IN VITRO DETERMINATION OF THE ILEAL DIGESTIBILITY OF PROTEIN AND AMINO ACIDS IN NEW ZEALAND BARLEYS

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ABBREVIATIONS

AA	amino acid
Ala	Alanine
Arg	Arginine
ANF	anti-nutritional factor
Asp	Aspartic acid
APU	Animal Physiology Unit
CP	crude protein
CF	crude fibre
cw	compare with
Cys	Cysteine
Da	daltons
DM	dry matter
DP	digestible protein
EHC	enzymically hydrolysed casein
EAAL	endogenous amino acid loss
EL	endogenous loss
EPL	endogenous protein loss
FCR	feed conversion ratio
FIA	flow injection analysis
GI	gastro-intestinal
Glu	Glutamic acid
Gly	Glycine
HA	homoarginine
His	Histidine
HPLC	high performance liquid chromatography
Iso	Isoleucine
IV	intravenous
kgs	kilograms
Leu	Leucine
Lys	Lysine

MBM	meat and bone meal
Met	Methionine
MWCO	molecular weight cut off
N	Nitrogen
OMIU	O-methylisourea
Phe	Phenylalanine
Pro	Proline
SD	standard deviation
Ser	Serine
SAPU	Small Animal Production Unit
SPF	specific pathogen free
Thr	Threonine
Tyr	Tyrosine
UDM	undigested dry matter
Val	Valine
VFI	voluntary food intake
w/w	weight for weight

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Abstract

The aim was to evaluate a recently developed *in vitro* digestibility assay for predicting the apparent ileal digestibility of protein and amino acids in barley, and secondly to evaluate the statistical prediction of apparent ileal digestibility of protein in barley based on chemical and physical measurements.

Seventeen barleys were collected from six growing regions from throughout New Zealand in 1995. Ten of these were selected to provide a range in crude protein from 8.5 to 13.3% (DM basis). The ten barleys were subjected to several physical and chemical measurements, and to the *in vitro* assay. The barleys were given as sole sources of protein to growing rats (n=6) and ileal digesta were collected at slaughter and nitrogen and amino acid digestibility determined with reference to the marker, chromic oxide. Six of the barleys were treated with O-methylisourea to convert lysine to homoarginine, a synthetic analogue of lysine, to allow determination of endogenous ileal lysine and protein flows.

Mean *in vivo* apparent ileal digestibility of nitrogen ranged from 71.4% to 80.3%. *In vivo* true lysine digestibility ranged from 73.2% to 100% while endogenous protein loss ranged from 6.4 to 44.8 g/kg DMI.

Physical measures made on the barley included grain bulk density (kg/hecto litre), screenings (%) and 1000 seedweight (g) and were highly variable. They provided no significant ($p>0.05$) predictive ability for protein digestibility or endogenous ileal protein loss. Chemical measures included CP (%), NDF (%), ADF (%), lignin (%), total β-glucans (%) and gastro-intestinal (GI) extracted β-Glucans (%) and were also highly variable. True lysine digestibility was able to be predicted based on the levels of GI extracted β-glucans and crude protein ($r^2= 0.97$). *In vivo* endogenous ileal protein loss was predicted based on total β-glucans ($r^2 = 0.77$).

In vitro protein digestibility was not significantly correlated with *in vivo* values. The *in vitro* technique requires more development before it can be used for the routine evaluation of digestible protein in barleys.

INTRODUCTION

In modern pig production, accurate diet formulation and the subsequent efficient use of available feeds requires an assessment of digestible energy and digestible amino acid profiles. These vary widely between and within feedstuffs.

Variation in digestibility is related to both chemical and physical factors, which may be related to the cultivar and variety. Climatic conditions also affect cereal quality. β-glucan levels in barley, for example, are known to be influenced by the climate (Hesselman and Thomke, 1982).

Nutrient digestibilities are currently determined using expensive and time-consuming animal trials involving the target species. Limited physical and financial resources mean, however, that the number of feedstuffs that can be evaluated is limited. A recent development has been the use of the laboratory rat as a model animal for determining ileal amino acid digestibilities in the pig. This has reduced the expense of feedstuff evaluation, however the same shortcomings remain, albeit on a reduced scale.

Due to these cost and time restrictions there is a compelling need for the feed industry to establish rapid and reliable ways to determine energy, protein and amino acid digestibilities for monogastric farm animals. Determination must be accurate, rapid and relatively inexpensive if it is to find a place in regular commercial practice. It should demonstrate an efficiency of resources to the extent that evaluation of energy, protein and amino acid digestibilities become routine in the assessment of cereal harvests.

One such possibility is the use of *in vitro* digestion technologies. There are many types of *in vitro* assay in current use and each has its own strengths and shortcomings (Boisen and Eggum, 1991). The critical outcome for an *in vitro* digestibility assay system is low cost, high repeatability and accurate correlation with *in vivo* data. This dissertation addresses the application of an *in vitro* digestibility assay within the New Zealand pig industry.

It is of current interest to the New Zealand pork industry to streamline production in any way possible as it competes against the lower priced imports of

Canadian and Australian pig meat. Feed expenditure accounts for around 70% of the total production costs for a pig producer. Therefore an increase in the efficiency of diet formulation, hence reducing the cost per unit of pig meat produced, is vital to New Zealand maintaining a competitive front. The foremost way this increase in efficiency can be secured is by diet formulators developing a better awareness of the quality of the feedstuffs they are dealing with.

This work is designed to assist in the achievement of that goal and will focus on protein and amino acids in barley. Barley constitutes a major component of pig feed in New Zealand, and although it is principally used as an energy source, it nevertheless makes a significant contribution to dietary protein. The importance of barley is paramount in New Zealand where around 100,000 hectares is planted annually (Statistics New Zealand, 1996a). Most barley in New Zealand is used in stock feed, with some being malted for the brewing industry. Barley will comprise up to 85% of breeder pig diets in the South Island, but typically much less in the North Island, (<62%) where a reasonable amount of maize is used (I.M. Barugh, personal communication). Barley is included principally as an energy source in New Zealand, however its contribution to dietary protein is also significant.

In this case, the presently described studies were conducted using New Zealand barleys collected from six different growing regions to investigate the possibility of predicting protein and AA (amino acid) digestibilities with a new *in vitro* digestibility technique (Boisen and Fernandez, 1995). *In vivo* apparent and true ileal digestibility data were determined in the laboratory rat.

The new *in vitro* technique (Boisen and Fernandez, 1995) was investigated to meet the aforementioned objectives of establishing a rapid, accurate, inexpensive and reliable way of determining protein and amino acid digestibilities for pigs with particular application to New Zealand barley varieties.

There are other potential applications of the present work. It is envisaged that the results could also provide millers and grain merchants with a quality assessment indicator on which a payment schedule could be based.

Further application of this technology is shown in the possibility of accelerating cereal breeding. Breeders may be able to evaluate new varieties earlier in the selection process without the requirement to multiply the offspring to quantities large enough to evaluate in animal assays and therefore a larger number of cultivars

could be screened. Reduction in the generation interval and an increased selection intensity could therefore result in a more efficient cereal selection programme. A final objective of the present work was to add to a bank of digestibility data for New Zealand feedstuffs.

