed ions (Bebot-Brigaud et al., 1999; De Stafano et al., 2002). It might also affect the activity of phytase. Misset (2003) indicated that the affinity of *A. niger* phytase for 2-IP_1 is low, which is the phosphate group in the axial position (with Na). This can be the result of the different position of this phosphate group, compared to the other five phosphates.

The formation of protein-phytate complexes in the stomach of animals is due to binary protein-phytate binding. In the small intestine, these complexes cannot be formed, and will mostly dissolve. At a pH of about 7 and higher, ternary complexes can be formed (Cheryan, 1980). This pH occurs in the distal part of the small intestine. Thus it is more likely that protein-phytate complexes formed in the stomach affect protein digestibility than complexes formed in the small intestine. On the other hand, complexes formed in the small intestine are more likely to be lost from the small intestine, because they are less likely to re-dissolve (Cheryan, 1980).

CONCLUSIONS

In this thesis, the impact of phytase on protein digestibility and energy utilization in poultry and pigs were investigated. From the results, the following is concluded:

- Protein forms insoluble complexes with phytate at low pH. Proteins in these complexes can be hydrolyzed by pepsin, but peptides > 12 kD remain bound to phytate. Phytase prevents formation of these complexes, or releases protein from such complexes faster, which probably increases the efficacy of pepsin.
- Dietary phytase supplementation improves amino acid digestibility in poultry. Also AME is improved, mainly as the result of increased digestibility of protein and fat.
- The feed compounder can incorporate the effect of phytase on protein digestibility and AME for poultry, in the matrix of feed formulation programs. This permits to maximize the value in the animal production chain.
- In piglets, an effect of phytase on post-absorptive energy utilization could not be shown. A number of effects were observed that indicate metabolic activities are affected. Simultaneously, both an increase and a decrease in heat production with phytase seem to exist, resulting in the absence of a net effect on energy retention.
- In pigs, an improvement of energy utilization by phytase is probably the result of post-absorptive effects, rather than of an improvement in nutrient digestibility.
- Phytase increases the digestibility of sodium and potassium.
- Phytase reduces the ileal recovery of gastric mucin. This is probably a secondary effect of lower secretion of pepsin.
- At a high level of phytase inclusion, phytate can be almost totally degraded in pigs. Mineral digestibility can be increased further than realized with the current industry standard (500 FTU/kg).

RECOMMENDATIONS FOR FUTURE RESEARCH

After finishing this thesis, a number of areas remain open for further investigation. The most important ones are:

- The effect of phytase on endogenous protein production in poultry, but especially in pigs; the effect on production of pancreatic secretions being a priority.
- Improved understanding of the physical chemistry of phytate-mineral-protein interactions under physiologically relevant conditions, helping to explain the observed effect of phytase on protein digestibility.
- Investigation of the effect of phytase on absorption of nutrients and its kinetics, using (e.g.) portal vein measurements.
- The physiological explanation for the positive effect of a very high phytase dose on mineral digestibility, in both poultry and pigs.

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Summary

Phosphorus (P) is an essential mineral for animals. It is mainly found in bones, but is also indispensable for growth and maintenance of other tissues. In vegetable feedstuffs that are used in animal feeds, a large proportion of the P is stored in phytate. Phytate is the salt of phytic acid; this is inositol (a kind of sugar) with six phosphate groups. It cannot or hardly be digested by monogastric animals, such as poultry and pigs. By supplementing feeds for pigs and poultry with phytase, digestibility of phosphorus contained in phytate can be increased. This enables feed compounders to reduce the use of inorganic phosphates, thereby reducing P-output in manure, and possible pollution of the environment.

The additional improvement in animal performance, unrelated to the effect on P-digestibility, was an additional benefit. This effect may be explained by an improvement in digestibility or post-absorptive utilization of nutrients, or by a reduced maintenance requirement of the animals. Understanding the mechanisms of these effects helps the feed compounder to maximize the impact of supplemented phytase, and further reduce feed costs. In this thesis, the effect of phytase on digestibility of protein (amino acids) and on energy utilization, both in pigs and poultry, are quantified, and potential mechanisms are investigated.

