Quantification of Inositol Phosphates Using ^{31}P Nuclear Magnetic Resonance Spectroscopy in Animal Nutrition

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A ^{31}P NMR method for quantitative determination of inositol phosphates in simple incubation samples of sodium phytate and *Aspergillus niger* phytase and in different types of complex samples, such as diets, digesta, and feces, is described. The inositol phosphates in complex samples were extracted with HCl, concentrated, and purified using freeze-drying and filtration and subsequently determined at pH 12.6 in aqueous solution using a ^{31}P NMR method. The ^{31}P NMR technique has as its main advantages over the HPLC techniques that it does not necessitate standards that may cause background matrix effects and that the spectra of inositol phosphates and orthophosphate appear in the same run without further sampling errors. The results of inositol hexaphosphate analysis with HPLC can be confirmed by this ^{31}P NMR method. Contents of inositol tetra-, tri-, di-, and monophosphate in the biological samples appear to be quantitatively not important. The ^{31}P NMR method can be applied for use in animal nutrition in general and studies of using phytase in diets for farm animals in particular, by measuring the content of inositol phosphates in feed ingredients, complete feeds, ileal contents, and feces of pigs and poultry.

Keywords: *Inositol phosphates; ^{31}P NMR; feed; digesta; feces; pigs; poultry*

INTRODUCTION

Many studies have been performed to study the beneficial effects of microbial phytase from *Aspergillus niger* on the utilization of phosphorus in diets for pigs and poultry [reviewed by Jongbloed et al. (1996) and Kornegay (1996)]. Phytase renders phosphorus bound in phytate available for absorption by hydrolyzing the orthophosphate groups from the inositol hexaphosphate molecule (IP6). The hydrolysis of dietary IP6 generally is not complete (Jongbloed et al., 1992; Mroz et al., 1994; Kemme et al., 1999). Nevertheless, an increase of the apparent digestibility or absorbability of phosphorus by ~20% is seen when 500 or more phytase units (FTU) per kilogram of diet are supplemented to diets with low intrinsic phytase activity.

Only in a limited number of studies was the disappearance of IP6 in sections of the gastrointestinal tract of pigs and poultry investigated (Jongbloed et al., 1992; Mroz et al., 1994; Kemme et al., 1998; Van der Klis and Versteegh, 1999). In these studies, no information was obtained about the production and disappearance of inositol phosphates with fewer than six phosphate groups. When phytase was incubated together with sodium phytate, the breakdown of IP6 by microbial phytase followed a stepwise pattern in which the intermediate inositol phosphates gradually appeared and disappeared during the time course (Frølich et al., 1986; van Loon et al., 1997). However, it can be questioned whether the in vitro pattern of breakdown is also valid for in vivo situations in which feed and digesta are the milieu of enzyme and substrate interaction. To monitor the action of phytase under in vivo conditions, inositol phosphates in complex matrixes have to be analyzed.

The present investigation describes a ^{31}P NMR method for the determination of inositol phosphates and orthophosphate in feed ingredients, complete feeds, ileal digesta, and feces of pigs and poultry. In addition, the on-line pattern of IP6 breakdown by *A. niger* phytase was registered. It was also investigated whether IP6 analysis with HPLC can be confirmed by the ^{31}P NMR method.

MATERIALS AND METHODS

Materials. (2-Aminoethyl)phosphonic acid and deuterium oxide (D_2O) were obtained from Acrõs Organics (Geel, Belgium), and phytic acid (dodecasodium salt from corn) was from Sigma Chemical Co. (St. Louis, MO). Deionized water was prepared using a Barnstead NANOpure water system and purified to a specific resistance of ≥18 MΩ·cm and a specific conductivity of ≤0.056 μS/cm. *A. niger* phytase (Natuphos) was kindly provided by The Royal Gist-brocades BV (Delft, The Netherlands). All other reagents used were of analytical quality.

