STUDIES ON THE EXTENT OF RUMINAL DEGRADATION OF PHYTATE FROM DIFFERENT FEEDSTUFFS

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ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Ca	Calcium
CP	Crude protein
CSM	Cottonseed meal
Cu	Copper
DM	Dry matter
DMI	Dry matter intake
DNA	Desoxyribonucleic acid
f:c	forage:concentrate ratio
Fe	Iron
FTU	Standard unit to express phytase activity
GfE	Gesellschaft für Ernährungsphysiologie
hRSM	Heat treated rapeseed meal
K	Potassium
IEP	Isoelectric point
InsPs	*Myo*-Inositol phosphates
InsP	*Myo*-Inositol monophosphate
$InsP_2$	*Myo*-Inositol bisphosphate
$InsP_3$	*Myo*-Inositol trisphosphate
$InsP_4$	*Myo*-Inositol tetrakisphosphate
$InsP_5$	*Myo*-Inositol pentakisphosphate
$InsP_6$	*Myo*-Inositol hexakisphosphate, here: phytate
$InsP_6$-P	Phoshorus bound in $InsP_6$
Mg	Magnesium
Mn	Manganese
MNaP	Monosodium phosphate
Na	Sodium
NRC	National Research Council
P	Phosphorus
Pi	Inorganic phosphorus
PSV	Protein storage vacuole
R^2	Coefficient of determination
RNA	Ribonucleic acid
RSM	Rapeseed meal
Rusitec	Rumen simulation technique
SBM	Soybean meal
TMR	Total mixed ration
Zn	Zinc

CHAPTER 1

EXTENDED INTRODUCTION

1 EXTENDED INTRODUCTION

Phosphorus (P) is an essential element for the metabolism of animals. 80 to 85% of the total body P is located in the bones as hydroxylapatite ($Ca_5(PO_4)_3OH$), where it ensures bone stability and serves as an important P reserve, from which P can be mobilised in times of deficiency. The remaining P is found in the soft tissue, where it is essential for diverse enzymatic reactions especially in energy metabolism and transfer as a component of ATP, ADP, AMP, and creatine phosphate (Karn, 2001). Phospholipids are essential compounds of cellular membranes and P containing nucleic acids represent the basis of genetics as components of DNA and RNA (Pfeffer et al., 2005). In ruminants, P needs of the rumen microorganisms also have to be considered as ruminal fermentation, digestibility of organic matter and protein synthesis can decrease when the P requirements of the microorganisms are not met (Kincaid and Rodehutscord, 2005). Besides the requirements of P for maintenance, growth, and reproduction of the animal, dairy cows additionally require P for milk production.

To ensure a sufficient P supply, diets for dairy cows are commonly supplemented with mineral P, often including a safety margin which leads to dietary P concentrations exceeding the science based recommendations given by the Gesellschaft für Ernährungsphysiologie (GfE) or the National Research Council (NRC). This, however, is of environmental concern. Between P intake and faecal P excretion there is a linear relationship leading to higher excretion of P at high levels of P intake (Wu et al., 2000; Wu et al., 2001; Ekelund et al., 2005). As the P uptake is regulated by the gut according to the needs of the body, the absorption of excessive P is reduced (Wu et al., 2000) and surplus P is excreted. Excessive P from supplemented diets is excreted in water-soluble forms (Dou et al., 2002) and thus contributes to the eutrophication of surface water when applied to the farmland with manure in excessive amounts. Furthermore, the known global phosphate deposits are finite resources (Mengel, 1997) and the European Commission (EC) recently added rock phosphate to the list of Critical Raw Materials (European Commission Press Release, 2014) emphasising its economic importance and concomitant supply risk. Therefore, ruminant nutrition should aim for minimising the use of mineral P sources by optimising the utilisation of organic P.

In plant seeds and grains, organic P is mainly bound in inositol phosphates (InsPs) which consist of an inositol ring with six carbon atoms, each associated with a hydroxyl group. Depending on the number of attached phosphate groups InsPs are denominated as InsP, $InsP_2$,

$InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$, also known as *myo*-inositol (1,2,3,4,5,6) hexakis(dihydrogen phosphate) or phytic acid. Phytic acid, however, occurs primarily as salts of mono- and divalent cations (Reddy et al., 1989) which are summarised under the term phytate. As the salt-free form of $InsP_6$ is unlikely to exist in nature (Shears and Turner, 2007), $InsP_6$ is used as abbreviation for phytate in the present thesis.

In the mature seed, P bound in $InsP_6$ represents the major fraction of total P, the amount however, varies distinctly between species (e.g. 42% in rye (Rodehutscord et al., 2016) and 85% in maize (Ravindran et al. 1994)). Detailed information about $InsP_6$ concentrations in different feedstuffs are provided in several studies and reviews (Reddy et al., 1989; Eeckhout and de Paepe, 1994; Ravindran et al., 1994; Ravindran et al., 1995; Kasim and Edwards, 1998; Rodehutscord et al., 2016). The concentrations of $InsP_5$ and lower phosphorylated InsPs in grains are only small, if there are any detectable at all (Kasim and Edwards, 1998; Rodehutscord et al., 2016). In milling products, the amounts of $InsP_5$ and lower InsPs tend to increase, an effect that can probably be ascribed to the fact that intrinsic phytase comes in contact with $InsP_6$ during the milling process and starts the hydrolysis process (Kasim and Edwards, 1998). Significant concentrations of $InsP_5$ and lower InsPs accompanied by smaller amounts of $InsP_6$ can be found in further processed plant products as processing conditions can cause extensive hydrolysis of $InsP_6$ and $InsP_5$ (Kasim and Edwards, 1998).

$InsP_6$ accumulates in most cereals and legumes during the ripening process in electron-dense spherical particles, the globoids, which are located in the protein storage vacuoles (PSV) of the seeds. In cereals, the globoids are predominantly found in the aleurone layer with the exception of maize, where $InsP_6$ is located in the germ (O'Dell, 1972). In oilseeds and legumes, the globoids are distributed throughout the kernel (Ravindran et al., 1995). The composition and structure of globoids depends on the plant species. Some plants are lacking globoids and $InsP_6$ is stored in the PSV without compartmentalisation (Reddy et al., 1989). Besides protein and $InsP_6$, high concentrations of several minerals, mainly K, Mg, Ca, and Fe, can be found in the globoids (Reddy et al., 1989; Bohn et al., 2008). Again, the concentrations of minerals found associated with globoids differ between plant species indicating the presence of different phytates in different seeds. The solubility of $InsP_6$, however, is determined by the associated mineral (Jackman and Black, 1951). Furthermore, $InsP_6$ is capable of binding proteins in some seeds and the binary protein-phytate complexes seem to be less susceptible to hydrolysis (Selle et al., 2012).

The cleavage of the phosphate residues from $InsP_6$ requires phytate-degrading enzymes (phytases). Phytases (*myo*-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases) are defined as phosphatases with the capability to catalyse the stepwise hydrolysis of $InsP_6$ to orthophosphate and lower InsPs (Konietzny and Greiner, 2002). Phytases can be categorised according to their catalytic mechanisms, their pH optima (acid or alkaline phytases) or the position of the carbon in the *myo*-inositol ring at which dephosphorylation is initiated (3-phytases, E.C.3.1.3.8; 6-phytases, E.C. 3.1.3.26; 5-phytases, E.C. 3.1.3.72; Greiner and Konietzny, 2006). The carbon atoms of the inositol ring are numbered following the rules recommended by the International Union of Pure and Applied Chemistry (IUPAC) based on Bernard Arganoff's turtle (Figure 1).

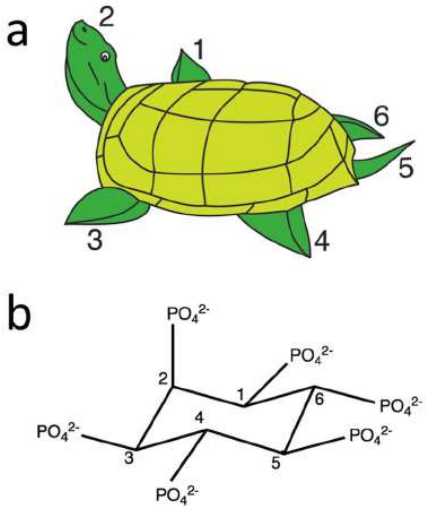

FIGURE 1. a) Agranoff's turtle and b) $InsP_6$ numbered according to Agranoff's turtle with one axial and five equatorial hydroxyl groups (Ariza et al., 2013)

The D-numbering starts with the carbon representing the right flipper of the turtle and proceeds anticlockwise around the inositol ring whereas the L-numbering starts with the carbon on the left flipper and proceeds clockwise. 3-phytases initialise the dephosphorylation at the D-3 position of $InsP_6$, 6-phytases from plant seeds at the L-6 (D-4) position, and 5-phytases at the D-5/L-5 position. The current rule is to use the D-numbering, thus 6-phytases from plant seeds should correctly be classified as 4-phytases, however, 6-phytase is still commonly used. In the present work, InsPs were analysed using high-performance ion chromatography (HPIC) which co-elutes enantiomers. As a result, Ins(1,2,3,4,5)P_5 for example could be D-Ins(1,2,3,4,5)P_5 or D-Ins(1,2,3,5,6)P_5 (equivalent to L-Ins(1,2,3,5,6)P_5 or L-Ins(1,2,3,4,5)P_5, respectively),

hydrolysis products of 4- or 6-phytases. To facilitate understanding, only the term '6-phytase' is used to describe phytases generating Ins(1,2,3,4,5)P_5 in the present thesis.

Phytases are widely distributed in plants, microorganisms and some animal tissues (Greiner, 2007). Mucosal phytase activity is described for pigs (Hu et al., 1996) and broiler (Selle and Ravindran, 2007; Huber et al., 2015), but for ruminants data regarding mucosal phytase activity are lacking. The few studies describing $InsP_6$ hydrolysis in the small intestine of ruminants, however, indicate that mucosal phytate activity seems to be physiologically irrelevant in ruminants (Humer and Zebeli, 2015).

Plant phytases are generally considered to be 6-phytases, because the predominant *myo*-inositol pentakisphosphate generated from phytases of rye, barley, spelt, oat, wheat bran, and rice has been identified as Ins(1,2,3,4,5)P_5 (Konietzny and Greiner, 2002). The activity of intrinsic phytase differs considerably between plant species. While phytase activity, for example, is very low or not detectable in maize and oats (≤ 143 U/kg DM), rye, triticale, and wheat show high phytase activities in the range of 1193-5130 U/kg DM (Eeckhout and de Paepe, 1994; Rodehutscord et al., 2016).

To date, 3-phytases represent the largest group of phytases and are generally found in fungi, bacteria and yeasts. However, activity of 3-phytases cannot be exclusively ascribed to microorganisms and 6-phytases do not only occur in plants. Greiner et al. (2000) and van der Kaay and van Haastert (1995) found indications of 6-phytase activity in microorganisms such as *Echerichia coli* and *Paramecium*, and activity of 3-phytase has been identified in soybean seeds (Konietzny and Greiner, 2002). Raun et al. (1956) demonstrated for the first time that rumen microorganisms produce phytase and concluded that the majority of phytase present in the rumen originates from rumen bacteria. A survey of phytase activity in cultures of the predominant rumen bacteria showed measurable activity in the strains of *Selenomonas ruminantium, Megasphera elsdenii, Prevotella ruminicola, Mitsuokella multiacidus,* and *Treponema* spp. (Yanke et al., 1998). Further phytase producing strains have been reported by Lan et al. (2002; *Mitsuokella jalaludinii)* and Nakashima et al. (2007*; Selenomonas lacticifex*).

5-phytases are described only for lily pollen (Barrientos et al., 1994), *S. ruminantium* subsp. *lactilytica* (Puhl et al., 2008), and *Bifidobacterium pseudocatenulatum* (Haros et al., 2009).

The potential of rumen microorganisms to hydrolyse $InsP_6$ seems to be high. Over the years, several *in vivo* studies measuring faecal $InsP_6$ excretion confirmed a total tract $InsP_6$

disappearance in the range of 93 to 99% for ruminants (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992; Ray et al., 2013). Other studies, however, determined lower values for $InsP_6$ disappearance. Ray et al. (2012) found that total tract $InsP_6$ disappearance from a high forage diet was only 83%. Yanke et al. (1998) observed higher phytase activity of rumen microorganisms in high grain diets indicating that $InsP_6$ hydrolysis could be impaired in high forage diets. Knowlton et al. (2007) and Brask-Pedersen et al. (2013) supplemented concentrate rich diets for lactating cows with exogenous phytase. Apparent total tract digestibility of P increased for the supplemented diet (46 vs. 39%; Knowlton et al., 2007) as well as ruminal and total tract $InsP_6$ disappearance (96 vs. 86% and 94 vs. 90%, respectively; Brask-Pedersen et al., 2013). This is in accordance with the findings of Bravo et al. (2002), where phytase supplementation of a high concentrate diet increased *in situ* P disappearance from soybean meal (SBM) and rapeseed meal (RSM) but had no effect in high forage diets. This could indicate that the ruminal passage rate influences the extent of ruminal $InsP_6$ hydrolysis as suggested by Kincaid et al. (2005) and exogenous phytase might have the potential to increase ruminal $InsP_6$ hydrolysis when a high ruminal turnover is given by high concentrate diets. An incomplete total tract $InsP_6$ disappearance was also observed by Mathur (1953), where values ranged from 57 to 61% when the amount of Ca in the concentrate was high. A decreased $InsP_6$ hydrolysis after Ca supplementation was observed also in pigs (Sandberg et al., 1993), broiler (Tamim et al., 2004), and *in vitro* (Akter et al., 2015). In a semi-continuous culture system (Rusitec), Godoy and Meschy (2001) observed a reduction of ruminal $InsP_6$ disappearance of approximately 18% when inorganic P replaced organic P as the main P source in the buffer. The repressing impact of high phosphate conditions on the synthesis of phytases is known, however, the extent of suppression seems to differ markedly between microbial species (Vats and Banerjee, 2004).

In vitro and *in situ* studies identified differences in extent and rate of $InsP_6$ hydrolysis between individual feedstuffs. Wheat middlings, hominy, rice bran, SBM, and dried distillers grain (Morse et al., 1992) as well as wheat (Brask-Pedersen et al., 2011) showed a rapid $InsP_6$ disappearance which was almost complete or completed between 6 and 8 h of *in vitro* incubation, while a similar extent of $InsP_6$ disappearance was observed only at later incubation times for cottonseed meal (Morse et al., 1992) or rapeseed cake (Brask-Pedersen et al., 2011). Further processing of feedstuffs also affects $InsP_6$ disappearance. Park et al. (1999) and Martín-

Tereso et al. (2009) showed that formaldehyde treatment suppresses *in situ* ruminal $InsP_6$ disappearance. The same effect was observed for heat treatment (Konishi et al., 1999).

Despite the observations of incomplete $InsP_6$ hydrolysis in ruminants, systematic studies on this subject are scarce. Better knowledge about differences in $InsP_6$ hydrolysis between feedstuffs and diets as well as factors that might affect the extent of $InsP_6$ hydrolysis, could be helpful to improve the utilisation of organic P in ruminant nutrition by dietary means.

CHAPTER 2

OVERVIEW AND RESEARCH QUESTIONS OF THE INCLUDED MANUSCRIPTS

2 OVERVIEW AND RESEARCH QUESTIONS OF THE INCLUDED MANUSCRIPTS

The general objective of the present thesis was to gain better knowledge about $InsP_6$ hydrolysis in ruminants and factors that might have the potential to affect the extent of $InsP_6$ hydrolysis. Different methodical approaches were used which allowed investigating the $InsP_6$ hydrolysis from total mixed rations (TMR) and different concentrates commonly used in ruminant nutrition. Furthermore, this enabled the estimation of the extent of ruminal $InsP_6$ hydrolysis as well as the determination of total tract disappearance of $InsP_6$. Concentrations of $InsP_6$ were analysed in the *in vivo* and *in vitro* studies and $InsP_5$, $InsP_4$, and $InsP_3$ concentrations additionally in the *in situ* experiment. However, a decrease in the analysed concentrations of InsPs does not necessarily imply a complete degradation of $InsP_6$ with the release of all phosphate groups as $InsP_2$ and InsP still might have been present. Hence, the term 'disappearance' rather than 'degradation' is used throughout the result sections of the manuscripts and the general discussion of the present thesis. In all experiments, the influence of dietary $InsP_6$ concentration and additional mineral P was examined.

Three manuscripts arouse from the studies which are presented in Chapter 4. The objectives can be summarised as follows.

MANUSCRIPT 1: Effect of mineral and rapeseed phosphorus supplementation on phytate degradation in dairy cows

Published in *Archives of Animal Nutrition*

The extent of total tract disappearance of $InsP_6$ in ruminants seems to be influenced by the composition of the diet. However, the results of different studies are hard to interpret as the effect on $InsP_6$ hydrolysis could also be ascribed to different passage rates of the digesta caused by differences in DMI and forage to concentrate ratios. Therefore, the first objective of this study was to compare *in vivo* total tract disappearance of $InsP_6$ in lactating dairy cows from a diet with or without supplementation of mineral P and two diets with high $InsP_6$ concentrations at a constant forage to concentrate ratio. The second objective was to estimate the ruminal disappearance of $InsP_6$ from the diets by incubating three diets with a rumen simulation technique (Rusitec).

MANUSCRIPT 2: Ruminal phytate degradation of maize grain and rapeseed meal *in vitro* and as affected by phytate content in donor animal diets and inorganic phosphorus in the buffer

Published in the *Journal of Animal Physiology and Animal Nutrition*

The ruminal P disappearance differs considerably between feedstuffs. With $InsP_6$ being the major storage form of P, the different localisation and distribution of $InsP_6$ in plant seeds may be responsible for differences in ruminal $InsP_6$ hydrolysis and thus in the disappearance of P between feedstuffs. The first objective of the study was to compare the *in vitro* degradation of $InsP_6$ from maize and RSM and to determine the effect of $InsP_6$ concentration in the diets fed to donor animals of rumen fluid. The second objective was to study the effect of mineral P supplementation on *in vitro* $InsP_6$ degradation from maize and RSM. $InsP_6$ concentrations were analysed in both feed residues and fermenter liquid to examine if eluted $InsP_6$ from the feed residues disappears or accumulates in the fermenter liquid.