Four potential mechanisms for an effect of phytate on protein digestibility were identified: 1) naturally occurring protein-phytate complexes, 2) *de novo* dietary protein complexing to phytate, 3) complexing of phytate with free amino acids, especially lysine and 4) complexing of proteolytic enzymes to phytate. In **Chapter 1**, the interaction between protein, phytate and phytase was studied *in vitro* in protein extracts from corn, canola meal, rice pollards, soybean meal, sunflowerseed meal and casein. In extracts from the first five feedstuffs, the occurrence of protein-phytate complexes was quantified. It appeared that most protein and phytate were not recovered in the same extract. In the water-soluble fraction of the feedstuffs, protein and phytate were not bound. With the possible exception of rice pollards, the results suggested that complexes of soluble proteins and phytate do not exist, or exist in small amounts only, in feedstuffs. Under acidic conditions, protein precipitates with phytate. The ratio of protein to phytate in the complexes revealed that, in a practical diet containing 1% phytate (expressed as phytic acid), phytate could potentially bind dietary protein up to 20% or more. Prior degradation of phytate by phytase prevented the development of such complexes. Phytase also increased the rate and the extent of protein hydrolysis by pepsin. Using electrophoresis, it was shown that pepsin could hydrolyze protein in a protein-phytate complex, but that the peptides > 12 kD remained bound to phytate. When phytase was added, protein was readily released from phytate. The experiment suggests that *de novo* formation of protein-phytate complexes may be of special relevance, and that phytase prevents formation of this complex, or aids in its degradation.

In **Chapter 2**, the effect of phytase supplementation on ileal amino acid digestibility and on apparent metabolizability of dietary energy (AME) in broilers is described. The diet was designed to be adequate in available P, but limiting in lysine. Phytase improved performance of the animals. Amino acid digestibility increased by about 3 and 4%-units with phytase supplementation of 500 and 1000 FTU/kg, respectively. The maximal increase in AME content due to phytase supplementation was 0.5 MJ/kg feed (3.5%), observed at a level of 750 FTU/

kg of feed. Lysine supplementation did not affect AME of the diet. Therefore, increase of the AME by phytase was not related to the lysine deficiency of the diet. Digestibility of macronutrients was not investigated in this experiment, but in the general discussion it is hypothesized that increased protein and fat digestibility explains most of the reported increase in AME.

Chapter 3 describes the evaluation of digestibility experiments and the assessment of the nutritional value of phytase to be used in least-cost formulation software by the feed compounder. In addition, the effect of phytase on animal performance using P-adequate diets, which is the original basis for this thesis, is evaluated. In most experiments, the digestibility of amino acids and the AME are not significantly improved by phytase. In a meta-analysis using all data available, it was shown that phytase improves digestibility of all amino acids, which was significant in most cases. A similar conclusion was drawn for the effect on AME in poultry. In an example it was shown that, using the assigned matrix-values, dietary phytase supplementation could reduce the costs of diets for laying hens and broilers by 2 and 6 Euro/ton, respectively. Also, the costs of pig feeds could be reduced up to 2.50 Euro/ton. If a diet requires a limitation of its maximum P level, the saving can be considerably higher.

Two experiments were conducted to evaluate the effect of phytase on energy metabolism of piglets. The first of these (**Chapter 4**) was performed using *ad libitum* fed, newly weaned piglets. Several improvements were observed in the phytase-supplemented group. Feed intake was higher in the piglets that received the phytase-supplemented diet. This effect was large (23%) in the first two weeks, but disappeared in the third week post-weaning. Various other variables (e.g. growth rate, heat production, energy retention) followed the same pattern. It is an indication that phytase facilitates the adaptation of piglets to their post-weaning state. Heat production related to physical activity was somewhat increased with phytase. Most differences observed were not significant, however. To reduce variation in a subsequent experiment, it was decided to restrict feed intake. Because this is difficult to realize in newly weaned piglets, the experiment started 3 weeks post-weaning.

In this experiment, described in **Chapter 5**, the effect of phytase supplementation to a diet with high phytate content (3.8 g phytate-P/kg) was tested. Energy partitioning was measured using indirect calorimetry. In addition, digestibility of different nutrients, minerals and energy, different blood variables and the weight of organs after the three-week experiment were measured. Energy digestibility increased with phytase, but energy metabolizability was similar to that of the controls. Production of methane was slightly increased with phytase. Energy retention and heat production were not affected by phytase supplementation. A number of effects observed, however, indicated that changes occurred in the piglets on the phytase diet. For example, the weight of the pancreas and the pH of venous blood were significantly reduced and CO_2 pressure was significantly increased on the phytase diet. It was suggested that phytase reduced energy expenditure of tissues of the digestive tract. Oxidative metabolic activity in visceral organs, 4 hours after feeding, was increased. It was hypothesized that a reduction of heat production by phytase was counterbalanced by increased energy expenditure for the increased absorption of minerals, and their subsequent urinary excretion. Using a mathe-