Sample Preparation. The sample preparation was based on the method of Bos et al. (1991), with some modifications. Samples of 3.0 g of freeze-dried and ground feed ingredients (1 mm sieve), complete diets, ileal digesta, and feces were extracted with 15.0 mL of 0.75 M HCl during 2 h at 20 °C, under agitation. The extracts were centrifuged, and 3.0 mL of

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the clear supernatant was heated during 15 min at 100 °C, under agitation. After cooling, the sample solutions were centrifuged. Subsequently, 4.0 mL of ethylenediaminetetraacetic acid (EDTA; 30 mg/mL) was added to 2.0 mL of the supernatant. The pH of the solutions was adjusted to 6.0, using 2 M NaOH, and then freeze-dried. The freeze-dried samples were dispersed in 5 mL of water and filtrated (0.45 μm), and the pH was adjusted to 12.6, using solid NaOH. Subsequently, the samples were freeze-dried again.

Sample Analysis. Analysis of the samples was based on the ^{31}P NMR method used by Frølich et al. (1986). ^{1}H-decoupled ^{31}P NMR spectra were recorded at 162 MHz at 295 K on a Bruker AMX 400 wb using either a 20 mm (incubation of sodium phytate and phytase) or a 10 mm probe (all other samples). Spectrometer settings were as follows: 7 s relaxation delay; 20 μs 90° pulse on ^{31}P; 265 μs 90° pulse for ^{1}H decoupling, using a GARP pulse sequence; 6000 Hz spectral width; 160 free induction decays per experiment, averaged in 8K memory. NMR data were processed using exponential multiplication (1 Hz line broadening), zero-filling to 16K memory and Fourier transformation. Chemical shifts for ^{31}P spectra were quoted in parts per million (ppm) downfield from 1 M H_3PO_4 (external reference). For quantification of ^{31}P signals, (2-aminoethyl)phosphonic acid (1.25 mg/mL) was used as an internal reference.

The content of total P in parallel subsamples was determined colorimetrically according to the vanadomolybdate procedure (AOAC, 1980). Phytic acid (IP6) in parallel subsamples of feed ingredients, complete diets, and ileal digesta was assayed with ion-exchange HPLC according to the method of Bos et al. (1991). Other inositol phosphates are not analyzed by this method.

Origin of Samples of Feed Ingredients, Complete Diets, Ileal Digesta, and Feces. The feed ingredients used in this experiment had been tested earlier for their P digestibility in pigs (Šebek et al., 1989) or their P absorbability in broiler chickens (Van der Klis and Versteegh, 1999). The complete diets used had been tested in digestibility experiments with pigs by Jongbloed et al. (1995) and Kemme et al. (1997, 1999) and contained ≤6.0 g of Ca/kg of diet and no phosphate supplements. Complete diets and ileal digesta of broilers were derived from the experiment by Van der Klis and Versteegh (1999). The broiler diets were classified according to four supply levels, the levels being 1.8 and 5.0 (supply level 1), 3.0 and 6.8 (supply level 3), and 3.6 and 8.1 (supply level 4) g of absorbable P and Ca, respectively. Supply level 2 contained 2.5 g of absorbable P and 5.6 g of Ca/kg, but no ^{31}-NMR spectra of these diets were measured. Complete diets and ileal digesta of laying hens came from Van der Klis and Versteegh (1996). The layer diets varied in feedstuff composition and had an average Ca concentration of 32 g/kg. Ileal digesta of pigs originated from the experiment by Kemme et al. (1999). They were classified according to five treatments as follows: (1) no addition; (2) addition of sodium phytate; (3) addition of microbial phytase; (4) addition of microbial phytase and lactic acid; and (5) addition of sodium phytate, microbial phytase, and lactic acid. Feces of pigs originated from a trial of Kemme et al. (1997).