Chapter 1

LITERATURE REVIEW

Introduction

The present review is oriented towards barley as a feedstuff for pigs in New Zealand. Topics covered include a review of digestion in the pig, and *in vivo* and *in vitro* methods for determining amino acid digestibility.

1.1 Protein digestion in the pig

An understanding of the digestive processes in pigs is of fundamental importance but is also necessary for managing the nutrition of domestic pigs in a commercial production setting. It is essential also in the development of computerised technologies such as growth modelling (Moughan and Verstegen, 1988).

This section (1.1) seeks to review the current concepts in and contemporary understanding of protein digestion in the pig, including both physiological and biochemical aspects and the morphology of the digestive tract.

The pig is an omnivore, with a functional caecum (Moughan, 1996), secreting numerous digestive enzymes (Fuller, 1991).

Many species of animal exhibit the simple stomach system found in pigs and in fact the rat has been demonstrated to be a suitable animal model (Moughan *et al.*, 1984) for digestion in the pig, and the pig as an animal model for certain aspects of protein nutrition for humans (Moughan *et al.*, 1994).

The morphology of the digestive tract.

1. The mouth

This is a source of endogenous protein from sloughed cells and α-amylase. There is no protein digestion here, only gross mechanical disruption of the food (Low and Zebrowska, 1989).

2. The stomach

The digestion of protein begins in the stomach where pepsin and HCl acid act to hydrolyse the proteins. The nature of the diet influences the rate of gastric emptying (Snook, 1973). The source of protein has been shown to exhibit an influence, perhaps due to differing solubilities of proteins (Rogers and Harper, 1966). Buraczewski *et al.* (1971) found that carbohydrates also delay the passage of protein from the stomach. According to Low (1990) almost half the nitrogenous material leaving the stomach is in the form of peptides of less than 10 amino acids (AA). This is in contrast to reports in the literature that suggest that only a small proportion of protein is digested in the stomach (Zebrowska, 1980). There is no evidence of amino acid or peptide absorption from the stomach (Zebrowska, 1980).

In his review, Snook (1973) cites evidence of five potential pepsins in the stomach of pigs. Pepsin hydrolyses peptide bonds, mainly those next to aromatic or dicarboxylic L-amino acids (Zebrowska, 1980). Pepsins are secreted in the inactive form as pepsinogen. Pepsinogens are activated to pepsin by hydrolytic removal of a peptide from the N terminal end of the molecule (Low and Zebrowska, 1989). Pepsin A is the most abundant, with pepsin C being the next most abundant enzyme(Low and Zebrowska, 1989).

In addition to the initiation of digestion, food is structurally disrupted in the stomach making it more susceptible to enzyme attack in the latter tract.

3. The small intestine

The small intestine is divided into the duodenum, jejunum and ileum with each being approximately one third of the organ. There are no clear histological changes demarcating the three sections and the products of protein digestion are absorbed in all sections of the small intestine (Friedrich, 1989). The ileum has a large digestive capacity, but it is in the proximal jejunum where most peptide absorption occurs (Grimble and Silk, 1989).

The protease activity in the small intestine is influenced by the rate at which enzymes are secreted and then inactivated. An increase in dietary protein intake induces more pancreatic proteolytic enzyme release (Boisen and Eggum, 1991).

Dietary protein may also increase protease activity by inhibiting the inactivation of enzymes (Snook, 1973).

The rate of digestion in the duodenum varies for different proteins (Zebrowska, 1980). Also, the retention time in the small intestine is variable. The retention time for soya bean protein was shown to be 160 minutes for the liquid phase and 290 minutes for the particulate fraction, but for milk protein it was 170 and 180 minutes for the liquid and solid phases respectively (Asche *et al*, 1989).

Leibholz (1982) reported 18% N absorption at the duodenum and 70% N absorption between the duodenum and jejunum. The definitions of the three sections in Leibholz's work were one third of the length of the small intestine each.

In contrast to only 18% of N absorption in the duodenum (Leibholz, 1982), there is intense protein digestion in this section (Zebrowska, 1980). Free amino acids are absorbed from the first part of the duodenum faster than peptides (Zebrowska, 1980).

There is a microbial population in the small intestine, shown by the fermentation of monosaccharides (Boisen and Eggum, 1991). There was evidence of fermentation by duodenal bacteria in the *in vitro* incubations of Löwgren *et al.* (1989) who used inoculum from the duodenum. Bacterial degradation in the small intestine is not as great as the large due to a relatively shorter passage time (Fuller, 1991).

4. The large intestine

The large intestine is split anatomically into two sections, the caecum and the colon. The colon is as wide as the caecum and tapers off towards the rectum. This is where the greatest effect of the gut microflora is found. Evidence for microbial activity is given by Just *et al.* (1981) who demonstrated decreases in fibre, N free extract and gross energy digestibility at the caecum-colon when antibiotics were administered. Further evidence of microbial activity is shown by decreases in the overall digestibility of CP and amino acids when microbial activity was depressed due to the cessation of microbes assimilating or metabolising the protein in the hind gut.

Enzymes from the small intestine that escape digestion may still hydrolyse protein in the lumen of the large intestine. Löwgren *et al.* (1989) found that *in vitro* incubations with inoculant from duodenum, ileum and faeces provided similar

patterns of DM digestibility, given enough time. They suggested that the issue of digestion by the enzymes is a matter of availability of the substrate in the different compartments of the intestine.

Ammonia is absorbed from the large intestine, but this is of no nutritional value to the pig (Low and Zebrowska, 1989). This is the major form of N uptake from the large intestine. Just *et al.* (1981) found that the caecum-colon can digest infused nutrients, but they found that the value of absorbed N is very low. Furthermore they found some evidence that free lysine is not absorbed as such in the large intestine.

Amino acids that are poorly digested in the small intestine show the greatest proportional disappearance in the large intestine (Tanksley and Knabe, 1984). More than half of them are essential AAs (Taverner and Farrell, 1981b).

It is estimated that a pig may acquire up to one third of its energy requirements from the large intestine (Mason, 1980). However nitrogenous compounds are not absorbed to any important nutritional extent from the large intestine. Mason (1980) cites workers who demonstrated that there is no appreciable secretion of proteases into the lumen of the large intestine by the mucosa and that trypsin and chymotrypsin are inactivated by the large intestinal microflora.

Anti nutritional factors

Different plants contain various factors (anti-nutritional factors, ANF) that act to decrease the value of the plant as a feed for monogastric species. This is well documented (e.g. Chubb, 1982) and a large part of nutrition research has been dedicated in recent times to an understanding of this area. The various factors that are present in plants include non starch polysaccharides (NSP), trypsin inhibitors, tannins, lectins, etc and also toxic chemical compounds, (e.g. mycotoxins and aflatoxins).

β-glucans are an ANF in barleys and exist also in some other cereals, e.g rye and triticale, wheat, maize, sorghum and oats (Jørgensen and Aastrup, 1988).

Annison (1993) discussed, with reference to NSP, how certain criteria must be met, to prove an ANF effect. These are:

1. The response should correlate with dose of the isolated NSP.
2. Synthetic analogues should produce the same response.

3. Destruction of NSP should reduce the response.

4. Inclusion level in the feedstuff should match response.

These criteria are useful for evaluating an NSP, and should in fact apply to the classification of all ANF compounds.