matical model, the energy cost of increased absorption of nutrients and of deposition and excretion of minerals was estimated at 4.6 kJ/($kg^{0.75}$.d), or about 1% of the energy required for maintenance. The simultaneous existence of both increases and decreases in heat production processes resulted in the absence of a net effect on energy retention. The increase in heat production may be in part explained by mineral metabolism, but other factors may also play a role, such as blood pressure and possible loss of energy-rich compounds in urine. These were, however, not investigated.

Chapter 6 describes an experiment in which the effect of gradual phytase supplementation of a P-deficient diet on mineral digestibility was investigated in piglets (8-28 kg BW). The doses applied were gradually increased from 0 to 15,000 FTU/kg. Phosphorus digestibility increased from 34 to 84%, at 0 and 15,000 FTU/kg, respectively. The amount of P liberated by phytase was calculated to be 1.76 g digestible P/kg of feed and the calculated digestibility of phytate-P was 85%. Sodium and potassium digestibility increased both by 10%-units, at the highest phytase dose compared to the unsupplemented diet. This agrees with observations made in the experiment of Chapter 5. The continuing increase of P digestibility above a supplementation of 1,500 FTU/kg was not in agreement with earlier reports. Mineral utilization can, thus, be more improved than realized at the present day industrial standard of 500 FTU/kg, further reducing the mineral load on the environment. The economic optimum, however, needs to be evaluated per case.

The increase in protein digestibility by phytase could be the result of a lower production, and subsequent lower net losses, of endogenously secreted proteins. Mucin is an important endogenous protein source. In **Chapter 7** the effect of phytase supplementation on net ileal recovery of mucin proteins in pigs (40-70 kg BW) was examined, using two basal diets containing a low and high phytate content (2.2 and 4.8 g phytate-P/kg, respectively). Phytase supplementation reduced ileal mucin recovery, regardless of the phytate content of the diet. This difference could totally be attributed to a lower recovery of gastric mucins; recovery of intestinal mucins was similar. On average, 3.3% of the ileal amino acid flow originated from mucin. This value was higher for serine (8%) and threonine (12%) than for other amino acids. It was calculated that mucin proteins affected apparent ileal AA digestibility with 0.8%-units, but for serine and threonine this effect was larger (2 and 4%, respectively). In this experiment, phytase increased amino acid digestibility by about 1%-unit on average (data not reported in this thesis). For threonine this was 1.3%. The reduced mucin production with phytase supplementation explained for most amino acids less than 10% of this difference, but for threonine this was 31%. These differences, although small, were in all cases significant. Energy expenditure related to the difference in mucin production was calculated to be 29 kJ/d lower on the phytase-supplemented diet.

In the **General Discussion,** various aspects of this work are evaluated. Firstly, the positive effect of phytase on protein digestibility was a recent topic of debate in the scientific community. Results of work reported after the analysis presented in Chapter 3 were evaluated, to test whether the values calculated are still valid. It was concluded that recent results are similar to earlier findings. It was analyzed that the arguments against a positive effect of phytase on

Fosfor (P) is een essentieel element voor dieren. Het komt vooral voor in botten, maar is ook onmisbaar voor de opbouw en instandhouding van andere weefsels. In plantaardige grondstoffen die in de diervoeding worden gebruikt, zit een groot deel van de P in fytaat. Fytaat is het zout van fytinezuur; dit is inositol (een soort suiker) met zes fosfaatgroepen. Het wordt niet of nauwelijks verteerd door eenmagige dieren, zoals pluimvee en varkens. Door het enzym fytase aan voeders voor deze dieren toe te voegen, kan de vertering van het fytaat-P worden verhoogd. Daardoor hoeft minder anorganisch fosfaat aan het voer te worden toegevoegd en wordt de fosfaatuitscheiding in de mest verlaagd, met als gevolg een lagere milieubelasting.