Calculations Made on the Datasets. The assayed contents of the inositol phosphates as determined with ^{31}P NMR were expressed by their P contents using their respective molar ratios. Also, the IP6 content measured with HPLC was expressed as phytic acid P, using the fact that phytic acid contains 28.2% P (w/w). The sum of the P contents, contained in inositol phosphates and orthophosphate and measured with ^{31}P NMR, was related to the total P content as measured colorimetrically in parallel subsamples. The same was done for P contained in IP6 and measured either with ^{31}P NMR or with HPLC. In this way, the balances of total P and phytic acid P could be made within one sample. Also, the standard errors of the observations were calculated within sample type.

RESULTS AND DISCUSSION

Hydrolysis of Sodium Phytate by *A. niger* Phytase. The hydrolysis of sodium phytate by phytase at pH 5.1 is shown in Figure 1. The incubation conditions were adopted from those of Frølich et al. (1986). The ^{31}P resonance assignments were done on the basis of simultaneously appearing and disappearing peaks during the hydrolysis time course. The addition of EDTA (complexing diamagnetic and paramagnetic metal ions) to the samples was essential to obtain the required narrow line widths in the spectra. The phytase activity of the incubation material of Frølich et al. (1986) was incorrectly given, because in this experiment using the same amount of phytase it took 10 times as long to find phytate breakdown patterns as described by Frølich et al. (1986). After 24 h of hydrolysis of phytate by phytase (Figure 1), a spectrum was obtained containing inositol hexa(6)-, penta(5)-, tetra(4)-, and tri(3)phosphates as well as orthophosphate (P). This seems to indicate that these species competed with each other as substrates for *A. niger* phytase. The inositol phosphate species annotated with a star in the spectrum recorded at 48 h (Figure 1) could not be assigned unambiguously. Because two of its resonances seemed to appear at the same time period as the identified inositol triphosphate species, they may well also originate from a minor inositol triphosphate species; the apparent third resonance could be, for instance, a shoulder on the left flank of the orthophosphate peak. The finding of a second inositol triphosphate species is compatible with literature data (Johnson et al., 1995). The breakdown of inositol tri- and diphosphates was much slower than

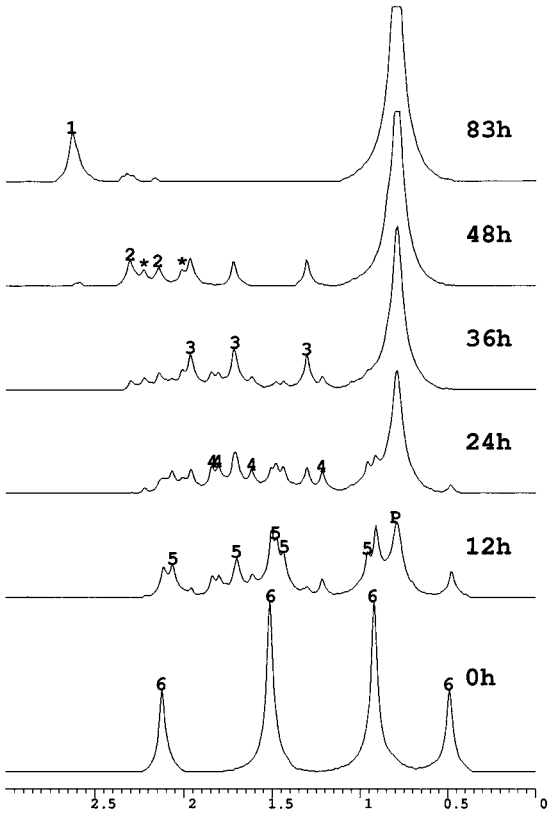

Figure 1. ^{1}H-decoupled ^{31}P NMR spectra showing the hydrolysis of pure sodium phytate at the time intervals indicated. Sample: total volume, 27 mL (20 mm probe); 200 mg of phytate; 0.25 M acetate; 80 mg of EDTA; 1.25 FTU of phytase; and 2 mL of 98% D_2O; pH was adjusted to 5.1. The numbered peaks represent resonances due to inositol hexa(6)-, penta(5)-, tetra(4)-, tri(3)-, di(2)-, and mono(1)phosphates and to orthophosphate (P); peaks marked with * probably represent an additional minor inositol triphosphate species.