Non starch polysaccharides (NSP) act to decrease the nutritive value of foods. For a long time they were considered unimportant, due to the fact that they are poorly digested. For example and in his recent review, Huisman (1989) did not include fibre in his definition of ANF. Recent understanding, however, shows that they can be considered as a major ANF. For example, lower apparent ileal digestibilities of barley compared to maize, sorghum and wheat are attributed to the higher fibre content in barley than the latter cereals (Tanksley and Knabe, 1984). Choct and Annison (1992) demonstrated that just 30g of isolated pentosans fed to poultry resulted in decreased liveweight gain (LWG) and protein digestion and apparent metabolisable energy.

There are two main suggested modes of action for the ANF effect of NSP. One is of an interaction with gut microflora. The second theory is perhaps the most widely accepted and strongly supported theory and is related to the viscosity of digesta increasing with inclusion of NSP. When Choct and Annison (1992) added pentosans to poultry diets they increased the viscosity of digesta. Decreasing the viscosity fourfold by enzyme hydrolysis of NSP resulted in the performance depression being overcome. The ANF effect seems to be associated with polysaccharide chain length. This is expected and is in line with the viscosity theory. With respect to poultry, Choct and Annison (1990) suggest that the effect is not due to nutrient dilution and increased intake. It appears that the diffusion of nutrients is actually hindered by the viscous digesta.

Summary

Protein digestion in the pig is based upon enzymatic cleavage of denatured proteins into peptides or AAs and their subsequent absorption. Anti-nutritional factors hinder digestion or absorption rendering the plant less nutritional than it would be based upon its proximate analysis.

1.2 Determination of amino acid digestibility

Accurate diet formulation requires not only the balancing of protein in the feed to the animal's requirements, but more importantly, the matching of the individual amino acids in the feed to the animal's requirements. Furthermore, it is not the gross amino acid composition in the feed that the nutritionist is concerned with but the useful, or "digestible" amino acid content. Determination of gross AA composition is relatively straightforward and can be achieved by HPLC analysis methods. The determination of AA digestibility, however, is somewhat more complex. There can be large differences between the gross AA content and the digestible AA content. This has been highlighted in the work of Meads *et al.* (1995) who demonstrated that blood meal containing as much as 92% CP may exhibit a digestibility of CP in rats as low as 17%. Regrettably, diet formulation is often still based on gross AA composition despite its acknowledged limitations (Moughan, 1996).

A further complication is that the calculation of digestibility for protein mixes based upon the digestibilities of individual proteins is not always possible due to ingredient interactions. Brulé and Savoie (1988) demonstrated this in an *in vitro* assay when they found a synergistic effect when mixing low digestibility wheat flour with highly digestible proteins.

The reasons for accurate, precision engineering of diets are mainly economic in origin, however, in recent times, environmental concerns are starting to provide motivation for accurate diet formulation. Protein synthesis ceases when the first limiting AA, usually lysine, is exhausted from cellular pools. Excess AAs may be preferentially catabolised for energy or deaminated and excreted. The N excretion from the pig fed a poorly formulated diet is considerable, and in geographical regions such as western Europe, it is becoming a major environmental concern.

The understanding of amino acid digestibilities assists in the ranking of feedstuffs for subsequent application of relative economic values for these feedstuffs (ie a payment schedule), and also for the formulation of diets where high cost proteins are interchanged with synthetic amino acids (Sauer and Ozimek, 1986).

It is not enough to determine the apparent digestibility of protein, but is necessary rather, to determine the apparent digestibilities of the individual amino acids. The reason for this is that supplying the required amount of protein may not

meet the requirements for the first limiting amino acids. Amino acid digestibilities vary widely, as demonstrated by Knabe *et al* (1989). Arginine was the most digestible in 25 feedstuffs from the 30 sampled by these authors. Threonine was least digestible in 11 samples, and tryptophan least digestible in 13 samples.

Availability vs digestibility

Availability has previously been estimated based on digestibility values or chemically available lysine tests such as the FDNB available lysine assay. An assumption of ileal digestibility assays has been that an amino acid not recovered at the terminal ileum has been absorbed in a form that is utilisable to the animal. This is now known not to be the case necessarily. For high quality proteins, it is often so, but not for low quality protein, or those that have been heat treated (Moughan *et al*, 1991).

Available AAs must not be confused with digestible AAs. Availability can be seen as the proportion of an amino acid in a feed that is absorbed from the gastrointestinal tract in a form suitable for utilisation.

Availability is a desirable measurement. However, currently there is no satisfactory method to determine available amino acids so digestible is sometimes used as a "best estimate" of availability (Moughan 1996).

The terms digestibility and availability of amino acids are sometimes used interchangeably in the literature, but this is erroneous. Digestibility gives no indication of whether or not the absorbed nutrient is biologically useful.

Ileal digestibilities may overestimate availabilities as was demonstrated by Bjarnason and Carpenter (1969) who found that rats absorbed E-N-propionyl-L-lysine from the small intestine. This would have been measured as lysine digestion, but it is not utilised by the rat, so therefore cannot be considered as being available. Batterham *et al.* (1990b) found that the digested lysine of soya bean meal had a retention coefficient of 0.75, whereas cottonseed meal digested lysine had a retention coefficient of 0.36. This means that much of the absorbed lysine in the cotton seed meal was either in an unutilisable form, or was metabolised (eg, preferential catabolism), but not retained and that only 25% of the digested lysine in soya bean meal exhibited this phenomenon. It was suggested by Batterham *et al.* (1990b) that a conformational change occurred at the peptide level.

Available lysine may be measured by the fluorodinitrobenzyne (FDNB) technique or the silcock available lysine technique (Van Barneveld, 1994a). Moughan *et al.* (1989) tested 20 meat and bone meals (MBMs) for *in vivo* digestibility (rats) and for FDNB available lysine. They found that MBMs with a high digestibility had low FDNB available lysine values, and conversely those low in *in vivo* digestibility had high reactive lysine contents. This suggests that lysine may be absorbed in a non available form and some chemically available lysine may not be absorbed (Moughan *et al.*, 1989). This highlights the fact that availability and digestibility are not synonymous terms. For processed foods evidence suggests that FDNB lysine overestimates availability, due to incomplete digestion and absorption (Moughan *et al.*, 1996).

Heat treatment and the determination of amino acid digestibility

Many feeds are routinely exposed to heat treatment during their processing, primarily to destroy anti-nutritional factors. Heat treatment helps destroy the ANF's, but at the same time renders the protein and amino acids less available to the animal. Despite much work in the area, there is still uncertainty as to the actual mechanisms of heat damage (Van Barnveld *et al.*, 1994a). However, much is known about the two common reactions, the Maillard reaction, and protein cross-linking. The Maillard reaction is named after a French scientist, Louis-Camille Maillard, (1878-1936), who studied the reaction in depth during the years 1912-1917. It is a non-enzymatic browning reaction that involves an amino group, typically an amino acid in food, and a reducing compound, such as a sugar.

Protein cross linking also happens under severe heat treatment. Both the Maillard reaction and protein cross-linking have the potential to change the availability of the amino acids to the animal, rendering amino acids such as lysine unutilisable. The Maillard reaction is the most common reaction to happen in the heating of feeds. Lysine is the amino acid usually most prone to heat damage and this is due to the readiness of its e-NH_2 group to react with other compounds in cross-linking.