MANUSCRIPT 3: Effect of dietary mineral phosphorus and phytate on *in situ* ruminal phytate disappearance from different concentrates in dairy cows

Accepted by the *Journal of Dairy Science*

Besides localisation and distribution of $InsP_6$, plant seeds differ markedly in their intrinsic phytase activity. The extent to which intrinsic phytase activity contributes to ruminal $InsP_6$ hydrolysis is unclear. Furthermore, knowledge about the main phytases active in the rumen and how they are affected is scarce. The first objective of this study was to determine the influence of P and $InsP_6$ concentration in the diet fed to dairy cows on *in situ* ruminal $InsP_6$ disappearance from five different concentrates differing in $InsP_6$ concentrations, localisations, storage forms, and intrinsic phytase activity (SBM, RSM, heat treated RSM, wheat, and maize). The second objective was to determine the main primary degradation products of $InsP_6$ for each concentrate and diet and, thus, draw conclusions about the involved phytases and how they might be influenced by the diet.

CHAPTER 3

GENERAL DISCUSSION

3 GENERAL DISCUSSION

Due to phytase activity of rumen microorganisms, ruminants are able to hydrolyse $InsP_6$ and hence utilise the released P. But the extent of $InsP_6$ hydrolysis seems to be influenced by several factors, which makes the efficacy of ruminal phytases of environmental interest. The necessity of supplementation of mineral P depends on the amount of released $InsP_6$-P that can be utilised to meet the animals' P requirements. Additionally, undegraded $InsP_6$ und thus unabsorbed $InsP_6$-P is excreted with the faeces, contributing to eutrophication. To date, there is a lack of studies examining dietary factors which have the potential to modulate $InsP_6$ hydrolysis in ruminants.

The characteristics of $InsP_6$ differ markedly between plant seeds. To understand how this might influence solubility and hydrolysis of $InsP_6$, the following section gives an overview of differences in localisation and binding of $InsP_6$ in plant seeds. Based on the results of the *in vivo*, *in vitro*, and *in situ* studies, differences in $InsP_6$ disappearance between feedstuffs (concentrates and TMR) and methods are discussed. Furthermore, the correlation between CP and $InsP_6$ disappearance is considered. As potential factors of influence on $InsP_6$ hydrolysis the $InsP_6$ and P concentrations in the diets fed to the animals used for the experiments were examined with each methodical approach. As the isomers of $InsP_5$ were described in Manuscript 3, their meaning for assessing the efficacy of different phytases in the rumen is discussed. Concentrations of lower InsPs ($InsP_4$ and $InsP_3$) are also included in the general discussion. Finally, consequences of the findings for ruminant nutrition and topics that require further research are addressed.

3.1 $INSP_6$ DISAPPEARANCE AND AFFECTING FACTORS

3.1.1 DISAPPEARANCE FROM DIFFERENT CONCENTRATES

SOLUBILITY OF $INSP_6$

Phytic acid is strongly negatively charged over a wide range of pH values and thus shows a high potential for chelating with positively charged minerals and proteins. These interactions can occur in the seed as well as in the gastrointestinal tract (Yu et al., 2012). The solubility of phytate complexes determines the hydrolysis of $InsP_6$, as phytate complexes are poorly susceptible to enzymatic dephosphorylation while undissolved (Schlemmer et al., 2009). The

main metals described in the context of phytate-mineral chelates in the literature are Ca, Zn, Mg, K, Fe, and Mn (Urbano et al., 2000; Ockenden et al., 2004; Bohn et al., 2008; Schlemmer et al., 2009). Despite the high affinity of Cu to phytic acid, Cu-phytates do not seem to be the primary storage facility for this element (Bohn et al., 2008) as Cu concentrations in globoids are low (Bohn et al., 2007). The relative stability and solubility of mineral-phytate complexes differs between the various chelates and depends on the pH value. Low solubility for Mg-phytates was reported at pH values >5 (Cheryan et al., 1983) or >7 (Jackman and Black, 1951), for Ca-phytates at pH >4 (Grynspan and Cheryan, 1983) or pH >6 (Jackman and Black, 1951; Scheuermann et al., 1988). Fe-phytates showed low solubility at a pH >5 (Jackman and Black, 1951) and Zn-phytates at pH values >4 (Scheuermann et al., 1988). The solubility of K-phytates (Cheryan, 1980) and Na-phytates (Scheuermann et al., 1988) is reported to be generally high. Experimental conditions such as temperature or $InsP_6$ to mineral ratios in the medium have further impact on the solubility of phytate complexes (Reddy et al., 1989) which might explain the deviation between the pH values given for Mg- and Ca-phytates. Solubility studies of mixed salts of phytic acid are lacking, but the solubility behaviour appears to be complex as for example the addition of Ca can reduce or increase Zn incorporation in the complex depending on the Zn:Ca ratio (Cheryan, 1980).

Unlike these studies, where the solubility of specific phytate-mineral chelates was examined, the solubility of $InsP_6$ from different concentrates was determined in the present work. In the course of the *in situ* study (Manuscript 3), the water-soluble fraction of $InsP_6$ was determined in the incubated concentrates (SBM, RSM (solvent extracted), hRSM (heat-treated RSM), wheat, and maize). The concentrates were soaked for one minute in double-distilled water and $InsP_6$ concentrations in the filter residues were analysed. The ranking of $InsP_6$ solubility values was maize (71%) > SBM (26%) > wheat (19%) > hRSM (8%) > RSM (-22%). The instantly soluble fraction of $InsP_6$ in a rumen fluid-buffer solution was determined for maize and RSM *in vitro* (Manuscript 2) which further confirmed a high solubility of $InsP_6$ in maize (57%) and a low solubility in solvent extracted RSM (7%).

In maize germ, concentrations of K and Mg are very high while only traces of Zn, Fe, Ca, and Mn occur (O'Dell, 1972; Lin et al., 2005), indicating that phytates primarily exist as highly soluble K- and Mg-phytates. When incubated *in vitro*, several studies confirmed a high $InsP_6$ solubility for maize. Scheuermann et al. (1988) incubated maize for 1 h in a buffer consisting of citrate, phosphate, borate, and hydrochloric acid at a pH range from 0.3 to 12. At pH 0.3,

solubility of $InsP_6$ was 100% but decreased to 38% between pH 1.5 and 2.5 before increasing again at pH 5, 6, 7, and 8 to 80, 90, 95, and 92%, respectively. De Boland (1975) incubated commercial and high lysine maize germ in distilled water for 18 h and found that 86% of $InsP_6$ were extracted at the end of incubation. The results were confirmed by O'Dell and de Boland (1976), who incubated the same samples in distilled water with a slightly different procedure and found 89 and 93% of $InsP_6$ in the extract after 6 h. On the other hand, only 33% of $InsP_6$-P were solubilised from maize in the study of Ton Nu et al. (2014) after 24 h of incubation in water. This could be due to the fact that the maize samples were soaked in a water:feed ratio of 3:1 while this ratio was 25:1 in the study of de Boland (1975) and 50:1 in the present *in situ* study. Stirring the samples at higher water:feed ratios might have extracted more $InsP_6$ and hence explain the low solubility of $InsP_6$ from maize in the study of Ton Nu et al. (2014).

Ca-, Mg-, and K-phytates are reported for soybeans (Lott and Buttrose, 1978). The presence of Ca-phytates seems to contribute to the lower $InsP_6$ solubility for SBM compared to maize (26% vs. 71%) which is in accordance with observations reported in the literature. After 18 h of incubation in water, about 20% less $InsP_6$ (69% vs. 86%) was solubilised from SBM compared to maize in the study of de Boland (1975). Han (1988) observed that 60% of $InsP_6$ in SBM was solubilised after 1 h of incubation in water, and neither pH value or temperature of the extracting media nor longer extraction time had a major impact on the $InsP_6$ amount extracted. Lower solubility values were given by Blaabjerg et al. (2010) and Ton Nu et al. (2014). Blaabjerg et al. (2010) soaked SBM in water and found an $InsP_6$ solubility of 19% after 2 h of incubation and of 33% after 24 h. In the study of Ton Nu et al. (2014), 8% of $InsP_6$ were solubilised after 2 h of incubation in water and 13% after 24 h of incubation (averaged over the treatments).

Ca-, Mg-, and K-phytates were also extracted from rapeseed (Gillberg and Törnell, 1976). However, the water-soluble fraction was lower for both incubated RSMs compared to SBM in the current work (8% hRSM, -22% RSM; 7% instantly soluble *in vitro*). Blaabjerg et al. (2010) found a very low solubility of $InsP_6$ from rapeseed cake as no $InsP_6$ was solubilised after 2 h of soaking in water and only 8% after 24 h. The solubility of $InsP_6$ in rapeseed products seems to be strongly dependent on the pH value of the medium. Gillberg and Törnell (1976) extracted $InsP_6$ from two different RSMs for 30 minutes in deionised water at different pH values. $InsP_6$ solubility decreased between pH 4.5 and 8 from >80% to about 10%, increased again slightly at pH 10 before dropping to almost 0% at pH 11. The effect was more pronounced in the heat

treated RSM. Serraino and Thompson (1984) observed a similar effect for rapeseed protein (RP) and rapeseed flour (RF). Without adjustment of pH (i.e. pH 5.8), $InsP_6$ solubility was 52% for RF and 31% for RP. Decreasing the pH to 5.15 and 3.5 increased solubility to 88% and 85% for RF and to 55 and 65% for RP, respectively. Increasing the pH value to 9 did not affect solubility from RF but decreased solubility from RP to 26%. The lower solubility of $InsP_6$ from RSM compared to SBM and the strong dependency on the pH value seems to be due to protein-phytate complexes rather than mineral-phytate complexes. $InsP_6$ has the potential for binding positively charged proteins and form binary protein-phytate complexes. Under acidic conditions, divalent cations like Ca^{2+} might interact as well and bind between two phosphate groups (Reddy et al., 1989) as shown in Figure 2.

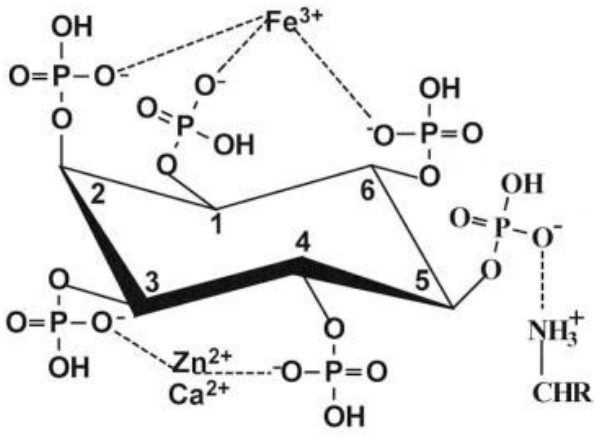

FIGURE 2. Illustration of a phytate-mineral or a phytate-protein complex, modified from Yu et al. (2012)

A tight association of $InsP_6$ and protein is reported for soybeans (Brooks and Morr, 1982) and rapeseed (Gillespie et al., 2005) as well as the existence of protein-phytate complexes in these seeds (Selle et al., 2012). While O'Dell and de Boland (1976) showed that $InsP_6$ is not bound to protein in maize, there is inconsistent information about protein-phytate complexes in wheat. A capability of $InsP_6$ to bind protein in wheat is reported (Selle et al., 2012) as well as little association between $InsP_6$ and proteins of wheat flour (Reddy et al., 1989). The solubility of protein-phytate complexes depends on the isoelectric point (IEP) of the proteins. In soybean, the protein-phytate complexes dissociate at a pH value higher than 4.5 (Grynspan and Cheryan, 1989), which is the region of the IEP for the major proteins in soybeans (Honig and Wolf, 1987). Rapeseed, however, has a complicated protein composition with proteins differing

widely in their IEPs (Gillberg and Törnell, 1976). While the IEP in 20 to 40% of rapeseed proteins is close to pH 11, it spreads out from pH 4 to pH 8 in other proteins (Gillberg and Törnell, 1976) which explains the dependence of $InsP_6$ solubility on pH value in rapeseed products.

Assessing the solubility of $InsP_6$ from wheat is difficult due to its high intrinsic phytase activity. Scheuermann et al. (1988) analysed the soluble $InsP_6$ in the supernatant after 1 h of incubation in a citrate buffer as described above for maize. They found that the solubility was up to 36% lower for wheat compared to maize between pH 4 and 12. The authors suggested that the low solubility between pH 4 and 6.5 might have been partly caused by an underestimation of solubility due to hydrolysis of $InsP_6$ through intrinsic phytase activity in this pH range. 19% of $InsP_6$ had disappeared from the solid fraction of wheat in this study and due to the short soaking time of one minute, this loss can presumably be attributed to solubility rather than $InsP_6$ hydrolysis. The lower solubility compared to maize and SBM could suggest the presence of some Ca- or Fe-phytates as the main minerals analysed in wheat globoids are K, Mg, Ca, and Fe (Bohn et al., 2007).

DISAPPEARANCE OF $INSP_6$

Studies on ruminal disappearance of $InsP_6$ from different concentrates are scarce. For this reason, *in vitro* studies examining $InsP_6$ disappearance from concentrates with phytase of different origin as well as *in situ* studies on ruminal P disappearance are included in the following section. Values for ruminal P disappearance can be adduced to draw conclusions on ruminal $InsP_6$ disappearance as $InsP_6$ represents the major part of total P in seeds. Hence, differences in P disappearance are predominantly due to effects on $InsP_6$ disappearance.

CEREALS

Besides the solubility of $InsP_6$, the localisation in the seed und thus the accessibility of phytases to the substrate seems to be decisive for the rate and extent of $InsP_6$ hydrolysis. While in maize about 90% of $InsP_6$ is located in the germ (88%, O'Dell (1972); 95%, Lin et al. (2005)), the main localisation site in most other cereals is the aleurone layer or the pericarp (O'Dell, 1972; Reddy and Sathe, 2002). Although the concentration of $InsP_6$-P in wheat germ is high compared to the rest of grain fraction (9.1 vs. 2.4 mg/g; Joyce et al. (2005)), only 13% of total $InsP_6$ are stored in the germ due to its small size (3% of kernel DM; O'Dell (1972)).

The *in situ* $InsP_6$ disappearance proceeded faster for maize than for wheat (Manuscript 3). After 8 h of incubation, 95% of $InsP_6$ from maize and 75% from wheat had disappeared. A fast $InsP_6$ disappearance from maize was also observed in the *in vitro* studies (Manuscript 2) where 83% of $InsP_6$-P had disappeared after 8 h of incubation and no more $InsP_6$ was found in the incubation system after 12 h of incubation. The results are comparable to those obtained by Ton Nu et al. (2014), who incubated ground maize with water and microbial phytase *in vitro*. After 8 h of incubation, no more $InsP_6$ was found in the maize residues. Morse et al. (1992) incubated hominy in a rumen fluid-buffer solution in a similar *in vitro* system as used in experiment 2 of Manuscript 2. After 6 h of incubation, 99% of $InsP_6$-P had disappeared from the solids. Concentrates are usually ground before incubation *in vitro* or *in situ* to simulate the effect of chewing. With $InsP_6$ being located in the germ, maize seems to benefit from grinding in particular. Cracking the seed renders the germ more accessible to exogenous phytase and might consequently increase the rate of $InsP_6$ disappearance. This is supported by the findings of Ton Nu et al. (2014), who compared the impact of screen size on $InsP_6$ disappearance and found an increase in $InsP_6$ degradation rate with decreasing screen size for maize. In wheat, exogenous phytase can access $InsP_6$ only after degradation of aleurone cell walls which are composed primarily of β-glucans and arabinoxylans (Steenfeldt et al., 1995; Regvar et al., 2011). Rumen microorganisms are capable of producing xylanases to hydrolyse the glycosidic bonds of arabinoxylans (Li et al., 2014) and make $InsP_6$ accessible to phytase. However, as the degradation of non-starch polysaccharides is a prerequisite for access of phytase to $InsP_6$, this might delay the onset of $InsP_6$ hydrolysis in wheat compared to maize.

The extent of ruminal $InsP_6$ hydrolysis seems to depend on phytase activity of rumen microorganisms rather than intrinsic phytase activity of seeds. Of all concentrates incubated in the present thesis, wheat is the only concentrate with high intrinsic phytase activity as the values given for maize, SBM, and RSM are negligible (Eeckhout and de Paepe, 1994; Viveros et al., 2000; Rodehutscord et al., 2016). However, the ruminal $InsP_6$ disappearance from wheat did not exceed that of SBM markedly and proceeded even slower compared to maize. Brask-Pedersen et al. (2011) incubated wheat in a rumen fluid-buffer solution and $InsP_6$ was completely hydrolysed after 6 h of incubation. The authors suggested that the rapid hydrolysis of $InsP_6$ was due to the large amount of intrinsic phytase of wheat. However, when wheat was soaked in water for 8 h only 21% of $InsP_6$-P had disappeared (Blaabjerg et al., 2007). This

indicates that the contribution of intrinsic phytase activity to ruminal $InsP_6$ hydrolysis is marginal.

OILSEED MEALS

In contrast to monocotyledons, where $InsP_6$ is associated with specific morphological components of the seed, $InsP_6$ containing globoids of dicotyledonous seeds are distributed throughout the kernel (Viveros et al., 2000). The hull or seed coat fraction contains only small amounts of $InsP_6$ if any (Reddy et al., 1989; Reddy and Sathe, 2002). Soybeans differ from other seeds in the localisation of $InsP_6$ in the PSV. Tombs (1967) reported that soybeans seem to lack globoids as $InsP_6$ is not located in specific areas but evenly distributed in the PSV. Lott and Buttrose (1978) found occasional, small globoids in soybean PSVs but also PSVs without any globoids. For *Brassicaceae*, however, numerous globoids surrounded by additional membranes have been reported which are connected to a crystalloid within the PSV (Gillespie et al., 2005). In the first hours of incubation, the ruminal *in situ* disappearance of $InsP_6$ from SBM proceeded faster than from RSM (64% vs. 21% after 8 h of incubation, Manuscript 3) whereas after 24 h of incubation the difference was almost equalised (98% vs. 95% RSM). Park et al. (1999) and Konishi et al. (1999) also observed differences between *in situ* ruminal $InsP_6$ disappearance of SBM and RSM. Effective degradability of SBM was higher than that of RSM at any calculated ruminal passage rate (SBM: 82% and 81%; RSM: 70%; averaged over passage rates). Han and Wilfred (1988) reported that $InsP_6$ from SBM was more readily and extensively hydrolysed than from cottonseed meal where $InsP_6$ is also segregated in globoids (Yatsu and Jacks, 1968). This indicates that the localisation of $InsP_6$ within the PSV affects the access of ruminal phytases to the substrate leading to differences in the pace and extent of $InsP_6$ hydrolysis between concentrates. As discussed in Manuscript 3, the localisation of $InsP_6$ in RSM suggests that enzymatic degradation of other cell components is required before phytase has access to $InsP_6$. This process seems to be unnecessary in SBM leading to an earlier onset of $InsP_6$ hydrolysis. The necessity of degradation of other cell components to enable $InsP_6$ hydrolysis in RSM is supported by the findings of Zyła and Koreleski (1993) and Newkirk and Classen (1998) who incubated RSM with crude and purified phytase *in vitro*. In both studies crude phytase hydrolysed $InsP_6$ more effectively than purified phytase indicating that non-phytase enzymes present in crude phytases enhance the ability of phytase to act on $InsP_6$ (Newkirk and Classen, 1998).