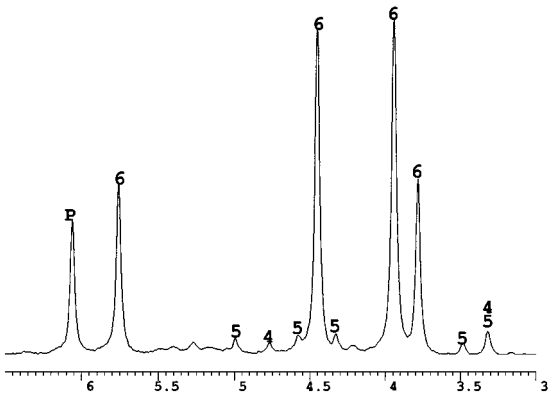

Figure 2. ^{1}H-decoupled ^{31}P NMR spectrum of a feed ingredient sample. Sample: total volume, 5 mL (10 mm probe); 0.4 mL of 98% D_2O; pH was adjusted to 12.6. The numbered peaks represent resonances due to inositol hexa(6)-, penta(5)-, and tetra(4)phosphates and to orthophosphate (P).

that of the higher inositol phosphates. No evidence was obtained for the breakdown of the inositol monophosphate species. This is well in line with the findings of Van Loon et al. (1997).

^{31}P NMR Spectra of Samples of Feed Ingredients and Ileal Digesta. *Feed Ingredients.* A feed ingredient sample containing a typical spectrum of inositol phosphates is shown in Figure 2. This experiment was performed at pH 12.6, and further specifications were as described in the figure legend. The inositol phosphate species were found to be stable at this pH. Experiments that are carried out at this pH do not have the disadvantage of pH shifting of resonances (except for orthophosphate), as is observed at pH 5.1. This is also why the positioning of the peaks and the scaling of the *X*-axes in Figures 1 and 2 are different. Also, the differences in chemical shifts compared to those of Frøhlich et al. (1986, 1988) can be directly related to differences in pH. The occurrence of multiple phosphates (each with 2 pK_a values) on one inositol molecule tends to influence pK_a values of the phosphates by electrostatic interactions due to the negative charges obtained through deprotonation. Therefore, different inositol phosphates have different pK_a values. Around pH 5 and between pH 11 and 12 this is particularly the case. The ^{31}P resonance assignments were done on the basis of a standard solution of IP6. The other peaks were assigned on the basis of comparison between spectra. In general, inositol pentaphosphates were found to be between 5 and 20% of the inositol hexaphosphate concentration. The fraction of inositol tetraphosphate, which could not be completely assigned, was in general <0.05 of the inositol hexaphosphate concentration. Lower inositol phosphates could not be detected and/or quantified unambiguously due to their very low concentrations. Table 1 shows the contents of IP6, IP5, and orthophosphate in various feed ingredients. In general, it appeared that cereals had very low contents of IP5; most of the P was bound in IP6 (70−80%) and orthophosphate. Wheat middlings contained much more P, IP6, IP5, and orthophosphate than wheat, but there were only slight changes in the relative distribution of the P-containing compounds. There were also no clear differences in this distribution among corn and hominy feed. Legumes contained small amounts of IP5, most of the P was bound in IP6, but the fraction of orthophosphate was relatively greater in these feed ingredients than in the cereals. There were only slight changes in the contribution of the P-containing compounds between full-fat soybeans and soybean meal. The byproducts of corn, sunflowerseed, and rapeseed contained more IP5 than the other feed ingredients tested. This is in line with the observations of Bos et al. (1993). The increased amount of IP5 in these feed ingredients is probably due to the wet processing they underwent, which enabled intrinsic phytase to hydrolyze part of the IP6 present in these feed ingredients.