It appears that heat may affect amino acid digestibility and availability in different ways. Heat is known to produce a change in overall chemical composition

(Van Barneveld *et al.*, 1994a). Little or no change was seen in the subsequent digestibility of amino acids by these authors, however, but the absolute content of lysine, arginine and cystine had all decreased due to Maillard compounds being formed. Interestingly in some cases heating actually increased digestibility (Van Barnveld *et al.*, 1994a), suggesting a conformational change that enhanced enzyme attack.

When heat damage to lysine occurs, the lysine is absorbed but not utilised, as mentioned previously. Van Barneveld *et al.* (1994b) demonstrated that the effect was increasingly pronounced as the severity of heating increased. Heating to 110°, 150° and 165°C resulted in lysine retention coefficients of 0.67, 0.59, and 0.48 respectively. This was in comparison to a retention of 0.87 in the raw product. With respect to the severity of treatment, Johns and James (1987) noted that as increasing heating times were applied to feed, all the amino acids decreased in digestibility.

It is arguable as to whether or not lysine availability actually decreases, because the absorbed compound from a Maillard reaction is no longer lysine.

Ileal vs faecal digestibility

It is well established (Low and Zebrowska, 1989) that faecal digestibility estimates are unacceptable. Breakdown of AAs in the hindgut to non-utilisable products means that digestibility measured at the faecal level will overestimate AA digestibility (Moughan *et al.*, 1987).

Diets that contain proteins with an overall poor digestibility highlight the differences between ileal and faecal digestibility estimates. Taverner and Farrell (1981b) showed with wheats, that those with the lowest DM digestibility showed the most difference between ileal and faecal CP digestibilities. This has also been demonstrated in a more digestible protein concentrate, MBM (Donkoh *et al.*, 1994c). This is due to relatively increased microbial action in the hindgut. The extent of microbial activity and hence the level of difference between ileal and faecal apparent digestibilities depends upon residence time in the digestive tract, and the size of the microbial population and the types of microbes present as well as the feedstuff type (Butts *et al.*, 1991b). Sauer and Ozimek (1986) reviewed much of the literature on the subject of sensitivity of the digestibility measure either at the terminal ileum or at the

faecal level. Subtle differences in protein digestibilities could be determined at the ileal level, but were not necessarily found at the faecal level. This was also demonstrated in the study of Sauer *et al.* (1977) which showed differences in the digestibility of amino acids between finely ground wheat and cracked wheat using the ileal measure but not with the faecal measure. Hence it is more accurate to use ileal digestibility measurements rather than faecal ones.

Faecal digestibility estimates are generally higher than ileal estimates (Sauer and Ozimek, 1986). Tanksley and Knabe (1984) demonstrated a net synthesis of methionine and lysine in the large intestine in half the grains that they studied, and also in most soyabean meals and in cotton seed meal. Taverner and Farrell (1981b) also found a net synthesis of methionine in the large intestine of pigs fed wheat or barley. Net synthesis of these AAs is due to the microbial metabolism in the large intestine.

Austic (1983) summarised the literature on differences between ileal and faecal apparent digestibilities for amino acids, and reported an average difference of 6.5%. Tanksley and Knabe (1984) reviewed the literature on the differences between ileal and faecal apparent digestibilities for several cereals. They found that the average apparent digestibilities for the essential amino acids were 4.5, 3.0, 4.5, 5.2 and 3.0% lower at the ileum than at the faecal level for wheat, maize, barley, oat groats and low-tannin sorghum, respectively. The within-cereal between animal variation was also high.

"True", "apparent" and "real" ileal digestibilities

Nutritionists try to match the animal's requirements for AAs with AAs contained in the feed. The AA levels in the feedstuffs are expressed in terms of either apparent, true or real digestibility. These are an advance upon crude values.

Apparent ileal AA digestibilities are being published more widely in the contemporary feed industry literature, as more and more producers, millers and merchants realise that formulating on the basis of CP leaves diets open to considerable and unmeasured deviation from the formulated values. However, apparent ileal digestibilities have drawbacks. For example they have been demonstrated to be influenced by dietary CP content (Donkoh and Moughan, 1994), therefore making

between-feed comparisons quite difficult. Donkoh and Moughan (1994) discussed work on the considered effect of dietary protein on apparent digestibilities of N and AAs, and how it is thought to be mainly due to the greater proportion of endogenous protein present relative to dietary protein at low protein concentrations.

Apparent digestibility of a nutrient is given as:

$$\text{Apparent Digestibility \%} = \frac{(\text{nutrient in diet} - \text{nutrient at the terminal ileum})}{\text{nutrient in diet.}} \times 100$$

Apparent protein digestibilities appear to be independent of the liveweight of the pig in the range of 20 -50 kg liveweight (Fernandez and Jørgensen, 1986).

True digestibilities are defined in the same manner as for apparent, excepting that a correction is made for amino acids appearing at the terminal ileum that are of endogenous origin. These endogenous losses are difficult to determine (Chapter 2.3) but the true measures are preferable to apparent ones. True digestibilities are independent of the assay dietary conditions (Darragh *et al.*, 1995), and specifically the dietary protein levels (Donkoh and Moughan, 1994). This allows comparison between feedstuffs even if they are consumed in different quantities, and true digestibilities should be additive (Donkoh and Moughan, 1994).

More recently the term real digestibility has been used, and applies mainly to feeds with high fibre or ANF levels present. It allows a correction for endogenous loss specifically induced by a given feed ingredient (Darragh *et al.*, 1995). This is on top of the basal level of endogenous amino acid loss (EAAL) that may be attributable to endogenous secretions bought about by normal feeding of proteins. Such readily digestible proteins as fishmeal will generally only elicit a basal secretion of EAAL.

Thus it is clear that the definition of digestibility will influence the numerical value given. In the process of diet formulation, the desired expression of nutrient digestibility will be determined by what the requirements are specified as. In an ideal situation both will be expressed in true terms, but in the contemporary situation most digestibility figures are expressed in terms of apparent digestibility. This is acceptable given the difficulties of routine measurement of true digestibilities. However, the nutrionist must appreciate the shortcomings of this approach, as outlined above.

Techniques to collect ileal digesta

Determination of ileal digestibilities requires sampling of digesta at the terminal ileum. There are many methods to sample the ileal digesta. The most common technique in pigs and poultry is the cannulation technique. This and other methods are described below.

Cannulation technology

Repeat sampling of digesta, continuous sampling or sampling over a long period of time is desirable as opposed to single sample collections to decrease the number of animals necessary in digestibility studies. This is achieved by the use of surgically fitted cannulae. There are three main types, the cannulae that go anterior to the ileo-caecal valve (simple T-piece, the ileo-caecal re-entrant cannula), those that may go anterior or posterior to the ileo-caecal valve (the ileo-colic re-entrant cannula), and thirdly the post valve T-caecum (PVTC) cannula. The method of re-entrant cannulation relies on the assumption of 100% marker recovery (Köhler *et al.* 1990) as opposed to a T-piece cannula recovering only part of the marker.

Köhler *et al.* (1991) examined the differences between the PVTC, re-entrant and simple T-piece cannulae. Nitrogen, dry matter (DM), crude fibre (CF), acid detergent fibre (ADF) and neutral detergent fibre (NDF) digestibilities were not significantly different between the three cannula types in diets that were either control, pectin rich, CF rich or semisynthetic. However, N and NDF in a fibre rich diet and N in the semi synthetic diet showed significant differences depending on the cannulation type. This is of significance when choosing a cannulation method. Blockages and leakages were observed around the ileal cannula when pigs were fed the fibre rich diet. Care must be taken to avoid these problems if any confidence is to be placed in the results.