In veel proeven met fytase verbeterde de dierprestaties (groei en voederconversie) door de fytasetoevoeging. Dat is logisch: de meeste van deze proeven werden uitgevoerd met voeders met een laag P gehalte, wat nodig was om het effect op de fosforvertering goed te kunnen meten. Maar ook onafhankelijk van het positieve effect op de fosforvertering verbeterde fytase de prestaties. Deze aantrekkelijke bonus zou kunnen worden verklaard door een betere vertering of benutting van nutriënten, of door een lagere behoefte van de dieren. Als dit werkingsmechanisme bekend is, kunnen voerfabrikanten fytase beter waarderen en daardoor de voerkosten verder verlagen. In dit proefschrift is de invloed van fytase op verteerbaarheid van eiwit (aminozuren) en op de energiebenutting bestudeerd in varkens en pluimvee. Deze effecten werden gekwantificeerd, en een aantal mogelijke mechanismen onderzocht.

Er zijn vier mechanismen beschreven hoe fytaat de eiwitvertering kan beïnvloeden. De vertering van aan fytaat gecomplexeerd eiwit kan moeilijker zijn dan van oplosbaar eiwit. Deze complexen kunnen van nature in plantaardige grondstoffen voorkomen (1) of gevormd worden door complexering van voereiwit (2) of vrije aminozuren (3) aan fytaat in het maagdarmkanaal. Door complexering van eiwitafbrekende (proteolytische) enzymen (4) kan de verteringscapaciteit worden beperkt.

In **hoofdstuk 1** worden de resultaten van "*in-vitro*" proeven naar de interactie tussen eiwit, fytaat en fytase beschreven, oftewel naar de eerste twee hiervoor genoemde mechanismen. Extracten van maïs, koolzaad, rijstevoermeel, sojaschroot, zonnebloemzaadschroot en caseïne werden gebruikt als eiwitbron. Caseïne dient vooral als voorbeeldstof; de andere vijf producten zijn gangbare voergrondstoffen. In de extracten van deze vijf grondstoffen werd het voorkomen van eiwit-fytaat complexen gekwantificeerd. In de meeste gevallen kwamen fytaat en eiwit niet in dezelfde extracten voor. In het wateroplosbare deel van de grondstoffen, bleken eiwit en fytaat niet aan elkaar te zijn gebonden. Dit suggereert dat in de onderzochte grondstoffen, mogelijk met rijstevoermeel als uitzondering, geen of weinig oplosbare eiwit-fytaat complexen voorkomen. Onder zure omstandigheden, zoals in de maag voor kan komen, sloeg eiwit neer met fytaat. De eiwit : fytaat-verhouding in het neerslag gaf aan dat 1% fytaat (uitgedrukt als fytinezuur), wat ongeveer het niveau is in praktische voeders, tot 20% of meer van het voereiwit kan binden. Na voorbehandeling met fytase werd geen neerslag gevormd. De snelheid en mate waarin pepsine eiwit van eiwit-fytaat neerslag af splitste, werden door fytase verhoogd. Met behulp van electroforese werd aangetoond dat pepsine eiwit in een eiwit-fytaat complex wel splitste in kleinere stukken, maar dat peptiden groter dan ongeveer 12 kD aan het fytaat gebonden bleven. Door fytase toe te voegen, werd het eiwit snel van het fytaat los-

gemaakt. Deze proeven duiden erop dat de vorming van een eiwit-fytaat neerslag in het maagdarmkanaal belangrijk is. Fytase kan de vorming van deze neerslagen voorkomen, of helpt om ze weer af te breken.

Hoofdstuk 2 beschrijft het effect van fytasetoevoeging aan het voer op de ileale aminozuren vertering en schijnbare energie omzetting ("AME") in kuikens. Het voer werd geformuleerd om voldoende beschikbaar P voor het kuiken te bevatten, maar was beperkend in het lysinegehalte. Fytase verbeterde de technische prestaties van de dieren. De verteerbaarheid van de aminozuren werd verbeterd met ongeveer 3 en 4%-eenheden, bij een toevoeging van 500 en 1000 eenheden fytase ("FTU"). Toevoeging van 750 FTU fytase gaf de maximale toename in AME: 0.5 MJ/kg voer (3.5%). Lysinetoevoeging verhoogde de AME niet. Daarom werd geconcludeerd dat het effect van fytase op de AME niet was toe te schrijven aan een indirect lysine effect. De verteerbaarheid van de macronutriënten werd in deze proef niet gemeten, maar in de algemene discussie wordt verondersteld dat een hogere eiwit- en vetvertering grotendeels de oorzaak zijn van de toegenomen energiebenutting.