Compared to cereals, the *in vitro* and *in situ* $InsP_6$ disappearance from oilseed meals proceeded slower in the first hours of incubation but the difference was less distinct for SBM. Bravo et al. (2002) calculated the ruminal P disappearance from SBM, RSM, and wheat (ruminal passage rate k=0.06 h^{-1}) with 75, 62, and 92%, respectively. As described above, $InsP_6$ is known to form protein-phytate complexes in soybeans and rapeseeds. The structural and chemical properties of the protein-phytate complexes might influence their response to phytase (Viveros et al., 2000), as, for instance, aggregated proteins could shield the $InsP_6$ rendering it less susceptible for hydrolysis by exogenous phytase (Selle et al., 2012). This would demand enzymatic protein degradation before $InsP_6$ can be hydrolysed leading to a delayed $InsP_6$ hydrolysis. Thus, in RSM, degradation of cell components and protein seems to be required before phytase has access to $InsP_6$ and hydrolysis can be initiated. This might be the reason for the increase in $InsP_6$-P concentrations in RSM in the first 6 h of *in vitro* (Manuscript 2, experiment 2) and 8 h of *in situ* incubation (Annex 1) by more than 50%. Even in the water soluble fraction of RSM, the $InsP_6$-P concentration increased (22%). This suggests that seed components other than $InsP_6$ are solubilised or degraded in RSM leading to a relative accumulation of $InsP_6$ in the bag residues. Additionally, DM disappearance in RSM proceeded slower compared to the other incubated concentrates (Annex 2) contributing to the negative values calculated for $InsP_6$ disappearance from RSM. This effect did not occur in experiment 1 of the *in vitro* studies (Manuscript 2). Though $InsP_6$ concentrations of RSM also increased in the first h of incubation, the extent was less distinct with 18% after 3 h and 6% after 6 h. The incubation procedure between the two *in vitro* experiments differed. While 1 g of feedstuff was incubated approximately in 17 ml of rumen fluid in experiment 1, the same amount was incubated in 8 ml of rumen fluid in experiment 2. Assuming that one milliliter of rumen fluid consists of 10^9-10^{10} bacteria, 10^5-10^6 protozoa and 10^3-10^4 fungi (McAllister and Cheng, 1996), the concentrates in experiment 1 were exposed to more ruminal microorganisms and thus to higher microbial enzyme activity which might have enhanced $InsP_6$ hydrolysis of RSM in experiment 1 compared to experiment 2. Furthermore, rinsing of the bags in experiment 2 and in the *in situ* study might have removed other feed particles contributing to the relative accumulation of $InsP_6$ as observed for the water-soluble fraction. Also, effects of the examined RSM used in the experiments cannot be excluded as the RSM did not come from the same lot. Although both RSM were solvent extracted and the chemical composition was similar, the production process might have influenced the disappearance of $InsP_6$. Additionally, analytical aspects have to be

considered in this regard. Enzymatic degradation of other cell components during the early incubation times might have improved the extraction of $InsP_6$ from RSM resulting in higher $InsP_6$ concentrations in the analysed bag residues compared to the concentrate.

Differences in *in situ* ruminal $InsP_6$ disappearance occurred for RSM and the heat treated RSM (hRSM). Heat treatment of concentrates reduces the ruminal degradability of nutrients such as starch and protein and, thus, DM disappearance from hRSM was lower compared to RSM. However, in the initial phase of incubation $InsP_6$ disappearance was higher for hRSM. As mentioned above, the $InsP_6$-P concentrations in RSM increased markedly in the first hours of incubation. This effect was less distinct for hRSM, which could be due to the fact that processing of concentrates transforms the cell structure and changes the chemical structure of the constituents (Blaabjerg et al., 2010). This might have improved the initial access of phytases to $InsP_6$ in hRSM. After 24 h, however, $InsP_6$ disappearance from hRSM was only 57% while 97% of $InsP_6$ from RSM had disappeared. A negative effect of heat treatment on $InsP_6$ hydrolysis of RSM was also observed in several other studies. To which extent, however, the heating procedure influences degradability of $InsP_6$ seems hardly predictable. Konishi et al. (1999) examined RSM heated at different temperatures *in situ*. Heating the RSM for 3 h at 133 and 143 °C significantly reduced the effective degradability of $InsP_6$ compared to the untreated RSM while it did not differ between the two heat treated RSM. Increasing the temperature further to 153 °C led to a further decrease in effective $InsP_6$ degradability. Park et al. (2000) fed RSM heated at 133 and 143 °C to sheep and determined the flow of $InsP_6$ to the duodenum. Compared to the untreated RSM, a significantly higher flow was found only for the RSM heated at 143 °C. The influence of heat treatment of RSM on the solubility of $InsP_6$ was examined by Gillberg and Törnell (1976). At pH 4.8, heat treatment of RSM at 90 °C for 5 min reduced the solubility of $InsP_6$ from 83 to 72%, but extending the heating time to 20 min did not have any further impact. Boiling of RSM in water for 4 min, on the other hand, decreased the $InsP_6$ solubility to 55% (±10).

Crean and Haisman (1963) observed the formation of less soluble Ca- and Mg-phytates in peas during cooking which replaced the otherwise present, completely water-soluble K-phytates. In concentrates with protein-phytate complexes such as SBM and RSM, however, the impact of heat treatment on protein structure seems to be more relevant. The structural changes of the proteins during the heating process shift the site of protein digestion from the rumen to the intestine (Dakowski et al., 1996). While this is desirable for the intestinal protein supply of the

ruminant, it seems to negatively affect ruminal $InsP_6$ hydrolysis from concentrates where protein is bound to $InsP_6$.

3.1.2 DISAPPEARANCE FROM TOTAL MIXED RATIONS

RUMINAL $INSP_6$ DISAPPEARANCE

Gaining *in vivo* results on ruminal hydrolysis is complex, thus, ruminal $InsP_6$ hydrolysis from diets has hardly been investigated so far. Ray et al. (2013) determined a ruminal $InsP_6$ hydrolysis of 85% from a diet with SBM, ground maize and dried beet pulp as main concentrates fed to fistulated lactating dairy cows. A similar $InsP_6$ disappearance was reported by Brask-Pedersen et al. (2013) where 86% of ingested $InsP_6$ from a TMR with rapeseed cake and dried beet pulp was hydrolysed in the rumen of lactating dairy cows. Addition of phytase to the diets increased ruminal $InsP_6$ disappearance to 96% indicating that even ruminants might benefit from exogenous phytase. In the study of Ray et al. (2012) only 68% of ingested $InsP_6$ were hydrolysed in the rumen. The examined diet was higher in forage compared to the diet in the study of Brask-Pedersen et al. (2013) (forage to concentrate ratio (f:c) 53:47 vs. 43:57) which makes a higher ruminal passage rate and thus less time for ruminal degradation an unlikely cause for the lower $InsP_6$ hydrolysis. Compared to the diet of Ray et al. (2013) which had almost the same f:c ratio (55:45), the $InsP_6$-P concentration of the diet was markedly lower (0.005 vs. 0.10% $InsP_6$-P of TMR DM). This suggests that the dietary $InsP_6$-P content might have influenced ruminal phytase activity, leading to a higher ruminal $InsP_6$ hydrolysis at higher $InsP_6$ concentrations in the diet. Raun et al. (1956) observed an opposite effect *in vitro* when the percentage of hydrolysed $InsP_6$-P decreased with increasing $InsP_6$ concentrations in the medium. They suggested that an excess of substrate might inhibit phytase activity. This assumption, however, does not seem to be valid as discussed in the following chapter (Chapter 3.1.3. AFFECTING FACTORS ON $INSP_6$ DISAPPEARANCE – HIGH $INSP_6$ CONCENTRATION IN THE DIET).

In the present work, ruminal $InsP_6$ disappearance from three different diets was determined *in vitro* with a rumen simulation technique (Manuscript 1). The diets differed in the composition of the concentrate mixture (cereal grains, legumes, and SBM with or without addition of mineral P vs. a RSM based diet with lower percentage of cereal grains and legumes) but ruminal $InsP_6$ disappearance was >94% for all incubated diets. Ekelund et al. (2003) incubated four different concentrate mixtures *in vitro* in a rumen fluid-buffer solution under anaerobic

conditions for 96 h and determined the disappearance of P. The concentrate mixtures included four different P sources, monosodium phosphate, wheat by-products (wheat middlings and bran), sunflower seed meal with palm expeller, and rapeseed concentrates (RSM, heat treated RSM, and full-fat rapeseed). *In vit*ro ruminal P disappearance differed between the diets and was 94, 90, 85, and 74%, respectively. The observed differences between the concentrate mixtures could be due to the fact that the rumen fluid:buffer ratio was 1:50. As discussed above for experiment 2 of the *in vitro* studies of this work, the concentrates in the study of Ekelund et al. (2003) were exposed to a relatively low number and activity of rumen microorganisms. In the wheat mixture with quickly degradable $InsP_6$, the enzyme activity might have been sufficient for $InsP_6$ hydrolysis but not in the concentrate mixture including rapeseed products with slow degradability and $InsP_6$ hydrolysis.

The potential of ruminal $InsP_6$ hydrolysis from different diets is high, however, in the cited studies 14 to 32% of ingested $InsP_6$ left the rumen undegraded and might be excreted if not hydrolysed postruminally.

POSTRUMINAL $INSP_6$ DISAPPEARANCE

Brask-Pedersen et al. (2013) observed that the flow of $InsP_6$-P decreased between the duodenum and ileum of lactating dairy cows from 3.8 to. 2.6 g/d. But no further hydrolysis of $InsP_6$ occurred behind the ileum. Ray et al. (2013) also found a reduction of $InsP_6$-P flow between the omasum and the ileum (2.7 vs. 1.4 g/d) in lactating dairy cows. With the faeces 0.98 g $InsP_6$-P were excreted per day, confirming that only very little hydrolysis of $InsP_6$ occurs behind the ileum. Park et al. (2002) determined the post ruminal $InsP_6$ degradation in sheep. The animals were fed a diet containing 20% RSM and digesta from the abomasum, jejunum, upper (middle of ascending colon), and lower large intestine (rectum) were analysed for $InsP_6$ and lower InsPs. 35% of dietary InsP-P had left the rumen undegraded and no considerable degradation of InsPs was observed between the abomasum and the jejunum where 32% of dietary InsP-P arrived. However, inflow of $InsP_3$ into the jejunum tended to be decreased. The authors suggested that duodenal mucosa which showed lower activity towards highly phosphorylated InsPs in pigs (Hu et al., 1996) might have degraded $InsP_3$ in the duodenum of sheep. 13% of dietary InsP-P reached the upper large intestine indicating microbial InsP hydrolysis behind the jejunum. Microbial phytase of the large intestine did not seem to be selective as all InsPs were hydrolysed similarly. No further decrease of InsP-P flow was

observed between upper and lower large intestine. Ray et al. (2012) suggested a fixed value for large intestinal phytate hydrolysis. In their study, 15% of phytate P disappeared on average in the large intestine irrespective of the amount of phytate P entering this segment. This value is similar to the disappearance of 19% observed by Park et al. (2002). Of nutritional significance is hydrolysis of InsP-P in the large intestine only if the released P is absorbed. The main absorption site in ruminants is the small intestine (Horst, 1986; Breves and Schröder, 1991). However, Ray et al. (2012) observed that inorganic P (Pi) released through hydrolysis of InsPs in the large intestine partially disappeared indicating net absorption of Pi. This is supported by studies in sheep where absorption of Pi in the large intestine was observed (Park et al., 2002).

It is noteworthy to mention that Park et al. (2000) found no phytate P in the soluble phase of duodenal digesta in sheep. Ruminally undegraded phytate P was exclusively found in the solid phase. This supports the results of the *in vitro* studies (Manuscript 2), where $InsP_6$ concentrations in the fermenter fluids were analysed to assess the hydrolysis of soluble $InsP_6$. $InsP_6$ was readily hydrolysed *in vitro* and the findings of Park et al. (2000) imply that these results are also valid *in vivo*.

TOTAL TRACT $INSP_6$ DISAPPEARANCE

Several studies confirmed a high potential for $InsP_6$ hydrolysis in ruminants when the faecal excretion of $InsP_6$ was determined. 99% and 100% of ingested $InsP_6$-P from diets based on cereals (sorghum, maize, oats) and SBM had disappeared in the digestive tract of calves and young steers (Nelson et al., 1976). Clark et al. (1986) also determined an $InsP_6$ hydrolysis of 98% in lactating dairy cows fed diets with SBM and cereal (maize, oats) based concentrate. A similar extent of total tract disappearance of $InsP_6$ in lactating dairy cows was shown by Morse et al. (1992) where 94 to 99% of ingested $InsP_6$-P from varying diets was hydrolysed. Only trace amounts of $InsP_6$ were detected in the faeces and the range of $InsP_6$ hydrolysis varied due to different markers used for calculation. Leytem et al. (2007) fed two different diets including either low phytate barley or conventional barley to sheep. The faecal excretion of $InsP_6$ did not significantly differ between the diets and the amount of excreted $InsP_6$ was negligible. In the aforementioned studies of Brask-Pedersen et al. (2013) and Ray et al. (2013) the total tract $InsP_6$ disappearance was 90 and 95%, respectively. 92% of $InsP_6$-P from Ca-phytate added to a basal diet had disappeared in the digestive tract of sheep (Tillman and Brethour, 1958). In the *in vivo* study of the present thesis, total tract $InsP_6$ disappearance from the control diet was 90%

(Manuscript 1). Lower values for $InsP_6$ disappearance were given by Kebreab et al. (2005) where 77 to 88% of ingested $InsP_6$-P was hydrolysed. The effects were ascribed to the incorporation of whole crop wheat which replaced grass silage at different levels. Whole crop wheat is resistant to microbial breakdown and can pass through the digestive tract intact. Kincaid et al. (2005) determined the $InsP_6$ disappearance from diets based on maize or barley grain. $InsP_6$ hydrolysis was 80 and 78%, respectively. Addition of exogenous phytase increased hydrolysis in both diets to 85%. The observed low $InsP_6$ hydrolysis and the effect of phytase addition were attributed to the high feed intake of the high-producing cows. Addition of phytase-cellulase to a high concentrate diet (f:c ratio 37:63) decreased faecal P excretion of lactating dairy cows in the study of Knowlton et al. (2007). The authors suggested that the addition of enzyme might have compensated for the shorter reaction time of ruminal phytase due to the high passage rate in high concentrate diets.

These results indicate that $InsP_6$ hydrolysis in ruminants is high but might not always be complete. Drawing conclusions on the cause of the effects by comparing different studies is not always possible as the experimental conditions might differ in more than one respect, e.g. DMI, f:c ratio, diet composition, or analytical methods. Barth and Hansard (1962), for example, observed *in vitro* a decreased $InsP_6$-P hydrolysis at wider Ca:P ratios which is confirmed by the *in vivo* findings of Mathur (1953) where total tract disappearance of $InsP_6$ was 61% at a Ca:P ratio of 1.2:1 and decreased further to 57% at a ratio of 2:1. The $InsP_6$ disappearance in the study of Clark et al. (1986), however, was 98% and increasing the Ca:P ratio from 1:1 to 1.5:1 did not influenced $InsP_6$ disappearance. The source of Ca was identical between the studies and the percentage of Ca in the diets similar, but diet composition as well as extraction of $InsP_6$ differed. Furthermore, precise information on DMI and f:c ratio lack in the study of Mathur (1953) which makes it hard to conclude which factor was the main cause of the differing results.

3.1.3 FACTORS AFFECTING $INSP_6$ DISAPPEARANCE

In the present thesis, the effects of high $InsP_6$ concentration and addition of mineral P on $InsP_6$ disappearance were examined as these factors had been suggested in the literature to have the potential to affect $InsP_6$ disappearance.

HIGH $InsP_6$ CONCENTRATION IN THE DIET

A positive effect of increasing the $InsP_6$ concentrations in the diets on disappearance of $InsP_6$ was observed *in vitro*, *in situ* and *in vivo* and the results are discussed in detail in Manuscript 1, 2, and 3. Some general thoughts, however, will be addressed in this section.