Köhler *et al.* (1990) reported that the T-cannula gave rise to greater variation compared to other techniques. Re-entrant cannulas gave higher digestibility values

than found with PVTC and T-piece cannulas. PVTC cannulation may cause less discomfort to the animals (Köhler *et al.*, 1990).

A further new technique has been proposed by Mroz *et al.* (1991). It involves fitting the pig with a T-cannula and the ileo-caecal valve is "steered" forward into the cannula by a thread at intervals through time for sampling of digesta. When the ileo-caecal valve is steered forward into the T-cannula a fitted ring blocks passage into the caecum and colon. This gave digestibility results that were lower than the PVTC cannula, interpreted by these authors to imply that the PVTC digesta were incompletely collected. However, this steered technique led to health problems in the animal including muscular hypertrophy, so requires further development before it can be accepted for routine use.

The use of cannulae is criticised on the grounds that they may disrupt normal physiology, digestion and absorption (Sauer and Ozimek, 1986). For example complete transection of the ileum will interfere with the myoelectric migration (Tanksley and Knabe, 1984). Moughan and Smith (1987) report conflicting evidence in the published literature with respect to effects of cannulation on pigs. However, fitting a simple T-piece cannula showed no difference in apparent faecal N and AA digestibilities in pigs, which suggests that the simple T-piece cannula did not alter the digestive physiology of the gastro-intestinal tract (Donkoh *et al.*, 1994a). However it has been shown that faecal estimates of digestibility are not as sensitive as ileal measures as discussed previously, therefore the reliability of the faecal measurement to evaluate the physiological affects of cannulation is questionable. Similarly, de Lange *et al.* (1989a) reported that T-piece cannulation of barrows did not result in any intestinal abnormalities.

With cannulated pigs, feed intake is generally restricted to avoid blockage of the cannula, and also to allow the hungry animal to consume all of the known quantity of marker. Whether a restricted feeding regime gives rise to digestibility values applicable to *ad lib* fed pigs is questionable (Sauer and Ozimek, 1986). However, there does not appear to be an effect of feed intake on the ileal digestibilities of crude protein and AAs in pigs fitted with simple or re-entrant cannulas (Sauer *et al.*, 1986). The latter authors concluded that simple or re-entrant cannulas do not significantly alter digestion processes in the pig. Moughan and Smith (1987) found no significant

differences ($p>0.05$) between intact pigs and simple T-piece cannula fitted pigs for apparent ileal amino acid digestibility in barley.

Ideal conditions for cannulation have been summarised by Darcy and Laplace, (1980) and Low (1990). These are: 1) the complete transection of the ileum must be avoided, 2) there must be a long post-operative recovery period, 3) the function of the ileo-caecal valve must be preserved, 4) passage of digesta in the cannula itself must be minimised and 5) the pig must have normal appetite/growth.

Central to the usefulness of cannulation technology is the choice of a suitable marker. A marker is necessary in all techniques where sampling is employed to recover digesta. A marker must exhibit good recovery, and not be absorbed or transformed. Cr_2O_3 is not without its limitations. Further evaluation of Cr_2O_3 needs to be undertaken. Snook (1973) reviews some problems with this marker. The main criticism appears to be evidence that the rate of Cr_2O_3 leaving the stomach does not match the rate of the feed dry matter material leaving the stomach. In one experiment reviewed by Snook (1973), one hour after a meal 17% more Cr_2O_3 remained in the stomach than its respective N it was supposed to travel with.

However, Cr_2O_3 is the most common marker used for the solid phase of diets and PEG is often used for liquid feeds and celite, a clay product, is also commonly used, amongst others, as a marker.

Ileo-rectal anastomosis

Another surgical technique that has been used in the past with pigs is ileo-rectal anastomosis, which involves the surgical removal or bypass of the large intestine. The ileum is transected 100 mm anterior to the ileo caecal valve (Green, 1988) and is sutured directly to the rectum. This allows the routine collection of digesta from the ileum through the rectum. Fuller (1991) reported pigs surviving for up to two years post-surgery.

However, removal of an organ from the digestive tract may result in alteration of the remaining tract, for example removal of the stomach has been suspected of inducing changes in the upper digestive tract (Snook, 1973). Removal of the large intestine will result in similar changes. Fuller (1991) demonstrated real histological changes in the small intestine 26 weeks post-surgery. The pigs also showed more

volatile fatty acid production in the digesta. There may be modification of the intestinal microflora (Köhler *et al.*, 1991), making the technique void in terms of avoiding microbial fermentation.

Green (1988) attempted to improve upon the ileo-rectal anastomosis technique by removing only the latter part of the large intestine, thus preserving the ileo caecal valve. There were no significant differences between methods, however, and so the modified procedure was discarded due to the complexity of the surgery. This technique raises serious ethical concerns.

The slaughter technique

The slaughter technique is possibly the best way to get accurate, and complete samples of digesta at the different stages of the intestine, due to the fact that there is minimal disturbance to the GI tract. The slaughter technique does not give more variable data. Moreover, there is no restriction on the type of foodstuff that can be fed (Donkoh *et al.*, 1994a), unlike cannulation methods. The slaughter technique was found by Donkoh *et al.* (1994a) to be no more variable than the T-cannulation for MBM, for apparent ileal N and AA digestibilities providing a regular feeding regime was observed.

The slaughter technique is expensive however, requiring higher numbers of animals. Apparent digestibilities determined with the slaughter technique are dependent on the time of euthanasia after a meal (Sauer *et al.*, 1977).

Donkoh *et al.* (1994a) worked on the slaughter technique with 30 kg pigs to identify the optimal sampling time, and site in the intestine. The effect of sampling time (hours after start of feeding) did not appear to effect the amount of digesta passing the ileum. Apparent digestibility increased when measurements were taken at 5, 7 and 9 hours after feeding commenced, being the highest (80%) at nine hours.

The site of sampling did not show any effect for the semisynthetic MBM based diet on apparent N digestibility. This is in agreement with work using lactic casein, (Kies *et al.*, 1986).

Routine approaches to determining AA digestibility

1. Near infrared reflectance (NIR) spectroscopy.

An exciting technique that is emerging, and with many biological applications is NIR spectroscopy. Compounds with similar chemical groupings will absorb IR radiation at certain wavelengths. This allows the identification of groups of chemicals within a feedstuff by their own identifying IR profile. Van Leeuwen *et al.*, (1991) demonstrated that the correlation coefficient r, was 0.9 and that the RSD was 4.3 units of digestibility when comparing *in vivo* estimates of apparent digestibility with NIR readings at a certain set of wavelengths. The match was not as good for some classes of feedstuffs but this was possibly due to an incorrect selection of filters on the wavelengths. This technique requires accurate and careful calibration, and this is critical to the success of the technology as is the mix of filters.