In **hoofdstuk 3** worden de resultaten van meerdere verteringsproeven gebruikt om de voedingswaarde van fytase te berekenen. Dit wordt uitgedrukt als "matrixwaarden": getallen die de veevoerfabrikant in het voerformulerings-programma kan gebruiken. Ook wordt het effect van fytase op de technische prestatie van dieren die P-adequate voeders kregen geëvalueerd. In de meeste proeven werden de vertering van aminozuren en de AME niet (statistisch) significant verbeterd. Indien alle proeven tezamen werden genomen bleek de vertering van alle aminozuren, in de meeste gevallen significant, te zijn verbeterd. Voor pluimvee was dit ook het geval voor de AME. In een voorbeeld werd berekend dat fytase, met de daaraan toegekende matrixwaarden, de kostprijs van leghennen- en kuikenvoer met ongeveer 2 en 6 Euro/ton kan verlagen. De kosten van varkensvoer kunnen tot ongeveer 2.50 Euro/ton voer worden verlaagd. Dit zijn grote bedragen. Als er een maximum P niveau vereist is, zijn de besparingen nog groter.

In twee proeven werd het effect van fytase op het energiemetabolisme van biggen gemeten. De eerste proef (**hoofdstuk 4**) was met *ad libitum* (onbeperkt) gevoerde, pas gespeende biggen. Een aantal verbeteringen werden waargenomen in de groep met fytaseverrijkt voer. De voeropname was in de eerste twee weken van de proef hoger (23%), maar dit verschil verdween in de derde week. Een soortgelijk effect werd waargenomen voor onder anderen groeisnelheid, warmteproductie en energieretentie. Dit is een indicatie dat het fytase de negatieve gevolgen van het spenen verminderde. Activiteitgerelateerde warmteproductie was hoger in de fytasegroep. De meeste effecten waren echter niet significant. Om in een vervolgproef de variatie te verkleinen, werd besloten biggen daarin beperkt te voeren. Omdat dit moeilijk kan met pas gespeende biggen, werd die proef drie weken na het spenen gestart.

In deze proef (**hoofdstuk 5**), werd het effect van fytase in een voer met hoog fytaatgehalte (3.8 g fytaat-P/kg voer) getest. De energieverdeling werd gemeten met behulp van indirecte calorimetrie. De vertering van verschillende nutriënten, mineralen en energie werd gemeten. Verschillende bloedwaarden en de gewichten van een aantal organen werden na de drie-weekse proef bepaald. De energievertering was hoger voor het fytaseverrijkte voer, maar de ener-

gie-omzetbaarheid was vergelijkbaar met die van de controle dieren. Er was geen verschil in energieretentie en warmteproductie. Sommige waarnemingen duidden er op dat er wel verschillen waren tussen de twee groepen biggen. Het gewicht van de alvleesklier en de pH van veneus bloed waren bijvoorbeeld lager en de CO_2-druk in het bloed was hoger in de dieren met fytaseverrijkt voer. Gesuggereerd werd dat fytase het energieverbruik van het maagdarmkanaal verlaagde. Vier uur na het voeren was de oxidatieve metabole activiteit in de verteringsorganen hoger. Er werd verondersteld dat een lagere warmteproductie van dieren die het voer met fytase kregen werd gecompenseerd door een hoger energieverbruik voor de verhoogde opname van mineralen en hun verhoogde uitscheiding in de urine. Met een wiskundig model werden de energiekosten voor de verhoogde opname van nutriënten en de opslag en uitscheiding van mineralen geschat op 4.6 kJ/($kg^{0.75}$.d), wat ongeveer 1% van de energiekosten voor onderhoud is. De gelijktijdige toe- en afname van warmteproducerende processen leidden er toe dat er netto geen verschil in energieretentie waar was te nemen. De toegenomen warmteproductie kan deels worden verklaard door het mineralenmetabolisme, maar andere factoren, zoals de bloeddruk of een mogelijk verlies van energierijke componenten in de urine, zouden ook een rol kunnen spelen. Dit werd echter niet gemeten in deze proef.