In the *in vivo* study, $InsP_6$ disappearance in the total tract of lactating dairy cows increased from 90% to 93 and 94% when the $InsP_6$-P concentrations of the diets were 0.5, 1.2, and 1.4 g/kg DM. Brask-Pedersen et al. (2013) fed a diet with an $InsP_6$-P concentration of 1.4 g/kg DM to lactating dairy cows and determined a total tract $InsP_6$ disappearance of 90%. They added low (2,023 FTU/kg DM), medium (3,982 FTU/kg DM), and high (6,015 FTU/kg DM) levels of microbial phytase to the diet and only for the high phytase level $InsP_6$ disappearance was 94% as observed in the present work at the same dietary $InsP_6$-P concentration. Brask-Pedersen et al. (2013) used rapeseed cake as main source of $InsP_6$ which arises from a different oil removal procedure than solvent extracted RSM as used in the present work. While rapeseed cake is the residual product of mechanical extraction, solvent extracted RSM is steam-heated during the extraction process. The possible effects of heating on accessibility of phytase to $InsP_6$ were discussed above and might have contributed to the slightly higher $InsP_6$ disappearance (94 vs. 90%) in the RSM based diet of the present thesis compared to the rapeseed cake based diet used by Brask-Pedersen et al. (2013). Ray et al. (2013) fed diets with $InsP_6$-P concentrations of 1.0, 1.8, and 2.9 g/kg DM to lactating dairy cows and from the reported excretion of $InsP_6$-P with the faeces a total tract disappearance of 95, 94, and 96% can be calculated. The increase in $InsP_6$ concentrations in the diets was obtained by using cottonseed meal (CSM). As mentioned above, the localisation of $InsP_6$ in cottonseed and rapeseed appears similar. Morse et al. (1992) determined the *in vitro* $InsP_6$ disappearance from CSM which proceeded similarly to that determined for RSM in experiment 1 of the *in vitro* studies (Manuscript 2; after 12 h of incubation: RSM 72%, CSM 72%; after 24 h of incubation: RSM 96%, CSM 100%). However, it has to be kept in mind that besides the differences in diet ingredients, other factors might contribute to the observed variances in $InsP_6$ hydrolysis between the studies. At high $InsP_6$-P concentrations in the diets (1.0 to 2.9 g/kg DM), the hydrolysis of $InsP_6$ in the total tract ranged from 90% (Brask-Pedersen et al., 2013) to 93 and 94% (Manuscript 1) and 96% (Ray et al., 2013). Morse et al. (1992) calculated the total tract $InsP_6$ hydrolysis using different markers and the results ranged from 94 to 99% which indicates the importance of considering methodical aspects when comparing different studies. Given the fact that the studies differed

in the markers used for quantification of faeces as well as in collection, preparation and analysis of the samples, comparing the results may not allow to draw conclusions on the impact of different diet ingredients on $InsP_6$ hydrolysis. However, besides the varying $InsP_6$-P concentrations in the diets and differences in performance between these studies, the results might suggest that when a certain level of $InsP_6$-P in the diet is achieved (about 1.0 g/kg DM) total tract hydrolysis of $InsP_6$ is higher than 90%.

Incubating the diet with high $InsP_6$-P concentration (1.4 g/kg DM) in a Rusitec led to converse results as the disappearance of $InsP_6$ was lower compared to the control diet (94% vs. 98%, Manuscript 1). Ekelund et al. (2003) also observed deviating results for P disappearance when diets with different P sources in the concentrate were fed to lactating dairy cows or incubated *in vitro*. While the *in vivo* total tract disappearance of P was not influenced by the P source in their study, the ruminal *in vitro* disappearance of P ranged from 74 to 94%, depending on the concentrate. In the Rusitec study of Manuscript 1, donor animals of rumen fluid were not fed with the incubated diets but with a low concentrate diet. Ekelund et al. (2003) did not describe the diet fed to donor animals of rumen fluid which suggests that they also received a standard diet commonly used for *in vitro* incubations rather than the incubated diets. As discussed in Manuscript 1, the microbial community adapts differently to different diets. Phytase activity, however, has only been demonstrated in amylolytic bacteria so far (Yanke et al., 1998; Lan et al., 2002) which are favoured in high concentrate diets. This in turn might influence the extent of $InsP_6$ hydrolysis. The impact of an adaption of the microbial community was also shown *in vitro* and *in situ*, when the $InsP_6$ disappearance from concentrates increased after feeding the high $InsP_6$ diets. While *in vitro* the $InsP_6$ disappearance from both maize and RSM was influenced, this effect was observed *in situ* only for oilseed meals. As discussed in Manuscript 3, it is likely that due to the high degradability of $InsP_6$ in grains, the ruminal microbial enzymatic activity was sufficient for prompt and complete *in situ* $InsP_6$ hydrolysis in the low $InsP_6$-P diet. Oilseed meals which are less prone to $InsP_6$ hydrolysis seem to benefit from changes in the ruminal microbial composition or enzyme activity caused by higher $InsP_6$-P concentrations in the diet. In the *in vitro* study, the rumen fluid was strained before incubation which might have retained particle associated bacteria and thus reduced phytase activity in the *in vitro* system. Thus, even maize with high $InsP_6$ degradability seems to have benefitted from a higher enzyme activity in the rumen fluid of donor animals fed the high $InsP_6$-P diet. Godoy and Meschy (2001), on the other hand, observed no influence on $InsP_6$ hydrolysis in a Rusitec

when diets differing in their $InsP_6$-P concentration were fed to the donor animals of rumen fluid. The diets contained 0.5 or 1 g $InsP_6$-P per kg DM, which is similar to the diets fed in Experiment 1 of the *in vitro* studies (0.15 vs. 1.26 g/kg DM, Manuscript 2). However, Godoy and Meschy (2001) determined the $InsP_6$ hydrolysis of commercial Na-phytate in the buffer which is, as discussed in Chapter 3.1.1, highly soluble. Thus, Na-phytate might generally be more readily hydrolysed than $InsP_6$ bound in seeds and concentrates independent of the microbial enzymatic activity in the rumen fluid.

ADDITION OF MINERAL P

The *in vitro* disappearance of $InsP_6$ from concentrates decreased with addition of Pi to the buffer (Manuscript 2) as did the *in vitro* and *in vivo* $InsP_6$ disappearance when Pi was added to the diet (Manuscript 1). Godoy and Meschy (2001) also found a lower $InsP_6$ hydrolysis when Pi was added to the buffer in a Rusitec. As discussed in Manuscript 1 and 2, the reasons for the observed decrease in $InsP_6$ hydrolysis remain unclear because the phytase activity of rumen bacteria does not seem to be influenced by high P concentrations (Yanke et al., 1998; Lan et al., 2002) as it is reported for phytases from moulds and yeasts (Shieh and Ware, 1968; Vats and Banerjee, 2004). For the *in vitro* studies might apply that *in vivo* conditions were not met. As discussed in Manuscript 3, the rumen fluid used as incubation medium *in vitro* might not reflect *in vivo* conditions exactly rendering the rumen microorganisms and their enzyme activity more susceptible to different treatments. But a decrease in total tract disappearance of $InsP_6$ was also observed *in vivo* after addition of Pi to the diet (85 vs. 90%, Manuscript 1). A similar though less distinct effect occurred in the study of Ray et al. (2013) where total tract hydrolysis of $InsP_6$ decreased slightly from 95 to 93% when Pi was added to a basal diet. The ruminal $InsP_6$ hydrolysis, however, did not differ between the treatments and was 85% for both diets. This suggests that not the ruminal but the postruminal $InsP_6$ disappearance might be affected by the dietary P content. This is supported by the findings of the *in situ* study (Manuscript 3) where ruminal $InsP_6$ disappearance from concentrates was not influenced by the Pi concentration of the diet. Though the rumen and the large intestine have some microbial species in common, the bacterial population is not exactly the same (Ray et al., 2012). One of the most abundant bacterial species found in the intestine of cattle are *Escherichia coli* (Maki and Picard, 1965) with a pH optimum of 4.5 for their phytase activity (Greiner and Konietzny, 2006). Faecal P excretion was higher when the diets with additional Pi were fed compared to the basal diet (86 vs. 60 g/d, Manuscript 1; 51 vs. 42 g/d, Ray et al. (2013)) indicating a higher

flow of total P in the intestinal digesta. Due to the buffering capacity of phosphate, the pH value in the digesta might have been higher leading to a lower phytase activity of the predominant *E. coli* in the intestine and thus to a lower total tract hydrolysis of $InsP_6$ *in vivo*.

3.1.4 FURTHER POSSIBLE INFLUENCING FACTORS

Besides the $InsP_6$ and Pi concentration of the diet or the incubation system, various other factor might affect the extent of $InsP_6$ hydrolysis in ruminants.

FORAGE TO CONCENTRATE RATIO AND DRY MATTER INTAKE

The DMI and the f:c ratio of the diet are closely connected and influence the ruminal passage rate and thus the time available for ruminal $InsP_6$ hydrolysis.

The amount of ingested feed is probably the most important variable concerning the retention time of the digesta in the gastrointestinal tract of ruminants (Colucci et al., 1990). Feeding diets with low f:c ratios increases DMI (Robinson and McQueen, 1997) and with increasing DMI the ruminal retention time of the digesta declines (Grovum and Williams, 1977; Colucci et al., 1982; Colucci et al., 1990). The low fibre content of concentrates compared to forages requires less chewing and ruminating activity leading to higher passage rates of the digesta (Robinson and McQueen, 1997; Tafaj et al., 2005). Lower ruminal nutrient degradability at higher DMI levels is mainly attributed to the increasing rumen outflow rate but higher amounts of rumen dry matter might also increase the time required for bacterial attachment to feed particles (Susmel et al., 1989). It is known that the degradability of protein is dependent on the rate at which the digesta leaves the rumen (Subuh et al., 1994). This might apply for the hydrolysis of $InsP_6$ as well and could be of special importance in diets containing concentrates with slow $InsP_6$ degradation.

The f:c ratio might have further effects on ruminal $InsP_6$ hydrolysis. Its impact on the composition of the mixed rumen microbiota and, therefore, on ruminal phytase activity was discussed in detail in Manuscript 1 and Manuscript 3. Yanke et al. (1998) determined the phytase activity of rumen microorganisms when diets with different f:c ratios were fed to donor animals of rumen fluid. Phytase activity of the bacterial fraction increased 6.25 fold when the f:c ratio was 50:50 compared to 90:10, and increased again 1.7 fold at a ratio of 10:90. This indicates that the f:c ratio has the potential to influence the ruminal $InsP_6$ hydrolysis *in vivo*. Furthermore, the change in the microbial composition caused by different f:c ratios might lead to a lower cellulolytic activity in the rumen. In high concentrate diets, a lower *in situ* CP

degradation was observed for different concentrates compared to low concentrate diets and the effects were ascribed to reduced cellulolytic activity (Ganev et al., 1979; Molero et al., 2004). This could apply for $InsP_6$ hydrolysis as well, especially when concentrates like RSM are fed where protein-phyate complexes are integrated in hardly accessible cell structures. However, the effect was only observed when the f:c ratios differed extremely and did not occur when the differences were less pronounced (Molero et al., 2004; Rotger et al., 2006).

Comparing different studies on $InsP_6$ hydrolysis could support the assertion that the effect of DMI is of great importance while the f:c ratios have to differ widely before an interference with $InsP_6$ hydrolysis occurs. The total tract $InsP_6$ disappearance in the studies of Brask-Pedersen et al. (2013), Ray et al. (2013), and the present thesis (Manuscript 1) ranged from 90 to 95% when f:c ratios were 43:57, 55:45, and 50:50 and DMI was 19 kg, 18 kg, and 23 kg, respectively. Kincaid et al. (2005) determined a total tract $InsP_6$ hydrolysis of only 79% (averaged over the treatments) when a diet with similar f:c ratio (48:52) was fed. With an average DMI of 29 kg, the feed intake of the animals was 4% of their body weight and the concomitant high ruminal turnover rate might have limited ruminal $InsP_6$ hydrolysis. However, as mentioned above, the studies differed in more than one aspect which does not allow to draw conclusions on only one possible effect on $InsP_6$ hydrolysis.

RUMINAL PH VALUE

As concentrates require little chewing activity and rumination, the excretion of saliva is reduced when ruminants are fed high amounts of concentrate. This affects the ruminal pH value because the reduced ratio of excreted salivary alkali to produced fermentation products (volatile fatty acids) causes the pH value to drop (Ørskov, 1994). Information on the optimum pH value for phytase activity of rumen microorganisms vary and are not very specific so far.

Known phytases produced by rumen bacteria are cysteine phytases (Huang et al., 2011) which are active at acidic pH with maximal activity at pH 4.5 (Puhl et al., 2008). Huang et al. (2011), however, analysed the ruminal genomic DNA from goats and cows and purified two cysteine phtytases with optimum pH values for phytase activity at 6.0 and 6.5. Both phytases retained much of their activity between pH 5.0 and 7.0. Phytase activity of the rumen bacterium *Mitsuokella jalaludinii* was observed between pH 5.7 and 7.3 with an optimum pH at 7.0 (Lan et al., 2002a). To date there is little knowledge about the multitude of active phytases and their properties in the rumen. The actual influence of the ruminal pH value on $InsP_6$ hydrolysis *in*

vivo might be less distinct as the conditions in the culture media are unlikely to reflect the complex interactions in the rumen adequately. But as the pH optimum for most of the reported phytases so far is in the acidic pH range (Vats and Banerjee, 2004; Greiner and Konietzny, 2006), this could suggest that ruminal $InsP_6$ hydrolysis might increase with higher amounts of concentrate in the diet due to the subsequent lower ruminal pH. Li et al. (2014a), on the other hand, suggested that ruminal microbes probably produce functional enzymes with different pH preference as the bacterial population increases, decreases and changes notably during a 24 h feeding cycle.

Presumably, the ruminal pH value did not have a major impact on the observed differences in total tract $InsP_6$ hydrolysis in the *in vivo* study of this work. The f:c ratio in the *in vivo* study was 50:50 for all diets which suggests that no major differences in ruminal pH values occurred.

INTERACTIONS WITH DIETARY CALCIUM

The *in vitro* $InsP_6$ hydrolysis of rumen microorganisms decreases with wider Ca:$InsP_6$ ratios (Barth and Hansard, 1962), an effect that was also observed *in vivo* when total tract disappearance of $InsP_6$ was determined in lactating cows (Mathur, 1953). According to Barth and Hansard (1962), a chemical reaction between $InsP_6$ and/or its hydrolysis products and Ca rather than an enzyme inhibition seems to cause this effect. As reviewed by Selle et al. (2009), Ca has the potential to influence $InsP_6$ hydrolysis in different ways. Depending on the pH value, Ca interacts with $InsP_6$ by formation of insoluble Ca-phytate complexes. Furthermore, limestone, a commonly used Ca source in diets, has an extremely high acid binding capacity and tends to increase the digesta pH in the gut. The pH value of the digesta, in turn, influences the activity of microbial phytases as discussed above because the majority of the phytases characterised to date show maximal activity in the acid pH range (Greiner and Konietzny, 2006). A direct inhibition of exogenous phytase activity through Ca by competing for active sites of the enzyme was also suggested but there is little tangible evidence to support this hypothesis at the moment (Selle et al., 2009).

Due to slightly acidic pH values in the rumen, ruminal precipitation of Ca-phytate complexes appears unlikely. However, as the pH value in the intestinal digesta increases, postruminal formation of insoluble Ca-phytates could be possible. This would suggest that the extent of the ruminal $InsP_6$ hydrolysis influences the intestinal precipitation. Studies of Yu et al. (2012) and Sandberg et al. (1989) showed that the reactivity of InsPs towards iron decreased with a lower

degree of phosphorylation which might apply for the formation of other metal chelates as well. Consequently, the more $InsP_6$ is hydrolysed in the rumen the less highly reactive substrate is available in the intestine to form insoluble complexes with Ca. This could explain why in the study of Mathur (1953) an effect of a wider Ca:P ratio was observed but not in the study of Clark et al. (1986) although the Ca concentrations were similar (0.6 or 0.9% of TMR DM, Clark et al. (1986); 1% of concentrate, Mathur (1953)). The generally low total tract $InsP_6$ disappearance (57 to 61%) observed by Mathur (1953) indicates a low ruminal $InsP_6$ hydrolysis, whereas the total tract $InsP_6$ disappearance of 98% suggests a high ruminal $InsP_6$ hydrolysis in the study of Clark et al. (1986). Similar conclusions could be drawn from the *in vivo* study of the present thesis (Manuscript 1). The Ca concentrations in the diets were 0.3% for the basal diet, 0.5% for the diet with additional Pi (Diet MP), and 0.7% for the high $InsP_6$ diets. In the high $InsP_6$ diets, the total tract $InsP_6$ hydrolysis was higher (93 and 94%) compared to the other diets (90% Diet C; 85% Diet MP) despite the highest Ca concentrations of all diets. This indicates that Ca concentrations did not affect $InsP_6$ hydrolysis in these diets. Due to the observed lower total tract $InsP_6$ disappearance for the basal diet and diet MP, however, a lower ruminal $InsP_6$ hydrolysis was suggested. Thus, the higher Ca concentration in diet MP compared to the basal diet might have favoured intestinal formation of Ca-phytates which could have contributed to the observed lower total tract $InsP_6$ hydrolysis for this diet.

FORMATION OF PROTEIN-PHYTATE COMPLEXES

According to Yu et al. (2012), the formation of protein-phytate complexes can occur in the PSV as well as in the gastrointestinal tract of animals and humans. As proteins have a negative net charge at pH values above their IEP, it is likely that ternary rather than binary complexes are formed in the small intestine by linking protein and phytate with a cationic bridge (Selle et al., 2012). However, the capability of protein to form complexes with InsPs is reduced by more than 4 times after dephosphorylation from $InsP_6$ to $InsP_5$ and is negligible for lower InsPs (Yu et al., 2012).

The intestinal digesta contains ruminally synthesised microbial protein, undegraded dietary protein, and endogenous protein which could possibly form complexes with $InsP_6$. To date, studies on the formation of such complexes in the gastrointestinal tract of ruminants are lacking. However, taking the present literature into account, the formation of protein-phytate

complexes might be dependent on the amount of ruminally undegraded $InsP_6$, the pH value of the digesta, and the availability of cations.

3.2 CORRELATION BETWEEN $INSP_6$ UND CP DISAPPEARANCE

$InsP_6$ is stored within the PSVs of the seed and its capability of binding proteins is proven for several seeds. Thus, it makes sense to examine the correlation between $InsP_6$ and CP concentrations and disappearance. The CP concentration in the bag residues was determined for all *in situ* incubated concentrates and linear regressions were performed for $InsP_6$ and CP concentration and disappearance for each concentrate. The values used for regression analyses are given in Annex 3.

For the correlations between $InsP_6$ and CP concentrations, the highest coefficient of determination ($R^2 = 0.93$) occurred for RSM (Figure 3). With decreasing concentrations of CP, the $InsP_6$ concentrations decreased as well. This reflects the close association of $InsP_6$ with CP.