2 The mobile nylon bag technique

This is a rarely used technique that involves digestion of food held within a nylon pouch that is administered to the pig and anchored or allowed to travel with the digesta (Low, 1990). A modified technique was described by Sauer *et al.* (1983) that involved a pre-digestion step before insertion to the duodenum via a cannula. The retention time in the stomach is variable, so oral administration of bags is not suitable (Sauer *et al.*, 1983). This method has been applied to a range of feedstuffs (Sauer *et al.*, 1983, Leibholz, 1991). With respect to cereals it ranked cereals lower using the conventional method (6.8 to 11.1 % units for wheat and barley, respectively) but ranked cereals identically using apparent faecal digestibilities. Identical ranking of feeds is an important consideration when comparing techniques for feed evaluation.

A possible drawback of this technique is that it does not account for feed/gut interactions (Sauer *et al.*, 1983). For example, Leibholz (1991) found that high fibre diets were producing lower digestibility results when in nylon bags than when measured directly. Accuracy could be improved by some form of correction for these feeds (Leibholz, 1991).

Summary

Determination of digestibility for amino acids should always be done at the ileal level, but this raises problems of digesta collection. Cannulation appears to be the preferred method for digesta collection in the pig, no doubt due to the advantage that pigs can be maintained after surgery for some time. Ileo-rectal anastomosis is not reported to be used so widely. Indigestible markers are necessary to correct for only partial digesta recovery with the cannulation and slaughter techniques, and this is a disadvantage.

1.3 Determination of endogenous amino acid loss

Endogenous protein material (endogenous loss, EL) is found throughout the gut (Schmitz *et al.*, 1991, Buraczewska, 1979, Leibholz, 1982). The secretion rate is higher in the first third of the small intestine and highest in the second third of the small intestine in comparison to the final one third (Moughan *et al.*, 1992a).

EL consists of saliva, gastric juice, pancreatic juice, bile, intestinal juice, mucopolysacharides and epithelial cells (Boisen and Moughan, 1996). Total EL were estimated to be 140g protein (N X 6.25) per day in a pig of 70 kg bodyweight by Horszczaruk *et al.* (1974) although this estimate can be criticised as it is based on only a single loop of the proximal small intestine that was sampled. In humans EL is estimated to be equal to10-20% of N intake (Grimble and Silk, 1989). Leibholz (1982) reported an endogenous N flow to the jejunum in pigs of 4.2 g N/d/kg DM intake.

Endogenous secretions into the lumen of the gastro-intestinal tract confound estimates of digestibility, especially as endogenous N is still subject to re-digestion (Souffrant, 1991). Using the ^{15}N technique, Souffrant (1991) reported that 70% of endogenous protein loss in the small intestine was reabsorbed by the end of the terminal ileum and 82% by the end of the large intestine.

Gut microbiology may have an influence on the form of endogenous nitrogen loss (EL) leaving the terminal ileum. It is widely acknowledged that the hindgut will distort the true picture due to microbial action (Chapter 2.1), and there is some evidence that up to 50% of non-absorbed endogenous N may be incorporated into foregut bacteria at the ileal level (Schulze, 1994). Furthermore, Schuttert *et al.* (1991) found evidence of microbial activity in the rat small intestine.

"True" measures of digestibility (Chapter 1.2) are necessary in order to accurately formulate diets according to the pig's needs and to avoid under-formulation of diets due to under-estimation of the animal's requirements. In order for the science of matching available nutrients to requirements to advance, there must be complete understanding of secretion levels, composition, and influencing factors on endogenous protein loss (EPL) and endogenous amino acid loss (EAAL). Therefore, much attention has been given in recent years to the task of establishing endogenous excretion levels, composition of the excreted nitrogenous compounds and also an investigation of the influencing factors.

Variation in endogenous N flow takes the form not only of absolute levels of endogenous secretions, but also the level of intact proteins vs. AA and peptides in the endogenous secretions. Most of the endogenous N at the terminal ileum is in the form of amino acids, peptides, or complete proteins (Moughan and Schuttert, 1991). Other sources of endogenous N at the terminal ileum are negligible, for example, Moughan and Schuttert (1991) found N from creatinine, urea and ammonia to contribute 0.73% of the total N in the ileal digesta of pigs fed a protein free diet.

N flow at the terminal ileum can be of endogenous or dietary origin, (i.e. undigested N). Other non dietary sources include ingested animal hair and micro-organisms (Moughan and Schuttert, 1991). Endogenous excretion is not constant, but is influenced by many factors, the primary one appearing to be dietary dry matter intake (Boisen and Moughan, 1996, Moughan, 1991). Butts *et al.* (1993b) found that increasing dry matter intake resulted in increasing EL levels in the 50 kg pig, the response being mostly linear, accept for lysine, glutamic acid and phenylalanine. There are other influencing factors such as fibre levels in the feed (e.g. Taverner *et al.*, 1981, Sauer and Ozimek, 1986) fibre viscosity (Larsen *et al.*, 1993), fibre type (Donkoh and Moughan 1994) and protein or peptide levels in the feed (Darragh *et al.*, 1990, Butts *et al.*, 1993a, Moughan and Rutherfurd, 1990, Donkoh and Moughan,

1994). There appears to be a linear relationship between EPL at the ileum and dietary fibre levels, the slope of this response will depend upon fibre type (Boisen and Moughan, 1996).

Moughan and Rutherfurd (1991) found no effect of the protein source itself, at least in the short term (8 hours), on the ileal flow of endogenous lysine in their work with 18 kg liveweight pigs when comparing purified soya bean protein based diets with purified gelatin protein. However, these are not natural sources when fed in this purified form, and Moughan and Rutherfurd (1991) suggest that protein sources such as non purified plant products were likely to exhibit an effect of protein source due to plant fibre and anti-nutritional compounds. This is confirmed in the review of Boisen and Moughan (1996) and also Moughan (1991).

Although it is likely that it is acceptable to apply a constant correction factor for endogenous loss when feeding highly digestible, high protein concentrates such as milk powder, casein, dried blood and fishmeal (Moughan and Rutherfurd, 1991), where there is not likely to be an influence of dietary fibre, such a constant correction factor will not apply to fibrous plant matter or ingredients containing anti-nutritional factors. Some individual feeds of plant origin do exhibit a "feed specific" influence on EPL levels. This is discussed by Boisen and Moughan (1996) to be typically 20-40 g/kg DM, and is composed of a feed specific loss on top of a basal secretion of approximately 20 g/kg DM intake, or 10-15 g/kg DM intake in a protein free diet situation.

This "source specific" EPL is likely to contribute in a mixed diet situation only proportionally to that feedstuff's inclusion level (Boisen and Moughan, 1996). This has important implications if a factorial approach is intended for mixed diet formulation.

Techniques for measuring EPL

It is necessary to distinguish between protein and AAs of dietary and endogenous origin when sampling the digesta at the terminal ileum. There are many techniques designed to assist in the determination of endogenous protein and AA excretions. These include feeding N-free diets, isotope labelling and dilution, regression analyses, the enzyme hydrolysed casein method (EHC), chemical labelling

by converting lysine to homoarginine and more recently (Boisen and Fernandez, 1995) an empirical calculation between *in vitro* and *in vivo* digestion systems. Boisen and Moughan (1996) review each of these, and conclude no difference in AA composition of the EPL determined under these systems. Moughan and Rutherfurd (1991) found no difference in endogenous loss composition when determined by the homoarginine technique or acetylated lysine with purified soya bean or gelatin protein. A further technique is possible, the use of lysine devoid feedstuffs, (Butts *et al.*, 1993a) though there are few such feedstuffs available.