Hoofdstuk 6 beschrijft een proef naar het effect van een toenemende fytasedosering (van 0 tot 15000 FTU/kg P-deficiënt voer) op de mineralenvertering in biggen (8-28 kg). De P vertering nam toe van 34 tot 84%, bij respectievelijk 0 en 15000 FTU/kg voer. De hoeveelheid extra verteerd P op het hoogste fytaseniveau was 1.76 g/kg. De berekende fytaatverteerbaarheid was 85%. Ten opzichte van het laagste niveau, namen de natrium- en kaliumvertering op het hoogste fytaseniveau beiden met 10%-eenheden toe. Dit komt overeen met de waarnemingen in hoofdstuk 5. De verdere toename van de P vertering bij een fytaseniveau hoger dan 1500 FTU/kg is niet in overeenstemming met eerdere proeven. De benutting van mineralen kan dus verder worden verbeterd dan momenteel wordt gerealiseerd met de (gangbare) toevoeging van 500 FTU/kg. Daardoor kan de mineralenbelasting van het milieu verder worden verkleind, al moet het economische optimum per geval worden bekeken.

De hogere eiwitvertering met fytase kan het resultaat zijn van een lagere productie en lager verlies van endogeen eiwit. Slijm is een belangrijke bron van endogeen eiwit. **Hoofdstuk 7** beschrijft een experiment naar het effect van fytase op de hoeveelheid slijm die in het ileum voorkomt. De proef werd uitgevoerd met groeiende varkens (40-70 kg) en met twee basisvoeders: één met laag en één met hoog fytaatgehalte (respectievelijk 2.2 en 4.8 g fytaat-P/kg). Fytasetoevoeging verlaagde de hoeveelheid ileaal slijm. Het verschil werd veroorzaakt door de kleinere hoeveelheid maagslijm; de hoeveelheid darmslijm was vergelijkbaar. Gemiddeld was 3.3% van de hoeveelheid aminozuren die het ileum passeerde afkomstig van slijm, maar dit was hoger voor serine (8%) en threonine (12%) dan voor de andere aminozuren. Berekend werd dat de schijnbare ileale aminozurenvertering met 0.8%-eenheden werd beïnvloed, maar dat dit meer was voor serine en threonine (2 en 4%). In deze proef verhoogde fytase de schijnbare aminozurenvertering met gemiddeld ongeveer 1% (niet gerapporteerd in dit proefschrift). Voor threonine was dit 1.3%. De lagere slijmproductie door fytasetoevoeging kon voor de meeste aminozuren minder dan 10% van het verschil in verteerbaarheid verklaren, maar voor

threonine was dit 31%; in alle gevallen was dit significant. Het werd berekend dat de energiekosten gerelateerd aan slijmproductie 29 kJ/dag lager waren voor het fytaseverrijkte voer.

In de **algemene discussie** zijn verschillende aspecten van het werk besproken. Onder wetenschappers is het effect van fytase op eiwitvertering onderwerp van debat. Daarom zijn eerst de resultaten van hoofdstuk 3 geëvalueerd, om te bepalen of de gerapporteerde waarden nog steeds juist zijn. Na opname van recentere proefgegevens in de berekeningen bleven de uitkomsten vergelijkbaar. Het werd geanalyseerd dat de argumenten tegen het positieve effect op twee hoofdpunten neerkomen. Het eerste is of resultaten die, in een proef, statistisch niet-significant zijn in een algehele analyse mogen worden gebruikt of niet. Het tweede is de validiteit van een bepaalde proeftechniek (de "protein efficiency ratio") voor het testen van fytase. De methode lijkt niet geschikt te zijn voor het testen van het effect van een enzym, zoals fytase, op de eiwitbenutting.

De energiebenutting lijkt door fytase niet op dezelfde wijze in pluimvee en varkens te worden beïnvloedt. De hogere AME waarden in pluimvee lijkt vooral het resultaat te zijn van een hogere eiwit- en vetvertering. In varkens blijkt de beïnvloeding van de energieverdeling door fytase gecompliceerder te zijn. Literatuur geeft aan dat energievertering en -omzetting niet verbeteren door fytasetoepassing. In de hoofdstukken 4 en 5 kon geen duidelijk effect van fytase op de energieverdeling worden aangetoond, maar er waren wel aanwijzingen dat het energiemetabolisme van de biggen veranderde. Dat deze veranderingen niet leidden tot een verbeterde energiebenutting zou kunnen worden toegeschreven aan het gelijktijdig voorkomen van processen die de warmteproductie verhogen en verlagen.

Slijmproductie was verlaagd door fytase. Omdat het onwaarschijnlijk is dat slijm aan fytaat complexeert wordt verondersteld dat dit een indirect effect is, namelijk via een effect op de pepsineproductie. Het mogelijke effect daarvan op de energiebenutting werd berekend op 135 kJ/dag, of 1.6% van de onderhoudsenergiebehoefte van een varken van 55 kg. Deze hypothese moet echter nog getest moet worden, evenals het effect van fytase op de productie van andere verteringsenzymen.