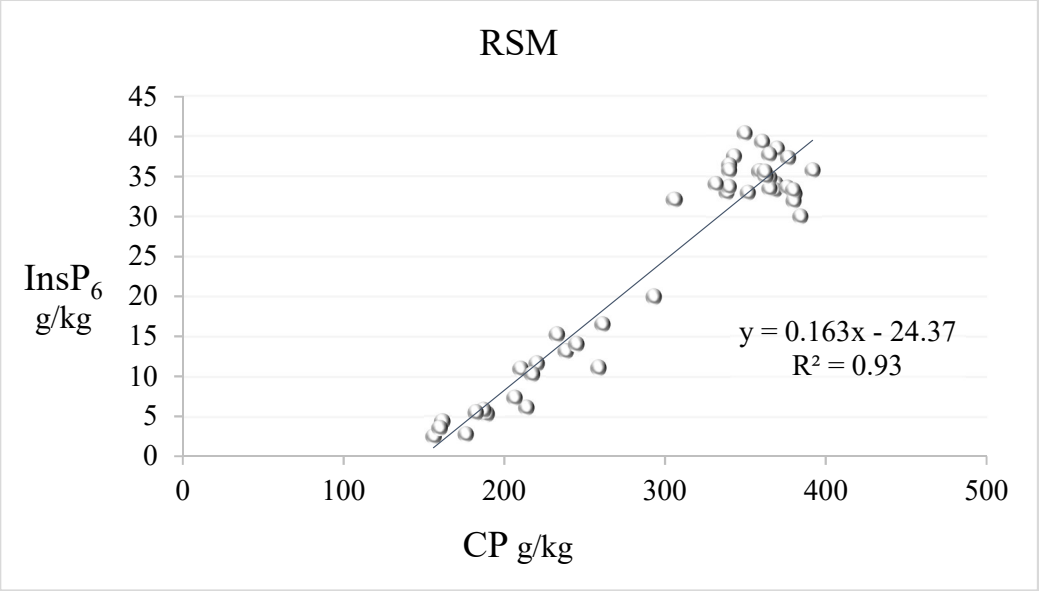

FIGURE 3. Linear regression for CP and $InsP_6$ concentrations (g/kg DM) in the bag residues of solvent extracted RSM

After the heat treatment of RSM, however, the correlation between CP and $InsP_6$ concentration changed to a negative value and the coefficient of determination of the regression was only $R^2 = 0.42$ (Figure 4).

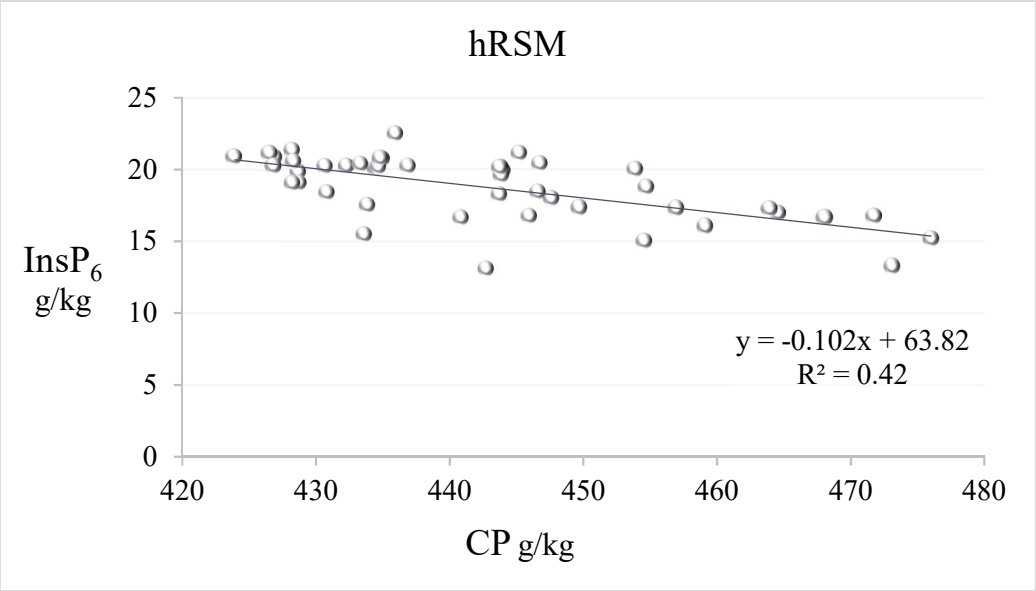

FIGURE 4. Linear regression for CP and $InsP_6$ concentrations (g/kg DM) in the bag residues of solvent extracted, heat treated RSM (hRSM)

As discussed in Chapter 3.1.1, heat treatment reduces the ruminal degradability of CP and affects the association of CP and $InsP_6$ in hRSM. Some $InsP_6$ in hRSM is still associated with the denatured protein mass and its hydrolysis depends on the degradability of the protein. However, due to changes of cell structures during heat application, some $InsP_6$ seems to be easily accessible to phytase after the heat treatment. Thus, the correlation between $InsP_6$ and CP concentrations in hRSM is more complex than in RSM, leading to a lower coefficient of determination for hRSM.

For SBM, too, the correlation of CP and $InsP_6$ concentration was not as strong as for RSM (R^2 = 0.57; Figure 5). In Chapter 3.1.1, the differences in binding and localisation of $InsP_6$ between SBM and RSM are discussed. The low coefficient of determination probably reflects the fact that although $InsP_6$ in soybeans is associated with protein, it is not stored in globoids within the PSV. This might render the $InsP_6$ concentration less dependent on the CP concentration compared to RSM, where $InsP_6$ is stored in globoids within the PSV.

In maize, $InsP_6$ does not form chelated complexes with protein and for wheat such complexes have not been reported either although $InsP_6$ is capable of binding wheat gluten at a certain pH value (Hill and Tyler, 1954). As expected, the linear regressions for wheat and maize did not result in in high coefficients of determination (wheat: $y = 0.073x – 1.26$, $R^2 = 0.42$; maize: $y = -0.051x + 6.43$, $R^2 = 0.47$).

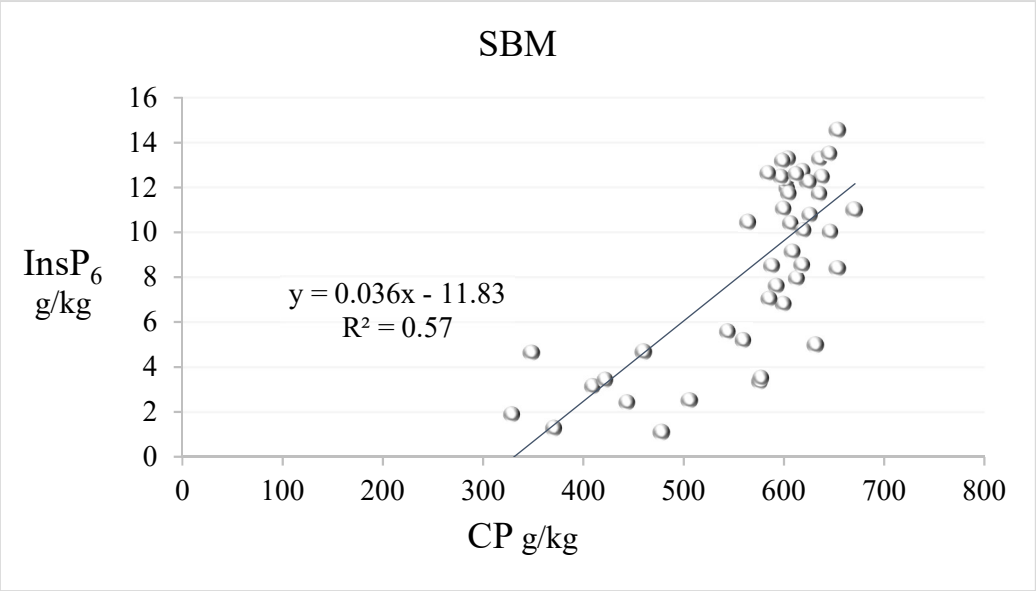

FIGURE 5. Linear regression for CP and $InsP_6$ concentrations (g/kg DM) in the bag residues of SBM

As the CP and $InsP_6$ concentrations in the concentrates were correlated to varying extents, linear regressions for CP and $InsP_6$ disappearances from concentrates were performed as well (Manuscript 3). The coefficients of determination were $R^2 = 0.97$ for RSM, $R^2 = 0.93$ for hRSM, $R^2 = 0.93$ for SBM, $R^2 = 0.83$ for wheat, and $R^2 = 0.70$ for maize. As discussed in Manuscript 3, the high coefficients of determination for oilseed meals reflect the existence of protein-phytate complexes in these concentrates. In oilseed meals, the disappearance of $InsP_6$ seems to be strongly dependent on the disappearance of CP. After evaluation of a bigger data set, a prediction of $InsP_6$ disappearance based on CP disappearance values might be possible, especially for RSM. Unlike analyses of $InsP_6$, CP degradation from concentrates is commonly determined. An estimation of $InsP_6$ hydrolysis based on CP degradation could help to avoid costly $InsP_6$ analyses but would still provide relevant data for e.g. diet calculations.

3.3 $InsP_5$ ISOMERS AND LOWER INSPS

In the course of the *in situ* study, the bag residues were analysed for isomers of $InsP_5$ and lower InsPs (Manuscript 3). The results can only be discussed based on the detected concentrations as isomers of $InsP_5$ and lower InsPs in the bag residues represent a net value. They can comprise undegraded InsPs originating from the concentrates as well as hydrolysis products of $InsP_6$ or lower InsPs hydrolysis. This makes it impossible to calculate the disappearance based on $InsP_5$ or lower InsPs concentrations in the concentrates as done for $InsP_6$.

Feeding a diet with increased $InsP_6$ concentration (Diet InsP) to the fistulated cows led to lower $InsP_5$ concentrations (∑of all detected $InsP_5$ isomers; Annex 4) compared to the other diets in the incubated oilseed meals. For hRSM, however, this effect occurred only for Diet InsP compared to Diet Basal. The higher disappearance of $InsP_6$ in oilseed meals when Diet InsP was fed (see Chapter 3.1.3), did not result in an accumulation of $InsP_5$. On the contrary, the lower $InsP_5$ concentrations suggest that the assumed positive effect of Diet InsP on phytase activity also improved the hydrolysis of $InsP_5$. With increasing dephosphorylation, however, the effect of the diet became less important. While the $InsP_4$ concentrations (∑ of all detected $InsP_4$ isomers; Annex 5) still tended to be lower when Diet InsP was fed ($P = 0.085$), the $InsP_3$ concentrations (∑ of all detected $InsP_3$ isomers; Annex 5) were not influenced by the diets. *In vitro* studies already suggested that the cleavage of the first phosphate group from $InsP_6$ is the decisive step in $InsP_6$ dephosphorylation and that further hydrolysis of lower InsPs follows soon after (Blaabjerg et al., 2007; Brask-Pedersen et al., 2011). Although a minor increase of $InsP_5$ and $InsP_4$ concentrations in pea seeds (Frias et al., 2003) and of $InsP_4$ and $InsP_3$ concentrations in lentil flour (Frias et al., 2003), wheat and rapeseed cake (Brask-Pedersen et al., 2011) was shown shortly after initiating $InsP_6$ hydrolysis, the concentrations of lower InsPs dropped quickly after a prolonged incubation time. Based on the $InsP_5$ concentrations in the bag residues and the fact that $InsP_4$ and $InsP_3$ isomers were only detected in the bag residues of hRSM, where substantial amounts were already found before incubation, the results of the *in situ* study of the present thesis support this assumption.

The capability of rumen microorganisms to produce phytase has long been known and strains of ruminal bacteria with measurable phytase activity have been identified. So far, however, little is known about the phytases that are active in the rumen and how they respond to dietary changes. Generally, microbial phytases are considered to be 3-phytases, but expression of a 5-phytase has been reported for *Selenomonas ruminantium* subsp. *lactilytica* (Puhl et al., 2008) and the phytase expressed by *Escherichia coli* was identified as a 6-phytase (Greiner et al., 2000). On the other hand, a 3-phytase was reported for soybean seeds (Konietzny and Greiner, 2002). By analysing the different $InsP_5$ isomers in the bag residues of the *in situ* study, a first step was taken towards assessing the main phytases active in the rumen (Manuscript 3). Three $InsP_5$ isomers were detected in the concentrates and bag residues of SBM, RSM, hRSM, and wheat: Ins(1,2,4,5,6)P_5, the hydrolysis product of 3-phytases, Ins(1,2,3,4,5)P_5, the hydrolysis product of 6-phytases, and Ins(1,2,3,4,6)P_5, the hydrolysis product of 5-phytases. In maize,

none of these $InsP_5$ isomers were detectable. In the processed concentrates (SBM, RSM, and hRSM), however, a fourth $InsP_5$ isomer was detected: Ins(1,3,4,5,6)P_5. Irvine and Schell (2001) described the presence of this isomer in avian erythrocytes and as predominant in mammalian cells and Pontoppidan et al. (2007) detected Ins(1,3,4,5,6)P_5 in untreated maize and barley. However, a 2-phytase has not been described to date and as this isomer was detected only in the oilseed meals, it probably arose during the production process as discussed in Manuscript 3.

The predominant $InsP_5$ isomers were Ins(1,2,4,5,6)P_5 and Ins(1,2,3,4,5)P_5, the hydrolysis products of 3- and 6-phytases. The concentrations of both isomers were influenced by the diet, leading to lower concentrations when Diet InsP was fed. For Ins(1,2,3,4,6)P_5, however, an interaction between diet, concentrate and time occurred, indicating that the 5-phytase reacted more substrate specific than 3- and 6-phytases. The conclusions from these findings are discussed in detail in Manuscript 3. Little is known about the properties of ruminal phytases, especially *in vivo*, and the great variety of findings makes it hard to apply the understanding of one phytase to another. Besides the differences in pH optima discussed in Chapter 3.1.4, the induction of phytase expression seems to differ as well. While the synthesis of *Mitsuokella jalaludinii* was induced by $InsP_6$ in the medium (Lan et al., 2002a), phytase of *S. ruminantium* subsp. *lactilytica* was expressed even when $InsP_6$ was excluded from the medium (Puhl et al., 2008). Taking the different effects of diet on concentrations of $InsP_5$ isomers into account, the data of the *in situ* study suggest that phytases of rumen bacteria might react differently to dietary changes. However, further research is required to assess the influence on actual phytase activity *in vivo*.

As discussed in Manuscript 3, several factors suggest that intrinsic plant phytases play only a minor role in the rumen. Although the *in situ* incubated concentrates were not analysed for their intrinsic phytase activity, it is well studied that wheat was the only examined concentrate with substantial endogenous phytase activity. Values for intrinsic phytase activity given in the literature are 1,193 and 1,850 U/kg DM for wheat (Eeckhout and de Paepe, 1994; Rodehutscord et al., 2016), 15 and 143 U/kg DM for maize (Eeckhout and de Paepe, 1994; Rodehutscord et al., 2016), 16 U/kg for RSM, and 8-40 U/kg for SBM (Eeckhout and de Paepe, 1994). $InsP_6$ concentrations for wheat and maize were similar (7.15 and 7.47 g/kg DM, respectively) and although maize shows hardly any intrinsic phytase activity, $InsP_6$ disappearance proceeded even faster compared to wheat. $InsP_6$ from SBM, RSM, and hRSM was also more or less

readily hydrolysed without having considerable intrinsic phytase activity. Furthermore, plant phytases are usually 6-phytases (Cosgrove, 1970) and the fact that Ins(1,2,3,4,5)P_5 concentrations were influenced by the diet suggests that microbial 6-phytases rather than intrinsic plant phytase contributed to this hydrolysis product. There is no evidence that intrinsic phytase activity might be influenced by increased amounts of substrate in the medium. However, it might have been possible that dietary changes influenced the degradation of the concentrate and, thus, influenced the access of intrinsic phytase to $InsP_6$. However, neither disappearance of DM nor that of CP was influenced by the diet which contradicts this possibility. Lastly, the pattern of $InsP_5$ isomers differed only slightly between the concentrates. In all concentrates, the highest percentage within $\sum InsP_5$ was calculated for Ins(1,2,4,5,6)P_5, followed by Ins(1,2,3,4,5)P_5. In wheat, the percentage of Ins(1,2,3,4,5)P_5 was only slightly, albeit significantly, higher compared to the other concentrates (see Manuscript 3).

The concentrations of $InsP_4$ and $InsP_3$ isomers do not allow for further interpretation of active phytases as the hydrolysis pathway of rumen phytases is almost completely unknown. The only pathway for a rumen microbial phytase that has been reported to date is that for *S. ruminantium* subsp. *lactilytica* (Puhl et al., 2008), which is described as follows:

$$InsP_6 \Rightarrow InsP(1,2,3,4,6)P_5 \Rightarrow InsP(1,2,3,6)P_4 \Rightarrow Ins(1,2,3)P_3 \Rightarrow Ins(1,2)P_2.$$

Known phytases of ruminal bacteria are cysteine phytases which are classified as acid phytases (Huang et al., 2011). The majority of acid phytases releases five of the six phosphate groups from $InsP_6$, continuing dephosphorylation adjacent to a free hydroxyl group (Greiner, 2007). This would explain the high percentages of Ins(1,2,5,6)P_4 on $\sum InsP_4$ (30%, Annex 6), which could be the next step of hydrolysis of a 3-phytase from Ins(1,2,4,5,6)P_5, and Ins(1,2,3,4)P_4 (29%) as the next hydrolysis product of a 6-phytase from Ins(1,2,3,4,5)P_4. However, in the present work $InsP_6$ concentrations were determined by High Performance Ion Chromatography (HPIC), which co-elutes enantiomers of InsPs and does not distinguish between D- and L-configurations. Thus, Ins(1,2,3,4,5)P_5 can be a D-Ins(1,2,3,5,6)P_5 derived from $InsP_6$ hydrolysis of a plant phytase or a L-Ins(1,2,3,4,5)P_5 originating from microbial 6-phytase hydrolysis. While this preliminary interpretation is feasible for this stage of the dephosphorylation process, it would be speculative to draw conclusions on $InsP_4$ and $InsP_3$ isomers from the data at hand.

3.4 METHODICAL ASPECTS

In order to gain better knowledge about ruminal $InsP_6$ hydrolysis and factors which could affect the extent of this hydrolysis, different methodical approaches were used in the present work. Each of these approaches has its advantages and disadvantages which are addressed in this chapter. Furthermore, methodical aspects observed during the experiments are discussed as they might be of relevance for the results.