When Donkoh *et al.* (1995) compared EHC, N-free and regression methods they found EHC gave results of increased EPL (11.7 mg/g DM intake) in comparison to the other two methods, (6.9 mg/g DM with N-free and 6.4 mg/g DM with the regression technique). Therefore, although there appears to be conflicting evidence on the influence of the technique for EPL measurement upon EPL composition, there does appear to be an effect on EPL quantity.

Protein free alimentation

Protein free alimentation has been the traditional way to determine EL and involves feeding a semi-synthetic diet that includes purified starch or cellulose to simulate fibre. Much has been learned about the N-free method. Negative N balance, as induced by the N-free method does not appear in itself to lead to a change in EL levels (Butts *et al.*, 1993a, Moughan, 1991). However, specific peptides or intact protein (as opposed to free amino acid) alimentation will increase EL, hence using an N-free diet may lead to underestimations of EL. In spite of this it is safe to assume that the principles reported by Moughan and Schuttert (1991) still apply, namely that under protein free alimentation the bulk of the endogenous N is in complete proteins, and that there is negligible non-protein N flow, even if it is bacterial protein (Schulze, 1994).

In spite of the fact that the feeding of protein in the diet results in increased EPL, parenterally infused AA resulted in a decreased EPL compared to a protein free diet (De Lange *et al.*, 1989b), and these authors suggest that feeding a protein free diet with intra-venous (IV) AAs is more accurate for the determination of EAAL than a protein free diet alone.

The three arguments presented in the literature against using a protein free diet are summarised by Skilton *et al.* (1988). Firstly, there is a reduced protein turnover in animals fed a diet devoid of protein. Secondly, lower gastric and pancreatic enzyme secretion is likely when the diet is protein free. Thirdly, any endogenous secretions in the protein free state are possibly subject to a greater breakdown and reabsorption. Therefore it has been generally considered somewhat unphysiological (Low, 1980) and therefore may have limited value in accurate balancing of diet formulations

All work based upon protein free diets needs to be interpreted with caution, since there is evidence that protein alimentation will increase endogenous N secretion, (e.g. Moughan and Rutherfurd, 1990, Donkoh *et al.*, 1995) as previously described. Moughan and Rutherfurd (1990) when using the EHC method, the complete homoarginine (HA) and the partial HA method to determine EL in rats fed gelatin protein and EHC peptides, reported that these all gave significantly higher EL of lysine than the N-free diet (521.8 ± 24.84 µg/g, 468.8 ± 27.94 µg/g, 506.2 ± 44.22 µg/g and 238.3 ± 9.43 µg/g respectively). There is an isolated report (Buraczewska, 1979) of protein free alimentation inducing approximately one third of the endogenous N loss of a 17 % protein diet (576 ± 131 mg/metre/day vs protein free giving just 167 ± 41 mg/metre/day in the ileum), but this is unusual. No other reports are known to be this extreme. Furthermore De Lange *et al.* (1989a) showed that feeding protein free diets may overestimate endogenous proline and glycine recoveries.

When N-free diets are fed, similar results of endogenous amino acid loss are found in comparison with animals that are fed synthetic industrial AAs as the sole N source but being devoid of the test amino acid. Skilton *et al.* (1988) found such results for eight non-essential amino acids (histidine, arginine, threonine, valine, isoleucine, leucine, tyrosine, and phenylalanine). Butts *et al.* (1993a) found that synthetic AA alimentation gave similar endogenous lysine flows to the protein free situation. Darragh *et al.* (1990) found no significant difference for all those amino acids reported by Skilton *et al.* (1988), and also lysine, methionine, proline and aspartic acid with respect to the EL induced from these synthetic amino acids and EL determined from a protein free diet.

This demonstrates that at least for certain non essential amino acids free synthetic AA alimentation does not induce EL (Moughan, 1991), unlike peptide

alimentation (Moughan *et al.*, 1992a, Butts *et al.*, 1993a). Thus Skilton *et al.* (1988) suggest that the protein-free situation may not be as unfavourable as is widely suggested. This also suggests that the N-free state *per se* did not influence EPL (Boisen and Moughan, 1996, Skilton *et al.*, 1988). Further evidence that the N free state does not lead to EAAL is given by de Lange *et al.* (1989b). Growing pigs fed a protein free diet showed no significant differences ($p<0.05$) in endogenous amino acid loss (EAAL) compared to those fed a protein free diet, but infused with IV amino acids.

The use of synthetic amino acids does not appear to have any difference on pancreatic enzyme activity in comparison with intact casein (Green *et al.*, 1973). This adds weight to the value of experimental work using synthetic AAs. In addition, synthetic amino acid supplementation overcomes the criticism of decreased protein turnover in a protein free diet in the pig (Butts *et al.*, 1993a), one of the arguments against protein free feeding experimentation.

Determination of EPL by regression.

Regression analysis involves feeding graded levels of a protein source and measuring the EPL at each of these levels. By plotting these values graphically an experimenter can extrapolate back to the level of zero protein inclusion. The intercept value at this point corresponds to EPL for that feedstuff. It has been claimed that this is more accurate than N-free determination (Boisen and Moughan, 1996). Donkoh *et al.* (1995), however, found that the protein free and regression technique with meat and bone meal gave similar results for EL, and quoted several other workers as having found the same result. Leibholz, (1982) also found the same result comparing regression and near protein free diets (0.9 g N/kg).

The regression approach assumes that EL remains constant with increasing levels of dietary protein intake, however some of the higher AA flows at higher levels of protein inclusion could be due to increased endogenous secretions, and it cannot be assumed that the relationship of EL to protein intake is linear (Donkoh *et al.*, 1995). The obvious shortcoming of this approach is the somewhat tedious and expensive task of conducting animal trials for each feedstuff.

Taverner *et al.* (1981a) used this approach with wheat and barley as the sole protein sources in two separate series of diets, and at two levels of dietary fibre (140 and 190 mg NDF/g). The results showed that there was no change in AA composition of the EL, and mucin appeared to contribute to the bulk of EPL.

EHC and ultra-filtration

This technique is relatively new and was first proposed by Moughan *et al.* (1990). It involves feeding the animal casein that has been cleaved by enzymes into peptides of <5,000 Da and also includes a centrifugation and ultra-filtration step in the treatment of the ileal digesta. This separates the fragments into a fraction <10,000 Da and another >10,000 Da. This way any undigested EHC peptides are not included in the EPL measurement (ie EPL is estimated by molecules with a MW>10,000). The fraction that is less than 10,000 Da may contain some endogenous loss, but this is not thought to be a significant proportion of the total endogenous lysine (Moughan and Schuttert, 1991). Butts *et al.* (1991a) found the endogenous free AAs plus small peptides in total AA flow to be less than 20% and Moughan *et al.* (1993) cites evidence of 11-21% of digesta N being endogenous free AAs and peptides. Darragh *et al.* (1990) found a very high correlation (r = 0.85) between EAAL composition for protein free and EHC diets, and they interpreted this to imply that most of the AA flow at the terminal ileum of the rat fed EHC is endogenous.