In de laatste paragrafen van de algemene discussie wordt gespeculeerd over fysiologische en fysische mechanismen die een verklaring kunnen geven voor het effect van fytase op energiebenutting en nutriëntenvertering. Ook wordt gespeculeerd op het mogelijke positieve effect van inositol mono-, di- of trifosfaat.

Samenvattend wordt in dit proefschrift aangetoond dat fytase helpt om eiwit-fytaat complexen af te breken, waardoor de eiwitvertering in pluimvee en varkens wordt verhoogd. In varkens was de betere eiwitvertering deels gerelateerd aan een lagere secretie van maagslijm. In kuikens is een hogere eiwit- en vetvertering oorzaak van de betere prestaties op fytase bevattende, niet in P beperkende voeders. In varkens kan dit echter slechts een deel van verbeterde dierprestaties (onder niet P-beperkende condities) verklaren. Voor de verklaring van het overige deel werd gehypothetiseerd over mogelijke effecten van fytase op het energiemetabolisme. Dit werd getest, maar leverde geen kwantitatief sluitend antwoord. Aanvullende metingen in deze experimenten toonden aan dat er wel belangrijke veranderingen in het metabolisme optraden na fytaseverrijking van het voer.

Appendices

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Training and Supervision Plan		**Graduate School WIAS**
Name	Arie Kies	
Group	Animal Nutrition	
Daily advisors	Martin Verstegen, Walter Gerrits	
Supervisor	Martin Verstegen	
Period	January 2000 - June 2005	

	Year	Cp
The Basic Package (minimum 2 cp)		
WIAS "Introduction course" (mandatory)	Exempted	1.0
WIAS course on philosophy of science and ethics (mandatory)	2003	1.0
SUBTOTAL		**2.0**
Scientific Exposure (minimum 5 cp)		
International conferences		
Int. Symposium on phytase in animal nutrition, Lublin, Poland (oral presentation)	2000	0.9
Western Nutrition Conference, Winnipeg, Canada.	2000	0.4
Poultry Science meeting, Atlanta, US 2000, 2001	2000-1	0.8
European Symposium on Feed Enzymes 3, Noordwijkerhout (oral presentation)	2000	1.1
American Society of Animal Science, Québec, Canada	2002	0.7
British Society Animal Science, York, UK (oral presentation)	2002	1.1
Antimicrobial Growth Promoters: Worldwide Ban on the Horizon? Noordwijk	2005	0.4
Seminars and workshops		
Symp. Occasion Retirement Dr. R.R. Marquardt, Winnipeg, Canada (oral presentation)	2000	0.7
Journée AFTAA, Rennes, France (oral presentation)	2002	0.7
Wageningen Centre for Food Science Roundtable (obesity)	2003	0.4
WIAS Seminar Plus " Dietary protein: Physiological constraints to nutritive value"	2004	0.6
SUBTOTAL		**7.8**
In-Depth Studies (minimum 4 cp)		
PHLO course "New Developments in Feed Evaluation"	1998	1.0
VLAG-WIAS course "Ecophysiology of the gastrointestinal tract"	2003	1.0
WIAS course "Biology underpinning animal sciences: Broaden your Horizon"	2003	0.8
VLAG/WIAS course "Nutrition & Sports"	2004	1.0
VLAG course "Reaction Kinetics in Food Science"	2004	1.0
SUBTOTAL		**4.8**
Professional Skills Support Courses (minimum 2 cp)		
Boertien & Partners course "Effectief beïnvloeden"	2001	0.4
WIAS course "Techniques for writing and presenting scientific papers"	2003	0.8
Zuidema course "Inzicht in invloed"	2003	1.0
EPT course "Primary communication programme"	2005	0.8
SUBTOTAL		**3.0**
Research Skills Training (optional)		
Preparing own PhD research proposal	2002	4.0
SUBTOTAL		**4.0**
Didactic Skills Training (optional)		
Lecturing		
University of Alberta, Canada. Lecture "Enzyme application in animal nutrition"	2000	0.2
PHLO course "Pluimveevoeding: nieuwe ontwikkelingen en praktijk"	2001	0.5
International PHLO course "Nutrition and climate, new developments" (poultry)	2003	0.2
PHLO course "Pluimveevoeding: nieuwe ontwikkelingen en praktijk"	2003	0.2
Supervising MSc theses		
Support to a group of "Beroeps Voorbereidend Blok", Wageningen University	2000	0.8
SUBTOTAL		**1.9**
Management Skills Training (optional)		
Organisation of seminars		
Organisation committee 3rd European Symposium Feed Enzymes	2000	0.4
Organisation + advisory committee AGP: Worldwide Ban on the Horizon?	2005	0.4
SUBTOTAL		**0.8**
TOTAL		**24.3**