The ruminal degradation of concentrates or diets can be assessed *in vitro*, *in situ* and *in vivo*. The term '*in vitro*' is used for many different methodical approaches. The two main classified types are batch cultures and continuous cultures (López, 2005) which can, according to the author, be differentiated as follows: Batch cultures are mainly used to estimate the digestibility or the extent of degradation in the rumen by single end-point or kinetic measurements in short- (hours) or medium-term (days) experiments. Continuous cultures simulate the rumen environment closer and enable long-term studies (weeks) as regular addition of buffer and nutrients and continuous removal of fermentation products allow for the establishment of a stable microbial population.

The greatest advantage of batch cultures is that a variety of questions can be examined easily as the effort keeps within reasonable limits. However, there is a lack of standardised procedures. As discussed in Manuscript 2, where the extent of *in vitro* $InsP_6$ disappearance differed between the two *in vitro* studies, incubation conditions as simple as the ratio of concentrate to rumen fluid seem to affect $InsP_6$ hydrolysis. This complicates the interpretation and comparison of results from different *in vitro* studies. For example: Different batch-culture experiments were conducted on ruminal $InsP_6$ hydrolysis *in vitro* by Brask-Pedersen et al. (2011), who incubated 0.5 g of concentrate in 50 ml of rumen fluid-buffer solution (diluted 1:5), while Morse et al. (1992) used 3 g of concentrate and 150 ml rumen fluid-buffer solution (diluted 1:4) which is similar to the second experiment of the *in vitro* studies of Manuscript 2 (3 g in 150 ml, 1:6). In experiment 1 of the *in vitro* studies, however, 6 g concentrate were incubated in 800 ml rumen fluid-buffer solution (diluted 1:2). Furthermore, all studies differed in the f:c ratios of the diets fed to the donor animals of rumen fluid. Fed diets, however, influence the properties of the rumen fluid and the composition of mixed rumen microbiota which, in turn, affects the *in vitro* hydrolysis of feedstuffs. Thus, it is hard to assess if the results of different studies are comparable or to which extent they were influenced by the experimental

conditions. This seems to be of particular importance when the ruminal degradation of diets is examined *in vitro*. As discussed in Manuscript 1, the examination of the ruminal *in vitro* disappearance of $InsP_6$ from different diets resulted in opposite effects as observed *in vivo*, when the total tract disappearance of $InsP_6$ was determined for the same diets. Similar differences between the results of *in vitro* and *in vivo* experiments were observed in the study of Ekelund et al. (2003). Feeding donor animals of rumen fluid with the *in vitro* examined diet, as suggested by Boguhn et al. (2013), seems to be crucial if *in vitro* results are applied to *in vivo* situations. A further issue that can limit the applicability of *in vitro* results is that *in vitro* studies do not necessarily reflect ruminal *in vivo* conditions. The rumen fluid, for example, is usually strained before it is used in *in vitro* incubations. In the case of measurements of ruminal $InsP_6$ hydrolysis, it remains unclear, how far this strained rumen fluid still reflects *in vivo* conditions (Brask-Pedersen et al., 2011) as phytase producing rumen bacteria are associated with the solid rumen fraction (Yanke et al., 1998). Due to the fact that an *in vitro* system cannot resemble the complex ruminal ecosystem *in vivo* in its entirety, effects on ruminal degradation found *in vitro* do not necessarily occur *in vivo* as well. In the *in vitro* studies (Manuscript 2), $InsP_6$ disappearance from maize and RSM was decreased when inorganic P was added to the buffer, an effect that did not occur when these concentrates were incubated *in situ* in the rumen. Furthermore, higher $InsP_6$ concentrations in the diet fed to donor animals of rumen fluid did increase *in vitro* ruminal $InsP_6$ disappearance from maize and RSM. *In situ*, however, this effect occurred only for RSM. *In vitro* systems might be more prone to changes and, thus, show effects which do not necessarily occur *in vivo*. Knuckles et al. (1989), for example, examined the effect of $InsP_6$ and its hydrolysis products on protein digestion *in vitro* and in rats *in vivo*. $InsP_6$ and lower InsPs inhibited the *in vitro* digestion of casein and bovine serum albumin by pepsin, but these effects were not reflected *in vivo* where it is likely that other enzymes initiated protein digestion. While *in vitro* studies are an appropriate approach to examine a variety of questions and to make comparisons that require well standardised conditions, *in vivo* studies are essential to verify if the *in vitro* results can be applied to *in vivo* conditions. Continuous cultures were hardly used to determine the ruminal $InsP_6$ hydrolysis so far. Godoy and Meschy (2001) determined the $InsP_6$ hydrolysis of Na-phytate in a Rusitec and also found a lower $InsP_6$ hydrolysis with increasing amounts of inorganic P in the buffer. However, due to the high solubility of Na-phytate (see Chapter 3.1.1), which was used as substrate in this study, it is hard

to compare these results with values for $InsP_6$ hydrolysis from concentrates. Thus, the suitability of a Rusitec to assess ruminal $InsP_6$ hydrolysis cannot be evaluated to date.

Compared to *in vitro* studies, the advantage of *in situ* studies is that ruminal digestion is examined in the animal and rumen conditions do not have to be simulated (López, 2005). Furthermore, the ruminal degradability of concentrates can be estimated for different passage rates. Several recommendations have been established to standardise the incubation procedure regarding bag size, pore size, sample size, or grinding size of the sample (Nocek, 1988; Vanzant et al., 1998; Seifried et al., 2015). However, this method also has some disadvantages. López (2005) described three main downsides: (1) not all material that has left the bag is actually degraded, (2) bag residues might not necessarily be undegradable matter originating from the feedstuff, and (3) the cloth of the bag represents a barrier which interferes with ruminal degradation leading to conditions in the bag that might differ from actual ruminal conditions. In the *in situ* study of the present thesis (Manuscript 3), five concentrates were examined when three different diets were fed to the fistulated animals. Defining the ruminal degradability of concentrates and their nutrients is of importance as this knowledge can be used for diet calculations. However, in the case of $InsP_6$, determining the *in situ* ruminal $InsP_6$ disappearance might not provide all required information to assess the actual *in vivo* hydrolysis of $InsP_6$. While addition of inorganic P to the diet decreased total tract $InsP_6$ disappearance *in vivo*, this effect was not observed *in situ*. Therefore, *in situ* studies cannot replace *in vivo* experiments completely. This seems to apply in particular, when the degradation of dietary ingredients with complex degradation patterns like $InsP_6$ is determined.

In vivo studies, albeit of high relevance, are effortful and costly, especially when the ruminal degradability of diets or concentrates is investigated in addition to total tract digestibility. To gain postruminal digesta, fistulated animals are a prerequisite. Furthermore, digesta is a heterogenous mixture of particulate matter and fluid and obtaining a sample that contains these constituents in the same proportions is difficult (France and Siddons, 1986). Thus, different markers associated with the different digesta constituents have to be administered to correct the unrepresentative sampling and determine the actual digesta flow at the sampling site (Faichney, 1975; France and Siddons, 1986). The animals used for the *in vivo* study of the present thesis were not fistulated, thus, the ruminal *in vivo* hydrolysis of $InsP_6$ was not determined. This, however, does not allow to draw conclusions from total tract digestibility of $InsP_6$ on ruminal $InsP_6$ hydrolysis. Thus, it remains unclear whether the observed effect of diet

was due to ruminal or postruminal changes in $InsP_6$ hydrolysis. This, in turn, implies that the actual extent of ruminal $InsP_6$ hydrolysis cannot be clarified conclusively and remains speculative if it is not determined by postruminal digesta sampling.

The chemical composition of the diets was determined from pooled samples of TMR, individual diet ingredients were not analysed. While the compositions of the TMR in Manuscript 1 and 2 did not differ widely from the calculated values based on earlier analyses of other batches of diet ingredients, the composition of Diet Basal in the *in situ* study (Manuscript 3) did. The P concentration of the diet was too high given the fact, that mainly concentrates with low P-content were used and no inorganic P (MNaP) was added. It cannot be ruled out that during the sampling or drying process of the TMR subsamples were confounded or traces of MNaP were accidentally mixed into Diet Basal. However, the focus of the *in situ* study was on $InsP_6$ disappearance from the concentrates, thus, the P concentration of the diet was not used for any calculations. Furthermore, the main effects were still apparent with high Pi concentrations in Diet Pi and high $InsP_6$ concentrations in Diet InsP compared to Diet Basal, suggesting that the interpretation of the results is not affected. In future studies, the chemical composition of all diet ingredients should be analysed which allows the calculation of the actual composition of the diet.

3.5 CONSEQUENCES FROM THE RESULTS

The findings of this work suggest that the present assumption, that $InsP_6$ is completely hydrolysed by ruminants, has to be reconsidered. The potential of rumen microorganisms to hydrolyse $InsP_6$ is unquestionable, however, there are factors that influence the extent of hydrolysis. So far, it is hard to draw clear conclusions from the results and determine reliably individual influence factors as complex interaction between different factors seem to exist.

The DMI of high producing cows is high (26 to 28 kg DMI/d at milk yields ranging from 35 to 51 kg/d (Boerman et al., 2015; Dann et al., 2015; Bahrami-Yekdangi et al., 2016)), which leads to a high ruminal turnover. This, in turn, decreases the time available for $InsP_6$ hydrolysis. Since for concentrates like RSM a slow progress of $InsP_6$ hydrolysis was determined *in vitro* and *in situ*, a high ruminal turnover could affect $InsP_6$ hydrolysis from these concentrates in particular. On the other hand, high producing cows are fed diets with high amounts of concentrates including $InsP_6$ rich concentrates like SBM or RSM. In the aforementioned studies of Boerman et al. (2015), Dann et al. (2015), and Bahrami-Yekdangi et al. (2016), SBM, RSM

and/or cottonseed represented between 14 and 17% of diet DM. In the present work, an increase of $InsP_6$ hydrolysis from concentrates as well as from TMR was observed *in vitro*, *in situ*, and *in vivo* with 14 to 18% RSM in the diets compared to diets with no RSM and, thus, low $InsP_6$ concentrations. Thus, an increased $InsP_6$ hydrolysis at high $InsP_6$ intake could balance a possible negative influence of high ruminal turnover on $InsP_6$ hydrolysis. However, other factors such as the concentration of mineral P or Ca in the diet or the pH value in the rumen or in the postruminal digesta, cannot be ruled out as affecting factors on $InsP_6$ hydrolysis for now, either.

The P stored in $InsP_6$ is a valuable P source in ruminant nutrition and achieving optimal utilisation of $InsP_6$-P is of interest for ecological reasons. High ruminal $InsP_6$ hydrolysis could help to save the finite global P resources as it seems to be possible to omit mineral P from ruminant diets completely. Furthermore, reducing the excretion of undegraded or unabsorbed P diminishes the risk of eutrophication by excessive manure application. However, before definite recommendations on diet calculations can be given, further research is required. The results of the present work indicate that *in vitro* and *in situ* studies might not be adequate to reflect the complex interactions in the rumen and, thus, further *in vivo* studies on ruminal $InsP_6$ hydrolysis are necessary to draw explicit conclusions.

To date, there are still inconsistencies regarding the values for the efficiency of utilisation of dietary P. The recommendations of the GfE in Germany gives an overall value of 0.70 for the efficiency of P utilisation (GfE, 2001). The NRC of the United States of America distinguishes between roughages and concentrates with values of 0.64 and 0.70, respectively (NRC, 2001). Suttle (2010) supports the differentiation between feeds but suggests 0.74 for dry roughages and 0.80 for grass, succulent forages and concentrates. The underlying thought and the resulting calculation for the value of utilisation proposed by the GfE is that 80% of dietary P are absorbable and that 90% of the absorbed P are used to meet the P net requirements (GfE, 1993). In the *in vivo* and *in situ* studies of the present work, differences in $InsP_6$ hydrolysis between concentrates became obvious. However, the extent of *in situ* hydrolysis after 24 h was high (>95%, averages of diets) for all concentrates (except for hRSM) rendering the released P absorbable in the intestine. This could suggest that the amount of absorbable P from concentrates is higher than 80% and, thus, that after further studies on this topic the recommendations of the GfE could be revised.

3.6 PERSPECTIVES FOR FUTURE RESEARCH

From the studies of the present work it is not possible to draw unambiguous conclusions on affecting factors of ruminal $InsP_6$ hydrolysis as the effect of these factors differed between the methodical approaches. However, both high $InsP_6$ and Pi concentrations in the diets fed to dairy cows seem to influence the hydrolysis process to some extent.

As the ruminal $InsP_6$ hydrolysis *in vivo* is of main interest, further *in vivo* studies are required to assess influencing factors. Comparing and interpreting results of different *in vivo* studies is often impossible as they differ in more than one respect. Thus, *in vivo* studies with similar experimental conditions are required allowing for comparisons of the respective results. After evaluating the results of the present thesis and the literature, ruminal passage rate as well as the composition of the diet seem to have the potential to affect the extent of ruminal $InsP_6$ hydrolysis *in vivo*. The passage rate may be of special importance when concentrates with slow $InsP_6$ degradation like RSM are part of the diet. Digestibility studies that use diets with different concentrate composition fed to animals in various stages of lactation and, thus, differing DMI and passage rates, would give further information on the relevance of these factors. Furthermore, the addition of exogenous phytase should be tested under different conditions. If $InsP_6$ hydrolysis is affected by passage rate or diet composition, the addition of phytase might have the potential to increase the extent of hydrolysis under certain conditions.

However, to gain further knowledge about actual ruminal $InsP_6$ hydrolysis, other *in vivo* approaches have to be considered as well. Postruminal $InsP_6$ degradation was observed by Park et al. (2002), Ray et al. (2012), and Ray et al. (2013) and although it might not be a major contributor to total tract $InsP_6$ hydrolysis, distinct conclusions on ruminal $InsP_6$ hydrolysis cannot be drawn from digestibility studies. Thus, analyses of postruminal digesta obtained from intestinally fistulated animals or by sampling of omasal digesta via rumen cannulas are required to assess actual ruminal $InsP_6$ hydrolysis.

Additionally, the determination of the true absorption coefficients of P for particular concentrates or feedstuffs would be of interest. Studies on this subject are elaborate, as the amount of endogenous P recycled via saliva has to be determined with a tracer. However, further knowledge about the true absorption coefficients of different concentrates or feedstuffs would help to provide more precise data on utilisation of P in ruminants and, thus, could help

to reduce unnecessary use of mineral P in ruminant nutrition as well as the excretion of excessive dietary P.

The high correlations between *in situ* CP and $InsP_6$ disappearance from oilseed meals demonstrated the close association between CP and $InsP_6$ in these concentrates. For certain concentrates it might be possible to create a model predicting the disappearance of $InsP_6$ from values for CP disappearance. Thus, further *in situ* studies on CP and $InsP_6$ disappearance from different concentrates should be conducted to gather a database, which allows to create such a model.

There is further need for research in regard to the microbial phytase activity in the rumen. Factors affecting ruminal $InsP_6$ hydrolysis might act in different ways in the rumen. Among others, changes of the microbial composition and hence the amount of phytase producing microorganisms in the rumen, the expression of phytase or the activity of phytase could be the reason for observed differences in ruminal $InsP_6$ hydrolysis. This suggests that it is important to learn more about phytase producing bacteria, their respective phytases and the properties of the expressed phytases. Huang et al. (2011) analysed ruminal DNA and found many sequences of previously unidentified phytases which indicates that the rumen is still widely unexplored in this respect. Furthermore, phytases are classified solely according to their capability of hydrolysing phytate *in vitro*, however, some enzymes might not be involved *in vivo* or have other functions as suggested by (Greiner, 2007). In this respect, Rusitec studies seem to be an appropriate approach as they enable to study long-term effects on microbial populations under controlled conditions (López, 2005).

3.7 CONCLUSIONS

- The potential of rumen microorganisms to hydrolyse $InsP_6$ is high. The ruminal *in vitro* and *in situ* disappearance of $InsP_6$ from different concentrates as well as the total tract $InsP_6$ disappearance from TMR was 90% and higher. However, the present assumption that $InsP_6$ is completely hydrolysed in general has to be reconsidered. As shown in the present work, differences in the pace of $InsP_6$ hydrolysis exist between concentrates. Furthermore, factors such as Pi and $InsP_6$ concentrations in the diet can affect the extent of $InsP_6$ hydrolysis from both concentrates and TMR.
- Identifying individual factors influencing the hydrolysis of $InsP_6$ is difficult as there seem to occur complex interactions. However, it is of environmental concern to further

investigate the efficiency and optimisation of ruminal $InsP_6$ hydrolysis. Drawing conclusions from the few so far published data on this topic is complicated by the fact that the studies usually differ in more than one respect. Thus, further research is required providing comparable conditions to examine particular factors.

- Using different methodical approaches to study $InsP_6$ hydrolysis in ruminants showed that *in vitro* and *in situ* studies may not be suitable to investigate all questions of concern. The applicability of results on *in vivo* situations seems not to be given in each and every case indicating the need of effortful studies on ruminal $InsP_6$ hydrolysis *in vivo*.
- To completely understand the processes involved in ruminal $InsP_6$ hydrolysis, it is necessary to use an interdisciplinary approach involving microbiologists. It is essential to identify further phytase producing rumen microorganisms, their respective phytases and especially the properties of their phytases *in vivo.* Ruminal $InsP_6$ hydrolysis can only be better understood when it is clarified how expression and activity of phytases actually react to differences in the ruminal environment *in vivo*.

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CHAPTER 4

INCLUDED MANUSCRIPTS

4 INCLUDED MANUSCRIPTS

4.1 MANUSCRIPT 1

Effects of mineral and rapeseed phosphorus supplementation on phytate degradation in dairy cows

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ABSTRACT

The objective of this study was to evaluate the effects of diet composition on phytate ($InsP_6$) hydrolysis in dairy cows. In experiment 1, four diets differing in the amount and source of phosphorus (P) were fed to 24 lactating dairy cows in a 4x4 Latin Square design. The control diet (Diet C) contained 4.18 g P/kg dry matter (DM). For Diet MP, mineral P was added (5.11 g P/kg DM). Diet RS contained rapeseed and rapeseed meal as organic P sources (5.26 g P/kg DM) whereas in Diet RSM organic P originated from rapeseed meal and rapeseed oil (5.04 g P/kg DM). Total P (tP) and $InsP_6$ excretion in faeces were measured. In experiment 2, a rumen simulation technique (Rusitec) was used to estimate ruminal disappearance of tP and $InsP_6$ from Diets C, MP, and RSM. In experiment 1, tP concentrations in faeces increased with tP intake and were highest for Diets RS and RSM. The source of supplemented P had no influence on tP digestibility, but tP digestibility was reduced for Diets MP, RS, and RSM compared to Diet C. The disappearance of $InsP_6$ decreased in Diet MP (85.0%) and increased in Diets RS and RSM (92.7 and 94.0%, respectively) compared to Diet C (90.0%). In experiment 2, P source influenced ruminal tP disappearance (Diet MP: 78.6%; Diet RSM: 75.3%). The disappearance of $InsP_6$ was higher for Diet C (98.1%) than for Diets MP and RSM (95.6 and 94.9%, respectively). The results confirmed the high potential of ruminants to utilise $InsP_6$, but differences in the composition of the diets influenced $InsP_6$ disappearance. Further studies of the site of $InsP_6$ hydrolysis are required to understand the relevance of $InsP_6$ hydrolysis for the absorption of P.