Table 1. Composition of Inositol Hexa- and Pentaphosphates and Inorganic Phosphate (Grams of P per Kilogram of DM) in Feed Ingredients Assayed with ^{31}P NMR

	n	IP6	IP5	P_i[a]	total P[b]
corn	3	2.4	0.2	0.7	3.3
corn germ, extracted	2	4.1	0.6	1.2	7.4
corn gluten feed	2	5.5	0.9	2.8	10.4
hominy feed	1	1.8	0.2	2.3	5.3
wheat	2	2.6	0.3	0.6	4.1
wheat middlings	1	8.7	0.5	1.5	13.5
beans (*Phaseolus vulgaris*)	2	1.9	0.4	1.6	5.4
peas	3	2.2	0.3	0.7	4.3
soybeans, full fat	2	2.8	0.3	1.1	5.5
soybean meal, extracted	3	3.9	0.3	0.9	6.8
sunflowerseed meal, extracted	4	8.5	0.7	1.4	12.7
rapeseed meal, extracted	1	10.0	0.8	2.0	12.7

[a] P_i = orthophosphate. [b] Total P was assayed colorimetrically in parallel samples by using the vanadomolybdate procedure (AOAC, 1980).

Ileal Digesta. Spectra of inositol phosphates and orthophosphate ileal digesta were similar to those obtained with feed ingredients (Figure 2). The finding of very limited amounts of lower inositol phosphates was in accordance with HPLC data from Sandberg and Ahderinne (1986) and Skoglund et al. (1997a) in studies with humans and with Skoglund et al. (1997b) and Schlemmer et al. (1997) in studies with pigs and/or broilers. Also, Kemme et al. (1998) calculated on the basis of the difference between duodenal phytic acid degradation and total tract digestibility of P that once-hydrolyzed phytic acid is almost completely degraded in the gastrointestinal tract of pigs. It was hypothesized that the velocity with which dietary IP6 comes into solution is governing the total process of hydrolysis of IP6 into inositol and orthophosphate groups. No accumulation of IP3, IP2, and IP1 was observed in ileal digesta of pigs, which is in contrast to the in vitro experiment with sodium phytate and microbial phytase reported in this paper. Possible explanations for this apparent contradiction are a relative shortage of inositol phosphates in solution in digesta that forces the phytase to attack the lower inositol phosphates and/or the possibility that intestinal phosphatases are able to efficiently breakdown the lower inositol phosphates in the gastrointestinal tract. The latter explanation seems to be likely because *A. niger* phytase is not capable of degrading IP1, whereas no accumulation of this inositol phosphate species was found in ileal digesta. Also, when ileal digesta of pigs and broilers were incubated in vitro with *A. niger* phytase, a decrease in IP6 content of the digesta was observed but no quantifiable increase in the levels of lower IPs could be demonstrated (data not shown). An alternative explanation could be that IP4−IP1 are not yet formed in the intestinal digesta of the pig because the reaction time is too short. Figure 1 showed that IP4 was first formed after 12 h of incubation. However, phytase activity in this incubation sample was 10 times lower than it is in gastric contents

Table 2. Average Composition of Inositol Hexa- and Pentaphosphates and Inorganic Phosphate (P_i) Assessed with ^{31}P NMR in Feed Ingredients, Complete Feeds, Ileal Digesta of Pigs and Poultry, and Feces of Pigs (Grams of P per Kilogram of DM; Mean ± SD) and Recoveries[a] of Total P and IP6 (Percent; Mean ± SD)