One credit point (cp) equals a study load of approximately 40 hours

Arie Karst Kies, werd op 7 mei 1957 geboren te Anna Paulowna. Na het doorlopen van de HAVO studeerde hij aan de toenmalige Bijzondere Hogere Landbouwschool (nu Van Hall Instituut) te Leeuwarden. Na het afstuderen in 1978 (onderzoeksdifferentiatie zoötechniek) ging hij naar de Landbouw Hogeschool te Wageningen (nu Wageningen Universiteit). Zijn studie zoötechniek werd in 1985 afgerond, met als afstudeervakken veevoeding, dierfysiologie, veefokkerij en bedrijfskunde.
Direct na de studie startte hij als (junior) nutritionist bij Hendrix' Voeders te Boxmeer (nu Hendrix-UTD, onderdeel van Nutreco). Tot de werkzaamheden behoorde onder andere het waarderen van grondstoffen, het berekenen van de nutritionele behoeften van dieren, het mede opzetten van voerprogramma's voor verschillende diersoorten en het maken van voersamenstellingen. In 1989 werd hij de varkensvoedingsonderzoeker bij Rhône Poulenc Animal Nutrition (nu Adisseo), in Commentry, Frankrijk. Met een team van 7-10 mensen werden proeven verricht met biggen, groeiende varkens en zeugen, onder andere naar de behoefte aan en effectiviteit van aminozuren, vitaminen, antimicrobiële groeibevorderaars en probiotica. In 1992 begon hij te werken bij Gist-brocades, bij het onderdeel dat nu DSM Food Specialties is. Hij werkte eerst twee jaar binnen de R&D organisatie, daarna 9 jaar als product development manager binnen de business unit Agri Ingredients. Hier werkte hij met name aan de toepassing van verschillende enzymen in de veevoeding. Zijn verantwoordelijkheden omvatte onder andere het dierkundige onderzoek en de technische ondersteuning van klanten in de Benelux landen. In deze periode werd het onderzoek verricht dat is gebruikt voor dit proefschrift. Het proefschrift zelf werd grotendeels in Arie's vrije tijd geschreven. Sinds de verkoop van de veevoederenzymen business door DSM Food Specialties werkt hij weer binnen R&D, nu als *nutritionist humane voeding, op het gebied voeding en gezondheid.*

Arie Karst Kies, was born on May 7th, 1957, in Anna Paulowna, which is in the flower-bulb growing area about 60 kilometers north of Amsterdam, The Netherlands. He graduated in 1978 from the Agricultural College in Leeuwarden, with a specialization in animal science research. The same year, he started to study Animal Science at Wageningen University. The study was finished in 1985, with the subjects being Animal Nutrition, Animal Physiology, Animal Breeding and Business Administration.
His working career started at Hendrix' Voeders, a large feed company now part of Nutreco Holding, as a (junior) nutritionist. The job included evaluation of the feeding value of feedstuffs, calculation of nutrient requirements of animals, development of feeding programs for different animal species and calculation of feed compositions. In 1989, he moved to Commentry, France, to work as the pig nutrition researcher for Rhône Poulenc Animal Nutrition (currently Adisseo). With a team of 7 to 10 people, experiments were performed with piglets, grower/finisher pigs and sows, to establish, among others, the requirements for and the efficacy of amino acids, vitamins, antimicrobial growth promoters and probiotics. In 1992, he moved back to The Netherlands, to work for Gist-brocades (now DSM Food Specialties). Firstly, he worked for two years within the R&D organization, then for 9 years as product development manager in the business unit Agri Ingredients. The work there was mainly related to the development of enzymes for application in animal nutrition. Responsibilities included animal research and technical service for customers in the Benelux area. Over this period, the investigations described in this thesis were performed. The thesis itself was mainly written in Arie's spare time. Since the sale of the feed enzyme business by DSM Food Specialties, he works again in the R&D organization, now as a human nutritionist, in the field of nutrition and health.

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