4.2 MANUSCRIPT 2

Ruminal phytate degradation of maize grain and rapeseed meal *in vitro* and as affected by phytate content in donor animal diets and inorganic phosphorus in the buffer

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ABSTRACT

In two *in vitro* studies the ruminal disappearance of phytate phosphorus ($InsP_6$-P) from maize grain and rapeseed meal (RSM) was determined. In experiment 1, two diets with different phosphorus (P) and $InsP_6$-P concentrations were fed to the donor animals of rumen fluid (diet HP: 0.49% P in dry matter; diet LP: 0.29% P). Maize grain and RSM were incubated in a rumen fluid/saliva mixture for 3, 6, 12, and 24 h. In experiment 2, a diet similar to diet HP was fed, and the rumen fluid was mixed with artificial saliva containing 120 mg inorganic P/l (Pi) or no inorganic P (P0). Maize grain and RSM were incubated with either buffer for 3, 6, 12, and 24 h. The concentrations of total P (tP) and $InsP_6$ were analysed in the fermenter fluids and feed residues. The disappearance of $InsP_6$-P from maize was completed after 12 h of incubation in both experiments. From RSM, 93% (diet LP) and 99% (diet HP) of $InsP_6$-P in experiment 1 and 80% (Pi) and 89% (P0) in experiment 2 had disappeared after 24 h of incubation. The $InsP_6$-P concentration in the fermenter fluids was higher for maize, but no accumulation of $InsP_6$-P occurred, indicating a prompt hydrolysis of soluble $InsP_6$. The results confirmed the capability of rumen microorganisms to efficiently hydrolyse $InsP_6$. However, differences between feedstuffs and diet composition as well as the presence of inorganic P in the *in vitro* system influenced the hydrolysis process. Further studies are required to understand how these factors affect the hydrolysis of $InsP_6$ and their respective relevance *in vivo*.

4.3 MANUSCRIPT 3

Effects of dietary mineral phosphorus and phytate on *in situ* ruminal phytate disappearance from different concentrates in dairy cows

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ABSTRACT

The first objective of this study was to determine the influence of the dietary composition on the *in situ* disappearance of phytate ($InsP_6$) from wheat, maize, soybean meal (SBM), and rapeseed meals (solvent extracted, without (RSM) or with (hRSM) heat treatment). The second objective was to assess the primary degradation products of $InsP_6$ in the rumen. In a 3x3 Latin Square, 3 diets with different phosphorus and $InsP_6$ concentrations (basal diet = 0.38% P in dry matter; high-P diet = 0.56% P; high-$InsP_6$ diet = 0.39% P) were fed to 3 ruminally fistulated lactating Jersey cows. Ground concentrates (sieve size = 2 mm) were incubated in polyester bags in the rumen for 2, 4, 8, 16, and 24 h. The bag residues were analysed for P, $InsP_6$, isomers of lower inositol phosphates ($InsP_5$, $InsP_4$, $InsP_3$), and crude protein. $InsP_6$ disappeared more rapidly from cereal grains than from oilseed meals, however, after 24 h of incubation ≥95% of $InsP_6$ had disappeared from all concentrates except hRSM (57%, diet average). Feeding the high-$InsP_6$ diet increased $InsP_6$ disappearance from oilseed meals, but not from maize and wheat. The predominant $InsP_5$ isomer in all bag residues was Ins(1,2,4,5,6)P_5 followed by Ins(1,2,3,4,5)P_5 and Ins(1,2,3,4,6)P_5. In the bag residues of both rapeseed meals, a further $InsP_5$ isomer (Ins(1,3,4,5,6)P_5) was detected. Feeding the high-$InsP_6$ diet led to lower concentrations of Ins(1,2,4,5,6)P_5 and Ins(1,2,3,4,5)P_5, whereas an interaction between diet, concentrate, and time occurred for Ins(1,2,3,4,6)P_5. The results confirm the high potential of rumen microorganisms to hydrolyze $InsP_6$. Increasing the amount of $InsP_6$ in the diet, however, can further enhance $InsP_6$ hydrolysis. This may be relevant when concentrates with slowly degradable $InsP_6$, such as RSM or heat treated concentrates, are fed to dairy cows. Based on the concentrations of $InsP_5$ isomers, 3- and 6-phytases appear to play a major role in the rumen. Conversely, intrinsic plant phytase activity appears to be less relevant. The percentage of its primary hydrolysis product, Ins(1,2,3,4,5)P_5, was only slightly higher in wheat known for high intrinsic phytase activity compared to the other concentrates. Additional information regarding the factors influencing the extent of ruminal $InsP_6$ disappearance will require further studies to determine the phytase activity of rumen microorganisms and the characteristics of their respective phytases.

CHAPTER 5

SUMMARY

5 SUMMARY

The predominant storage form of phosphorus (P) in plant seeds and grains is phytate, which is defined as any salt of phytic acid (*myo*-inositol (1,2,3,4,5,6) hexakis(dihydrogen phosphate) or $InsP_6$). To cleave the phosphate group and, thus, make the bound P available for absorption by the animal, the enzyme phytase is required. As rumen microorganisms show substantial phytase activity, P from $InsP_6$ has been considered available for ruminants so far. However, recent studies have suggested that the extent of $InsP_6$ hydrolysis in ruminants is variable, leading to an incomplete hydrolysis of $InsP_6$ in specific conditions followed by the excretion of P from undegraded $InsP_6$. As P is an essential element for the metabolism in animals it is important to ensure that the animals' requirements are met. Diets for ruminants are often supplemented with mineral P (Pi). However, the global phosphate resources are finite and the excretion of surplus P contributes to eutrophication of surface water when applied to the farmland with manure in excessive amounts. Thus, dietary P supply is of environmental concern. Better knowledge about ruminal $InsP_6$ hydrolysis could help to optimise the utilisation of $InsP_6$ and, thus, reduce the use of Pi as well as unnecessary excretion of P. Hence, the objectives of the present thesis were to examine the $InsP_6$ hydrolysis from different feedstuffs in ruminants and to identify factors that might affect the extent of $InsP_6$ hydrolysis.

In the first study (Manuscript 1), the total digestive tract disappearance of $InsP_6$ from diets differing in amount and source of P was determined in lactating dairy cows. The control diet (Diet C) contained 0.42% P in DM, for Diet MP, mineral P was added (0.51% P). Diet RS and Diet RSM contained organic P sources (Diet RS: rapeseed and rapeseed meal (RSM), 0.53% P; Diet RSM: RSM and rapeseed oil, 0.50% P). Compared to Diet C, the addition of mineral P in Diet MP decreased $InsP_6$ disappearance from 90 to 85%. For the diets with organic P sources, however, $InsP_6$ disappearance increased (93% Diet RS, 94% Diet RSM). For Diets C, MP, and RSM, the ruminal $InsP_6$ hydrolysis was also estimated in a Rusitec study. *In vitro*, the ruminal $InsP_6$ disappearance was 98% for Diet C, 96% for Diet MP, and 95% for Diet RSM. The results confirmed the high potential of rumen microorganisms to hydrolyse $InsP_6$, but the composition of the diet influenced the extent of hydrolysis *in vivo*. Although an influence of diet composition was also observed *in vitro*, this effect was in opposite direction compared to the *in vivo* study. While increasing the $InsP_6$ concentrations in the diets increased $InsP_6$ hydrolysis *in vivo*, ruminal $InsP_6$ hydrolysis decreased *in vitro*.

In the second study (Manuscript 2), two *in vitro* experiments were conducted in order to determine the $InsP_6$ hydrolysis from maize grain and RSM. In experiment 1, two diets differing in P- and $InsP_6$-P concentration were fed to the donor animals of rumen fluid (high P diet: 0.49% P, low P diet: 0.29% P). In experiment 2, a diet similar to the high P diet of experiment 1 was fed to the donor animals of rumen fluid (0.45% P) and the rumen fluid was mixed with artificial saliva containing Pi (PI: 120 mg Pi/l) or no Pi. Maize and RSM were incubated for 3, 6, 12, and 24 h in both experiments and the $InsP_6$ concentration was analysed in fermenter fluids and bag residues. $InsP_6$ disappearance from maize proceeded faster than from RSM. When averaged among treatments, $InsP_6$ from maize had disappeared completely after 12 h of incubation while $InsP_6$ disappearance from RSM was 96% (experiment 1) and 84.5% (experiment 2) after 24 h of incubation. The disappearance of $InsP_6$ was higher when the diet with high P concentration was fed (experiment 1) and lower when the rumen fluid was mixed with Pi containing buffer (experiment 2).

In the third study (Manuscript 3), the *in situ* disappearance of $InsP_6$ from five different concentrates was examined. Maize, wheat, RSM, heat treated RSM (hRSM), and soybean meal were incubated in the rumen of fistulated dairy cows fed with three diets differing in P- and $InsP_6$-P concentration. As in the *in vivo* study (Manuscript 1), mineral P was added to a basal diet (Diet Basal: 0.38% P, Diet Pi: 0.56% P) while P was exclusively of organic origin in the third diet (Diet InsP: 0.39% P). Concentrations of $InsP_6$ and isomers of $InsP_5$, $InsP_4$, and $InsP_3$ were determined in the bag residues after 2, 4, 8, 16, and 24 h of incubation. The disappearance of $InsP_6$ from cereals proceeded faster than from oilseed meals, however, averaged over the diets, after 24 h of incubation ≥95% had disappeared from all concentrates except for hRSM (57%). Feeding the diet with high $InsP_6$ concentrations (Diet InsP) increased $InsP_6$ disappearance from oilseed meals but not from cereals, while feeding Diet Pi did not influence ruminal $InsP_6$ hydrolysis from any concentrate. The results derived from analysis of lower InsPs suggested that intrinsic plant phytase activity plays only a minor role in the rumen and that active phytases in the rumen react differently to changes in the ruminal environment.

The results of the present thesis suggest that the composition of the diet fed to ruminants affects the extent of ruminal $InsP_6$ hydrolysis. While high $InsP_6$ concentrations have the potential to increase $InsP_6$ hydrolysis, a decrease of $InsP_6$ hydrolysis can occur after addition of Pi to the diet. However, the effects were inconsistent for the different methodical approaches used in the present work. Furthermore, differences in the pace of $InsP_6$ hydrolysis between concentrates

occurred which could be of importance at high ruminal passage rates when the time available for ruminal hydrolysis decreases. Further research is required to clarify the complex interactions in the rumen and how they affect $InsP_6$ hydrolysis before reliable recommendations for diet calculations can be given. Especially further *in vivo* studies would be of interest here, as *in vitro* and *in situ* studies do not seem to reflect ruminal and total tract hydrolysis of $InsP_6$ precisely.

CHAPTER 6

ZUSAMMENFASSUNG

6 ZUSAMMENFASSUNG

Phosphor (P) wird in pflanzlichen Samen und Körnern überwiegend als Phytat gespeichert. Unter dem Begriff Phytat werden alle Salze der Phytinsäure (*Myo*-Inositol 1,2,3,4,5,6-Hexakisdihydrogenphosphat oder $InsP_6$) zusammengefasst. Um die Phosphatgruppe abzuspalten und so den darin gebundenen P für die Absorption verfügbar zu machen, wird das Enzym Phytase benötigt. Da die Mikroorganismen im Pansen eine nicht unerhebliche Phytaseaktivität aufweisen, ging man bislang davon aus, dass der in $InsP_6$ gespeicherte P Wiederkäuern generell zur Verfügung steht. Studien der letzten Jahre zeigten allerdings, dass auch beim Wiederkäuer die $InsP_6$-Hydrolyse beeinträchtigt werden kann und entsprechend P aus nicht abgebautem $InsP_6$ ausgeschieden wird. Da P im tierischen Stoffwechsel eine essentielle Rolle einnimmt, ist es unabdingbar, den P-Bedarf des Tieres zu decken. Auf Grund von Unsicherheiten hinsichtlich der Verfügbarkeit von $InsP_6$ erfolgt bei der Rationsgestaltung für Wiederkäuer häufig eine Zulage von anorganischem P (Pi). Da die globalen P-Vorräte endlich sind und die Ausscheidung von überschüssigem P bei Überdüngung zur Eutrophierung von Oberflächengewässern beitragen kann, ist eine adäquate Versorgung der Tiere mit P auch von ökologischer Bedeutung. Genauere Erkenntnisse zur ruminalen $InsP_6$-Hydrolyse können dazu beitragen, die Verwertung von $InsP_6$ zu optimieren und dadurch sowohl den Einsatz von Pi als auch die Ausscheidung von überschüssigem P zu verhindern. Ziel dieser Arbeit war es daher, die Hydrolyse von $InsP_6$ aus unterschiedlichen Futtermitteln beim Wiederkäuer näher zu untersuchen und Faktoren zu identifizieren, die die $InsP_6$-Hydrolyse beeinflussen können.

Im Rahmen der ersten Studie (Manuskript 1) wurde der $InsP_6$-Abbau aus verschiedenen Rationen im Gesamtverdauungstrakt laktierender Milchkühe bestimmt. Die Rationen unterschieden sich hinsichtlich der P-Konzentration und P-Quelle. Zu einer Kontrollration mit einem P-Gehalt von 0,42% in der TM wurde für Ration MP mineralischer P zugelegt (0,51% P). In Ration RS und RSM wurden vergleichbare P-Gehalte mit organischen P-Quellen erzielt (Ration RS: Rapssaat und Rapsextraktionsschrot (RSM), 0,53% P; Ration RSM: RSM und Rapsöl, 0,50% P). Im Vergleich zur Kontrollration ging der $InsP_6$-Abbau bei Ration MP von 90 auf 85% zurück, während er bei den Rationen RS und RSM auf 93 bzw. 94% anstieg. Für die Rationen C, MP und RSM wurde zusätzlich der ruminale Abbau mittels Rusitec bestimmt. Der *in vitro* Abbau lag für Ration C bei 98%, für Ration MP bei 96% und für Ration RSM bei 95%. Die Ergebnisse bestätigen das hohe Potential der Mikroorganismen im Pansen zur $InsP_6$-

Hydrolyse, die Zusammensetzung der Ration beeinflusste allerdings das Ausmaß des Abbaus *in vivo*. Zwar unterschieden sich die Rationen auch im ruminalen *in vitro* Abbau von $InsP_6$, der Effekt der Rationen war allerdings umgekehrt. Während eine höhere $InsP_6$- Konzentration in der Ration *in vivo* zu einem Anstieg des $InsP_6$-Abbaus führte, ging der *in vitro* Abbau von $InsP_6$ zurück.

Für die zweite Studie (Manuskript 2) wurden zur Bestimmung der $InsP_6$-Hydrolyse aus Mais und RSM zwei *in vitro* Experimente durchgeführt. In Experiment 1 wurden die Spendertiere des Pansensaftes mit zwei Rationen angefüttert, die sich hinsichtlich der P- und $InsP_6$-Konzentration unterschieden (P-reiche Ration: 0,49% P, P-arme Ration: 0,29% P). In Experiment 2 wurden die Tiere mit einer Ration angefüttert, die mit der P-reichen Ration aus Experiment 1 vergleichbar war (0,45% P) und der Pansensaft mit Pi-haltigem (120 mg P/l) oder Pi-freiem Puffer gemischt. Mais und RSM wurden jeweils für 3, 6, 12 und 24 h inkubiert und anschließend die $InsP_6$-Konzentration in der Fermenterflüssigkeit und im Futterrückstand bestimmt. Die $InsP_6$-Hydrolyse verlief bei Mais schneller als bei RSM. Während bei Mais das $InsP_6$ nach 12 h Inkubation vollständig abgebaut war, waren bei RSM nach 24 h, gemittelt über die einzelnen Behandlungen, 96% des $InsP_6$ in Experiment 1 und 84,5% in Experiment 2 abgebaut. Die $InsP_6$-Hydrolyse war bei Fütterung der P-reichen Ration höher als bei Fütterung der P-armen Ration, die Zulage von Pi über den Puffer führte zu einem Rückgang der Hydrolyse.

In der dritten Studie (Manuskript 3) wurde der ruminale Abbau von $InsP_6$ aus fünf verschiedenen Futtermitteln untersucht. Die Inkubation von Mais, Weizen, RSM, hitzebehandeltem RSM und Sojaextraktionsschrot erfolgte bei Fütterung dreier unterschiedlicher Rationen. Ähnlich wie im ersten Versuch (Manuskript 1) wurde Pi zu einer Kontrollration zugelegt (Ration Basal: 0,38% P, Ration Pi: 0,56% P) während in der dritten Ration P gänzlich organischen Ursprungs war (Ration InsP: 0,39%). Die $InsP_6$-Konzentration sowie die Konzentrationen der Isomere von $InsP_5$, $InsP_4$ und $InsP_3$ wurden in den Beutelrückständen nach 2, 4, 8, 16 und 24 h gemessen. Der $InsP_6$-Abbau verlief bei Getreide schneller als bei den Ölschroten, nach 24 h Inkubation war jedoch bei allen Futtermitteln, gemittelt über die Rationen, ≥95% des $InsP_6$ aus den Beuteln verschwunden. Einzig bei Inkubation von hRSM waren nach 24 h lediglich 57% des $InsP_6$ abgebaut. Bei Fütterung der $InsP_6$-reichen Ration (Ration InsP) stieg der $InsP_6$-Abbau bei den Ölschroten an. Dieser Effekt war bei den Getreiden nicht nachzuweisen. Die Fütterung von Ration Pi zeigte dagegen bei

keinem der untersuchten Futtermittel einen Einfluss auf den ruminalen *in situ* Abbau von $InsP_6$. Die Konzentrationen der Isomere der niederen InsPs in den Inkubationsrückständen ließen vermuten, dass die endogene pflanzliche Phytaseaktivität im Pansen eine untergeordnete Rolle spielt. Zudem scheinen die im Pansen aktiven Phytasen auf Rationsänderungen unterschiedlich zu reagieren.