item	*n*	IP6	IP5	P_i	recovery of total P	recovery of IP6
feed ingredients	26	4.4 ± 3.0	0.4 ± 0.3	1.3 ± 0.7	83 ± 16	104 ± 25
complete diets, pigs[b]	11	3.6 ± 0.7	0.2 ± 0.1	0.8 ± 0.2	95 ± 7	94 ± 9
complete diets, broilers[c]						
supply level 1	9	1.9 ± 1.0	0.2 ± 0.0	1.8 ± 0.8	96 ± 12	88 ± 12
supply level 3	9	1.9 ± 0.9	0.1 ± 0.0	3.9 ± 1.0	108 ± 15	91 ± 14
supply level 4	3	2.6 ± 1.8	0.2 ± 0.1	2.5 ± 0.9	83 ± 5	81 ± 14
complete diets, laying hens	4	2.7 ± 0.7	0.2 ± 0.0	2.7 ± 0.5	98 ± 11	79 ± 7
ileal digesta, pigs						
treatment 1	4	9.7 ± 0.8	0.4 ± 0.1	1.7 ± 0.6	87 ± 5	84 ± 3
treatment 2	4	13.8 ± 1.8	0.5 ± 0.1	1.9 ± 0.3	89 ± 8	83 ± 8
treatment 3	4	6.7 ± 2.4	0.6 ± 0.1	1.4 ± 0.5	87 ± 7	85 ± 3
treatment 4	4	4.3 ± 1.4	0.6 ± 0.1	1.2 ± 0.2	71 ± 7	81 ± 5
treatment 5	3	6.7 ± 0.9	1.2 ± 0.3	1.5 ± 0.2	75 ± 4	87 ± 5
ileal digesta, broilers[d]						
supply level 1	36	5.7 ± 3.4	0.2 ± 0.1	1.0 ± 0.5	95 ± 12	108 ± 12
supply level 2	12	6.4 ± 1.9	0.2 ± 0.0	1.3 ± 0.3	81 ± 7	92 ± 5
supply level 3	79	6.9 ± 2.6	0.3 ± 0.1	2.2 ± 1.3	94 ± 10	100 ± 12
supply level 4	12	7.6 ± 2.4	0.3 ± 0.2	2.2 ± 0.9	89 ± 8.7	91 ± 8
ileal digesta, laying hens[e]	16	8.0 ± 4.1	0.5 ± 0.3	1.9 ± 1.1	89 ± 10.1	105 ± 23
feces, pigs[f]	14	0.8 ± 0.6	0.0 ± 0.0	18.1 ± 4.1	102 ± 7	nd[g]

[a] Recovery of total P was calculated as the sum of IP6, IP5, and orthophosphate as analyzed by ^{31}P NMR divided by the total P content of the sample as analyzed colorimetrically and divided by 100%. Recovery of IP6 was calculated as IP6 content as analyzed by ^{31}P NMR divided by IP6 content of the sample as analyzed by HPLC and divided by 100%. [b] Four of the pig diets contained IP5 levels that were just below the quantification level of 0.12 g of P/kg of DM, but the peaks were clearly detected; the contribution of these peaks to the recovery of total P was set at 0.12 g of P/kg of DM. [c] Six of the complete diets for broilers at supply levels 1 and 3 and two at level 3 contained IP5 levels that were just below the quantification level of 0.12 g of P/kg of DM, but the peaks were clearly detected; the contribution of these peaks to the recovery of total P was set at 0.12 g of P/kg of DM. [d] Numbers of observations of the recovery of IP6 were 18, 6, 40, and 6 for supply levels 1, 2, 3, and 4, respectively. [e] Number of observations of the recovery of IP6 was 8. [f] Data of feces of pigs receiving diets with and without added phytase were pooled. There were no differences in IP6 and IP5 concentrations among these groups but P_i concentration was higher in feces of pigs receiving the diets without phytase. [g] nd, IP6 content of feces of pigs was not determined with HPLC; thus, recovery of IP6 was not calculated.

of pigs fed 800 FTU of microbial phytase/kg of diet. This implies that at this dose (at similar conditions) IP4 will be first formed after 1.2 h. This time is much shorter than the average gastric retention time; thus, it can be concluded that a too short reaction time between phytate and phytases is not the reason for the absence of lower inositol phosphates in ileal digesta of pigs.

Applicability of the ^{31}P NMR Method for Characterization of Inositol Phosphates in Feed Ingredients, Complete Diets, Ileal Digesta, and Feces of Pigs and Poultry. *Variation in Composition of the Various Sample Types.* Among the various batches of feed ingredients, a high variation in IP6, IP5, and orthophosphate contents was shown, as may be expected (Table 2). Figures of the individual feed ingredients were already listed in Table 1. Concentrations of IP6 in the complete diets for pigs were somewhat higher and orthophosphate concentrations lower than in Dutch practical diets. IP5 concentrations were low in all pig diets, and some were below the quantification level of 0.12 g of P/kg of DM. The analyzed complete diets for broilers were split into three supply levels of absorbable P and Ca, because higher Ca (and P) levels may have a detrimental effects on the accuracy of the ^{31}P NMR method. Feed ingredient composition was similar for the diets at supply levels 1 and 3, except for limestone and feed phosphate, and thus their contents of IP6 and IP5 were similar. The IP5 levels were low or even below the quantification level. The complete diets for laying hens varied in feedstuff composition, which reflected their IP6 content (1.7−3.4 g of IP6 P/kg of DM). Again, IP5 concentrations were low. The level of orthophosphate in these diets was elevated due to supplementation of a feed phosphate. Addition of sodium phytate to the diet (treatment 2) caused an increase in IP6 content of the samples of pig digesta as compared to the diet without any addition (treatment 1), whereas addition of microbial phytase (treatment 3) caused a decrease of the IP6 content of the digesta. When lactic acid was added to the diet that was already supplemented with phytase (treatment 4), a further reduction in IP6 content of the digesta was observed. When all three supplements were added to the pig diet (treatment 5), IP6 content of the digesta remained at the same level as was observed for phytase addition only, but the content of IP5 was increased. In the ileal digesta of broilers, the concentration of orthophosphate depended on the supply level of absorbable P and so did the concentration of IP6. Again, concentrations of IP5 were low. The ileal digesta of laying hens showed some variation in IP6 content due to the variation present in IP6 content of the diets. IP5 and orthophosphate concentrations were comparable to those observed in ileal digesta of broilers at supply levels 3 and 4. The data for pig feces refer to feces from pigs receiving diets with and without added phytase. There were no differences in IP6 concentration among the two dietary groups, but orthophosphate concentration was higher in feces from pigs receiving the diets without phytase (data not shown). The concentration of IP6 was low, and IP5 was not even present in pig feces. This indicates that IPs are almost completely hydrolyzed by the bacterial microflora in the hindgut of the pig. The finding that there were high concentrations of orthophosphate present in feces of pigs fed below their P requirement, whereas the orthophosphate concentration in ileal digesta was low, supports the common opinion that there is no net absorption in the hindgut of pigs (Jongbloed, 1987).

Analysis of the Accuracy of the ^{31}P NMR Method. ^{31}P NMR spectroscopy is a fully quantitative technique that

measures the number of P nuclei as bound in compounds. For quantification of ^{31}P signals, (2-aminoethyl)phosphonic acid was added directly tot the sample prior to analysis as an internal reference. Breakdown of the marker under sample conditions did not occur within the time of sample analysis. Only after a time period of 24 h was a small reduction in the amount observed. Spectra of the inositol phosphates and orthophosphate remained stable for at least 64 h. The recovery of IP6 was tested by spiking a feed ingredient (corn gluten feed) sample with sodium phytate. The recovery of this IP6 standard in this sample was 106%, which was slightly higher than that measured with HPLC by Skoglund et al. (1997a). Variation of repeated measurements within one sample was well below the error made in the estimation of the integral. The error made in the estimation of the integrals was ~5% for the higher concentrated ones (in general, IP6 and orthophosphate) and ~10% for the ones with lower concentrations (in general, IP5 and IP4).