Die Ergebnisse der vorliegenden Arbeit legen den Schluss nahe, dass die Zusammensetzung der an Wiederkäuer verfütterten Rationen das Ausmaß der ruminalen $InsP_6$-Hydrolyse beeinflussen kann. Hohe $InsP_6$-Konzentrationen in der Ration scheinen den Abbau von $InsP_6$ zu steigern während eine Zulage von Pi einen negativen Einfluss haben kann. Die beobachteten Effekte waren bei den unterschiedlichen methodischen Ansätzen, die im Rahmen dieser Arbeit eingesetzt wurden, allerdings teilweise gegensätzlich. Des Weiteren waren Unterschiede hinsichtlich der Geschwindigkeit, in der der $InsP_6$-Abbau voranschritt, zwischen den untersuchten Futtermitteln zu erkennen. Dies könnte vor allem bei hohen Passageraten für das Ausmaß des ruminalen $InsP_6$-Abbaus von Bedeutung sein, wenn weniger Zeit zur Hydrolyse zur Verfügung steht. Bevor zuverlässige Empfehlungen zur Rationsplanung gegeben werden können, besteht allerdings noch weiterer Forschungsbedarf, um die komplexen Wechselwirkungen im Pansen aufzuklären.

CHAPTER 7

ANNEX

7 ANNEX

ANNEX 1. Concentration of $InsP_6$-P in the bag residues *in situ* in g/kg DM (n=9; averages of diets; means and SEM)

Incubation time	Concentrate*				
h	Maize	Wheat	SBM	RSM	hRSM
2	0.69^{Ca}	3.77^{Cc}	3.47^{Db}	9.43^{Cd}	5.74^{Cc}
	0.04	0.19	0.06	0.19	0.07
4	0.60^{Ca}	4.42^{Cc}	3.22^{Db}	10.2^{Cd}	5.56^{Cc}
	0.05	0.31	0.18	0.17	0.11
8	0.19^{Ba}	5.64^{Dc}	2.85^{Cb}	9.87^{Cd}	5.70^{Cc}
	0.02	0.50	0.26	0.25	0.11
16	0.06^{Aa}	1.53^{Bb}	1.68^{Bb}	3.71^{Bc}	4.91^{Bd}
	0.01	0.15	0.25	0.35	0.10
24	0.05^{Aa}	0.24^{Aa}	0.91^{Ab}	1.45^{Ac}	4.45^{Ad}
	0.02	0.04	0.18	0.24	0.19

* SBM, soybean meal; RSM, rapeseed meal; hRSM, heat treated RSM; ^{ab} Means with different lowercase superscripts differ significantly within row; ^{AB} Means with different uppercase superscripts differ within column

ANNEX 2. Disappearance of DM *in situ* in % (n=9; averages of diets, means and SEM)

Incubation time	Concentrate*				
h	Maize	Wheat	SBM	RSM	hRSM
2	31^{Ab}	67^{Ad}	37^{Ac}	31^{Ab}	28^{Aa}
	0.64	1.18	0.71	0.72	0.31
4	34^{Bb}	73^{Be}	41^{Bd}	36^{Bc}	30^{Ba}
	0.48	2.67	0.64	0.46	0.50
8	46^{Cb}	89^{Ce}	59^{Cd}	54^{Cc}	38^{Ca}
	1.05	0.64	1.43	0.89	0.34
16	61^{Db}	93^{CDe}	84^{Dd}	76^{Dc}	49^{Da}
	2.91	0.23	0.68	1.37	1.32
24	79^{Eb}	95^{Dc}	93^{Ec}	81^{Eb}	57^{Ea}
	2.65	0.05	0.65	0.48	0.78

* SBM, soybean meal; RSM, rapeseed meal; hRSM, heat treated RSM; ^{ab} Means with different lowercase superscripts differ significantly within row; ^{AB} Means with different uppercase superscripts differ within column

ANNEX 3. Concentration (g/kg DM) and disappearance (%) of CP and $InsP_6$ *in situ*

Concentrate*	Diet	Time	CP	$InsP_6$	CP	$InsP_6$
			g/kg		%	
SBM	1	2	604	11.95	23	33
SBM	1	2	604	13.26	24	27
SBM	1	2	639	12.46	18	29
SBM	1	4	602	12.28	29	36
SBM	1	4	623	12.24	29	39
SBM	1	4	636	13.25	23	29
SBM	1	8	619	12.72	43	49
SBM	1	8	647	10.04	45	62
SBM	1	8	653	14.56	54	55
SBM	1	16	588	8.51	81	88
SBM	1	16	544	5.55	81	92
SBM	1	16	564	10.44	83	86
SBM	1	24	600	6.82	88	94
SBM	1	24	443	2.41	92	98
SBM	1	24	348	4.62	96	98
SBM	2	2	599	13.15	30	33
SBM	2	2	597	12.46	23	29
SBM	2	2	625	12.23	24	35
SBM	2	4	605	11.73	32	41
SBM	2	4	607	10.42	29	46
SBM	2	4	645	13.47	29	34
SBM	2	8	626	10.79	54	65
SBM	2	8	620	10.11	54	67
SBM	2	8	671	11.00	46	61
SBM	2	16	593	7.62	80	89
SBM	2	16	560	5.18	78	91
SBM	2	16	586	7.04	83	91
SBM	2	24	577	3.35	89	97
SBM	2	24	410	3.15	93	98
SBM	2	24	460	4.67	94	97
SBM	3	2	585	12.61	27	30
SBM	3	2	600	11.06	24	38
SBM	3	2	636	11.71	20	35
SBM	3	4	608	9.13	27	52
SBM	3	4	613	7.95	27	58
SBM	3	4	613	12.56	27	34
SBM	3	8	632	4.98	42	80

Concentrate*	Diet	Time	CP	$InsP_6$	CP	$InsP_6$
			g/kg		%	
SBM	3	8	618	8.55	46	67
SBM	3	8	653	8.38	52	73
SBM	3	16	506	2.50	86	97
SBM	3	16	421	3.43	88	96
SBM	3	16	578	3.48	82	95
SBM	3	24	478	1.09	92	99
SBM	3	24	329	1.87	95	99
SBM	3	24	371	1.26	96	99
RSM	1	2	392	35.70	27	-17
RSM	1	2	381	32.84	28	-10
RSM	1	2	380	31.95	26	-9
RSM	1	4	369	38.41	36	-17
RSM	1	4	369	34.06	36	-5
RSM	1	4	359	35.59	33	-17
RSM	1	8	349	40.30	54	6
RSM	1	8	343	37.46	55	14
RSM	1	8	306	32.07	66	36
RSM	1	16	233	15.13	86	84
RSM	1	16	238	13.10	86	86
RSM	1	16	245	13.95	82	82
RSM	1	24	211	10.88	88	89
RSM	1	24	189	5.28	90	95
RSM	1	24	161	4.32	92	96
RSM	2	2	377	37.24	31	-21
RSM	2	2	384	30.03	23	-7
RSM	2	2	368	33.26	32	-9
RSM	2	4	360	39.24	36	-23
RSM	2	4	365	34.82	35	-10
RSM	2	4	358	35.62	37	-10
RSM	2	8	338	32.98	57	25
RSM	2	8	340	36.35	56	16
RSM	2	8	340	35.74	57	20
RSM	2	16	220	11.50	87	88
RSM	2	16	293	19.95	74	68
RSM	2	16	261	16.50	81	79
RSM	2	24	214	6.02	88	94
RSM	2	24	187	5.72	90	95
RSM	2	24	182	5.43	90	95
RSM	3	2	376	33.62	28	-14

Concentrate*	Diet	Time	CP	$InsP_6$	CP	$InsP_6$
			g/kg		%	
RSM	3	2	379	33.29	26	-15
RSM	3	2	365	33.49	33	-8
RSM	3	4	365	37.67	36	-17
RSM	3	4	363	35.01	35	-10
RSM	3	4	362	35.55	35	-13
RSM	3	8	351	32.96	51	18
RSM	3	8	340	33.69	57	24
RSM	3	8	331	34.04	58	23
RSM	3	16	207	7.22	88	93
RSM	3	16	217	10.22	87	90
RSM	3	16	259	11.02	82	86
RSM	3	24	156	2.46	92	98
RSM	3	24	160	3.51	92	97
RSM	3	24	176	2.70	91	98
hRSM	1	2	428	21.31	18	5
hRSM	1	2	429	19.82	16	9
hRSM	1	2	434	20.06	17	10
hRSM	1	4	428	20.53	22	12
hRSM	1	4	432	20.22	21	14
hRSM	1	4	433	20.36	15	7
hRSM	1	8	436	22.44	28	13
hRSM	1	8	444	19.91	27	23
hRSM	1	8	445	21.11	27	19
hRSM	1	16	448	18.00	41	44
hRSM	1	16	465	16.99	37	46
hRSM	1	16	454	20.01	30	28
hRSM	1	24	447	18.43	46	48
hRSM	1	24	476	15.19	42	57
hRSM	1	24	455	18.81	47	49
hRSM	2	2	427	20.87	19	7
hRSM	2	2	426	21.13	16	3
hRSM	2	2	431	20.21	16	8
hRSM	2	4	427	20.28	20	11
hRSM	2	4	429	19.06	20	16
hRSM	2	4	435	20.73	19	9
hRSM	2	8	435	20.16	29	23
hRSM	2	8	444	19.58	26	24
hRSM	2	8	447	20.39	24	18
hRSM	2	16	450	17.34	41	47

Concentrate*	Diet	Time	CP	$InsP_6$	CP	$InsP_6$
			g/kg		%	
hRSM	2	16	457	17.31	33	41
hRSM	2	16	464	17.28	32	41
hRSM	2	24	455	15.02	47	59
hRSM	2	24	459	16.06	44	54
hRSM	2	24	472	16.77	44	53
hRSM	3	2	424	20.89	18	5
hRSM	3	2	428	19.04	18	14
hRSM	3	2	437	20.21	17	10
hRSM	3	4	431	18.39	21	21
hRSM	3	4	434	17.51	18	23
hRSM	3	4	435	20.77	20	10
hRSM	3	8	444	18.26	28	31
hRSM	3	8	444	20.15	26	22
hRSM	3	8	444	20.14	26	21
hRSM	3	16	446	16.76	46	53
hRSM	3	16	441	16.66	43	49
hRSM	3	16	468	16.68	33	44
hRSM	3	24	443	13.14	52	66
hRSM	3	24	434	15.50	56	63
hRSM	3	24	473	13.26	45	64
Wheat	1	2	244	10.46	39	49
Wheat	1	2	253	11.58	37	44
Wheat	1	2	237	13.19	46	41
Wheat	1	4	274	16.49	61	54
Wheat	1	4	282	15.80	56	52
Wheat	1	4	166	20.95	69	23
Wheat	1	8	181	23.50	87	66
Wheat	1	8	171	15.82	84	70
Wheat	1	8	193	15.70	83	73
Wheat	1	16	118	5.83	95	95
Wheat	1	16	93	3.26	96	97
Wheat	1	16	113	6.21	95	94
Wheat	1	24	87	1.44	97	99
Wheat	1	24	71	0.51	98	100
Wheat	1	24	72	0.81	97	99
Wheat	2	2	283	15.70	50	45
Wheat	2	2	211	17.00	50	22
Wheat	2	2	212	14.55	48	30
Wheat	2	4	285	16.60	53	47

Concentrate*	Diet	Time	CP	$InsP_6$	CP	$InsP_6$
			g/kg		%	
Wheat	2	4	222	18.07	61	37
Wheat	2	4	161	9.57	48	40
Wheat	2	8	161	22.51	89	71
Wheat	2	8	177	22.30	85	63
Wheat	2	8	207	12.07	80	78
Wheat	2	16	118	6.64	95	94
Wheat	2	16	142	7.78	92	92
Wheat	2	16	131	6.16	93	93
Wheat	2	24	87	1.39	97	99
Wheat	2	24	70	0.45	97	100
Wheat	2	24	74	0.99	97	99
Wheat	3	2	235	12.89	39	35
Wheat	3	2	221	12.57	41	34
Wheat	3	2	250	12.73	40	40
Wheat	3	4	258	16.88	60	49
Wheat	3	4	210	12.21	48	41
Wheat	3	4	234	14.72	49	37
Wheat	3	8	201	15.80	82	73
Wheat	3	8	140	28.31	91	64
Wheat	3	8	144	24.43	91	71
Wheat	3	16	133	4.63	93	95
Wheat	3	16	111	2.96	95	97
Wheat	3	16	126	5.28	94	95
Wheat	3	24	95	0.82	96	99
Wheat	3	24	69	0.46	98	100
Wheat	3	24	76	0.65	97	100
Maize	1	2	91	2.26	33	79
Maize	1	2	94	2.47	28	76
Maize	1	2	96	1.95	29	82
Maize	1	4	93	1.95	36	83
Maize	1	4	95	2.34	36	80
Maize	1	4	95	1.85	33	83
Maize	1	8	98	0.40	47	97
Maize	1	8	97	0.59	38	95
Maize	1	8	97	0.80	42	94
Maize	1	16	109	0.14	62	99
Maize	1	16	105	0.19	48	99
Maize	1	16	104	0.32	46	98
Maize	1	24	124	0.08	65	100

Concentrate*	Diet	Time	CP	$InsP_6$	CP	$InsP_6$
			g/kg		%	
Maize	1	24	129	0.12	59	100
Maize	1	24	133	0.18	71	100
Maize	2	2	94	2.70	33	76
Maize	2	2	94	2.91	29	72
Maize	2	2	95	2.13	31	81
Maize	2	4	95	2.46	33	78
Maize	2	4	95	2.70	32	75
Maize	2	4	94	1.23	34	89
Maize	2	8	106	0.60	44	96
Maize	2	8	97	0.91	43	93
Maize	2	8	96	0.50	44	96
Maize	2	16	110	0.17	60	99
Maize	2	16	102	0.20	50	99
Maize	2	16	102	0.25	50	98
Maize	2	24	141	0.12	68	100
Maize	2	24	138	0.15	68	100
Maize	2	24	120	0.12	59	99
Maize	3	2	94	2.91	30	73
Maize	3	2	95	2.81	30	74
Maize	3	2	97	1.82	33	84
Maize	3	4	93	2.52	33	77
Maize	3	4	96	2.54	33	77
Maize	3	4	96	1.64	31	85
Maize	3	8	100	1.02	43	93
Maize	3	8	98	0.51	43	96
Maize	3	8	99	0.59	46	96
Maize	3	16	114	0.08	69	100
Maize	3	16	123	0.20	60	99
Maize	3	16	106	0.26	50	98
Maize	3	24	122	0.08	75	100
Maize	3	24	136	0.11	72	100

* SBM, soybean meal; RSM, rapeseed meal; hRSM, heat treated RSM

ANNEX 4. Concentration of ∑$InsP_5$ (∑ of all detected $InsP_5$ isomers) in the bag residues *in situ* in g/kg DM (n=9; averages of diets; means and SEM)

Incubation time	Concentrate*			
h	Wheat	SBM	RSM	hRSM
2	0.98^{Ca}	1.36^{Da}	4.57^{Bb}	10.1^{Bc}
	0.05	0.03	0.16	0.13
4	1.15^{Ca}	1.22^{CDa}	4.94^{Bb}	9.67^{Bc}
	0.09	0.10	0.13	0.27
8	1.42^{Db}	1.07^{Ca}	4.78^{Bc}	9.96^{Bd}
	0.15	0.11	0.13	0.17
16	0.31^{Ba}	0.65^{Bb}	1.77^{Ac}	8.87^{Ad}
	0.04	0.11	0.17	0.21
24	$-^{Aa}$	0.36^{Ab}	0.69^{Ac}	8.40^{Ad}
	-	0.08	0.21	0.59

* SBM, soybean meal; RSM, rapeseed meal; hRSM, heat treated RSM; ^{ab}Means with different lowercase superscripts differ significantly within row; ^{AB}Means with different uppercase superscripts differ within column

ANNEX 5. Concentrations of ∑$InsP_4$ (∑ of all detected $InsP_4$ isomers) and ∑$InsP_3$ (∑of all detected $InsP_3$ isomers) in the bag residues of heat treated RSM *in situ* in nmol/g DM when different diets were fed to the fistulated animals (n=3; means and SEM)

Incubation time	∑$InsP_4$			∑$InsP_3$		
			Diet*			
h	Basal	Pi	InsP	Basal	Pi	InsP
2	6127	6258	6358	1927	2068	1728
	455	527	378	62	106	906
4	6093	6164	5640	2312	2459	2000
	348	892	1133	285	362	533
8	6232	5750	5761	2183	1892	2118
	848	701	251	176	84	139
16	5592	5376	4778	1889	1744	1745
	943	325	437	237	66	261
24	5910	5094	4732	2116	1833	1558
	560	468	1045	263	179	466

*Basal: basal diet (0.38% P, 0.04% $InsP_6$); Pi: Diet Basal with additional inorganic P (0.56% P, 0.05% $InsP_6$); InsP: high $InsP_6$ concentration in the diet (0.39% P, 0.35% $InsP_6$)

ANNEX 6. Percentage of $InsP_4$ isomers in $\sum InsP_4$ ($\sum$ of all detected $InsP_4$ isomers) in the bag residues of heat treated RSM *in situ* in % (n=9; averages of diets; means and SEM)

Incubation time	$InsP_4$					
h	Ins(1,2,3,4)	Ins(1,2,4,5)	Ins(1,2,5,6)	Ins(1,4,5,6)	Ins(x)*	Ins(y)*
2	29	13	32	6	14	6
	0.29	1.74	1.66	0.25	0.42	0.28
4	29	13	30	6	16	6
	0.57	1.97	1.45	0.19	0.93	0.26
8	29	13	30	7	15	7
	0.60	2.00	1.47	0.26	0.79	0.42
16	30	13	30	6	14	6
	0.70	1.65	1.39	0.22	0.64	0.28
24	30	12	30	6	15	6
	0.59	1.68	1.16	0.17	0.78	0.33

*$InsP_4$ isomers have not yet been identified

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