Sources of Bias Influencing the Recoveries of Total P and IP6. The recovery of total P in the form of IP6 plus IP5 plus orthophosphate was generally well below 100% (Table 2). This can be explained by several factors involved in the analytical method. First, samples were concentrated 7.5 times during their preparation for the ^{31}P NMR method. Spillage of sample material during sample treatment (as caused by, e.g., foam formation during freeze-drying) will underestimate the levels of IP6, IP5, and orthophosphate. Second, P contained in DNA and phospholipids is not registered by the ^{31}P NMR technique, but these compounds contribute to the total P content of the sample. Third, lower inositol phosphate concentrations often were below the detection limit of the ^{31}P NMR method, but again they contribute to the total P concentration of the sample. The recovery of IP6 tended to be below 100%, which can also be explained by the errors mentioned for concentration of the samples for ^{31}P NMR analysis. On the other hand, IP6 concentrations may be overestimated when HPLC is used (Lehrfeld and Morris, 1992). IP6 appeared to be stable at pH <1 and 60 °C during 48 h. It can thus be excluded that IP5 concentrations were increased due to IP6 degradation during sample preparation.

Apart from the systematical errors mentioned above, the sampling error between parallel samples (0.13 g of P/kg of DM; Jongbloed, 1987) and the analytical error of the measurement of total P (0.12 g of P/kg of DM; Jongbloed, 1987) and IP6 (~8%) methods should be taken into account.

General Use of the ^{31}P NMR Method. Xu et al. (1992) have already stated that ^{31}P NMR techniques showed good prospective for the analysis of IP profiles in foods. However, since the experiments of Frølich et al. (1986) and Johnson et al. (1995), no clear progress in the inositol phosphate analysis with ^{31}P NMR techniques in foods and particularly in feed has been made. Most research in this field was focused on the development of HPLC and HPLC-derived techniques to solve the problem of quantification of inositol phosphates. The ^{31}P NMR technique has as its main advantages over the HPLC techniques that it does not necessitate standards that may cause background matrix effects and that the spectra of inositol phosphates and orthophosphate appear in the same run without further sampling errors. The main disadvantage is the relative insensitivity of the NMR technique. To overcome the insensitivity problem, we concentrated our samples with a factor 7.5, including two freeze-drying steps, which inevitably reduced precision and led to systematically lowered values. Still, for the various samples concentrations of lower inositol phosphates appeared to be below the quantification level (0.19 mmol of inositol phosphate/kg of DM in the material before sample preparation). The method developed can be used in complete diets for pigs and poultry and can be used for confirmation of HPLC methods. Levels of Ca up to 30 g/kg do not cause any decrease in the resolution of the method. However, variation in the recoveries of total P and IP6 with feed ingredients is still too high, and further studies are required to elucidate which factors cause this variation and disturb the accuracy of the method when applied on feed ingredients. Concentrations of IP4−IP1 in digesta of pigs and poultry generally were not detectable, but the P content that is incorporated in these compounds seems to be not important for animal nutrition. The accuracy of inositol phosphate analysis and the insight in the absolute contents of lower IPs in digesta may be improved in the future when a measurement system in which HPLC and ^{31}P NMR techniques are combined can be used for inositol phosphate analysis.

Conclusions. The ^{31}P NMR method described can be applied for quantification of inositol phosphate concentrations in feed ingredients, complete diets, intestinal contents, and feces of pigs and poultry. The concentration of inositol phosphates with four or fewer phosphorus ester bonds in these types of samples is too low for quantification with this technique, but this does not disqualify this technique for use in animal nutrition. The kinetics of breakdown of inositol hexaphosphate present in feed ingredients by phytase in the gastrointestinal tract of pigs and poultry are completely different from those of incubations of sodium phytate with phytase.

Results of the inositol hexaphosphate analysis in the mentioned sample types obtained by HPLC analysis were confirmed by the ^{31}P NMR method.

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