However it leads to the criticism that using this technique results in an underestimation of EAAL (Butts *et al.*, 1993a). The opposite criticism has been raised also, (Moughan *et al.*, 1992a), that using the unfiltered digesta may lead to overestimation of EAAL. In the work of Butts *et al.* (1991a) there were lower EAAL with the centrifuge/ultra-filtration technique than untreated digesta, though only significantly so ($p<0.05$) for lysine, glutamic acid and threonine. Nonetheless, the possibility remains of excluding small endogenous peptides and amino acids from the precipitate (Butts *et al.*, 1993a). This was shown to be the case in Butts *et al.* (1992a) where 46% of glycine, 36% of histidine, 28% of lysine and between 13-24% of other amino acids in the digesta ended up lost in the ultrafiltrate of digesta obtained after feeding rats a protein free diet after centrifugation/ultrafiltration. Moughan *et al.* (1992a) also demonstrated the loss of AAs into the supernate when they demonstrated

that the only AAs not significantly less after ultra filtration than before ultrafiltration were arginine, glycine leucine and phenylalanine. Lysine and aspartic acid were very significantly lower ($p<0.001$), the average amino acid figure was 14.9% less in the filtered digesta. This is further evidence that there is little free amino acid flow in the total N flow as discussed previously, as generally only limited amounts of the total N is lost into the ultrafiltrate due to being <10,000 Da.

The EHC method was also tested by treatment of digesta with a precipitating agent (Butts *et al.*, 1991a), namely perchloric acid or trichloroacetic acid. The value of TCA or PCA were questionable, and it was concluded that just the centrifugation step was sufficient, as it required less labour and no more chemicals. Büchmann (1977) similarly found that TCA only precipitated 4% more than centrifugation alone.

The difference between the EHC and N-free methods referred to earlier in the work of Donkoh *et al.* (1995) when examining the influence of technique for measuring EPL, demonstrates the point that peptide alimentation stimulates EPL. Hence, the EHC method is likely to give more accurate EPL values than N-free alimentation, as it simulates the products of digestion that would normally be found in the digestive tract in commercial practice. The EHC method gave generally higher EAAL values than the regression method in the growing rat (Donkoh *et al.*, 1995).

The EHC method has provided the key evidence to suggest that dietary peptides influence EPL and reabsorption (Boisen and Moughan, 1996). Butts *et al.* (1991a) found significantly ($p<0.05$) lower values for endogenous excretion levels for lysine and proline and highly significant ($p<0.01$) differences for threonine and alanine. Serine, glutamic acid, valine, isoleucine and leucine were also very highly significantly lower ($p<0.001$) with a protein-free diet while endogenous flows of tyrosine, phenylalanine, histidine, arginine and aspartic acid were not significantly less between the EHC and protein free diets. Moughan *et al.* (1992a) found that lysine, aspartic acid, threonine, serine, glutamic acid, valine, isoleucine and leucine were significantly lower ($p<0.05$) for the protein free diet compared to the EHC diet. Proline and glycine were however higher in the protein free diet, but only significantly so for glycine ($p<0.05$). The reverse trend for these two amino acids is possibly due to higher endogenous losses of proline and glycine which is frequently observed in the protein-free situation (Moughan *et al.*, 1992a). Taverner *et al.*, (1981a) suggested that this is due to a high reflux of the free amino acids after intracellular digestion.

The EHC method gives estimations of EAAL that strictly only apply to highly digestible proteins such as MBM which do not contain ANFs or fibre (Donkoh *et al.*, 1995). Such feeds with high fibre elicit an extra endogenous loss not seen with highly digestible feeds.

The Homoarginine method

If it were possible to label proteins in the diet then it would be possible to measure both undigested proteins and EPL and possibly EAAL at the terminal ileum. The labelling of lysine by means other than isotopes is a possibility.

The search for a suitable label of lysine has involved isotopes but these are subject to rapid re-secretion into the lumen (Simon *et al.*, 1983). However a non-returning label is available in the form of a guanidination of lysine side chains in proteins (Hagemeister and Erbersdobler, 1985). This converts the lysine to a synthetic analogue of lysine, homoarginine. Homoarginine (HA) is acid stable (Moughan and Rutherfurd, 1990) and does not occur naturally in protein. It is not synthesised by mammals, as it is not a substrate for protein synthesis (Schmitz *et al.*, 1991). Schmitz *et al.* (1991) showed the non-returning nature of HA in two key experiments, demonstrating that 0.12% of free HA infused into the jejunum is recovered at the terminal ileum, and that <0.2 % of IV HA appeared at the terminal ileum.

Schmitz *et al.* (1991) also demonstrated that HA and lysine are absorbed to the same extent as each other. This makes quantification of endogenous lysine very easy. There was shown to be a rapid proteolysis of the labelled protein and subsequent absorption of HA.

If complete conversion of lysine to HA is achieved then any lysine appearing at the terminal ileum must be endogenous in its origin. However complete conversion of lysine to HA is difficult to achieve. Schmitz *et al.* (1991) achieved 89.6-99.6% conversion, a very high figure indeed. Moughan and Rutherfurd (1990) achieved near total conversion of gelatin protein (95%).

Homoarginine can be converted by arginase to lysine (Prior *et al.*, 1975) However, use of completely guanidinated proteins to calculate EL relies on the assumption that there is negligible hydrolysis of HA by arginase in the digestive tract,

(Schuttert *et al.*, 1991). Schuttert *et al.* (1991) found this to be so when incubating *in vitro* preparations of rat ileal digesta with HA.

In the case of incomplete conversion, the difference in the ratio of lysine:HA in the diet and digesta is taken to be a measure of endogenous lysine secretion, the ratio of lysine:HA in the digesta being higher than that in the diet. Moughan and Rutherfurd, (1990) found no significant ($P>0.05$) difference in the level of EL when determined with both completely guanidinated gelatin protein, and partially guanidinated gelatin protein, (469 ± 27.9 μg/g and 506 ± 24.8 μg/g respectively) suggesting that complete guanidination is not necessary to use the HA technique.

HA can be used to measure the "feed specific" EPL that was discussed earlier (Boisen and Moughan, 1996). HA cannot be effectively applied to heat damaged proteins however. This is due to the Maillard reaction making lysine unavailable for reaction with OMIU. Schmitz *et al.* (1991) found heat damaged casein to show this, and make the assumption that heat damaged protein is less degradable. HA cannot be used for prolonged periods of time due to toxicity problems (Boisen and Moughan, 1996). It was observed by Moughan and Rutherfurd (1990) that rats fed a guanidinated diet decreased their VFI over the day.

Further, possible problems include the uncertainty of labelling. Where there is incomplete conversion it is possible that lysine was not transformed due to physical inaccessibility. This suggests that perhaps it is not absorbed to the same extent as the homoarginine. Another unmeasured possible effect is the effect of homoarginine on the factors that are normally anti-nutritional. Guanidination may affect the anti-nutritional properties.

Isotope dilution as a means of determining EPL

Isotopes, either stable or radioactive, can be used to label either dietary or somatic protein, ^{15}N being the most common procedure (Boisen and Moughan, 1996). The ^{15}N method relies upon a basic requirement of a steady state being achieved for ^{15}N uptake (Schulze *et al.*, 1995). The second main concern lies with selecting the right pool of proteins for labelling (Schulze *et al.*, 1995). After labelling a suitable somatic reference pool, and then subsequent feeding of an ^{15}N isotope labelled diet,

the difference in the ratios of ^{15}N to ^{14}N in the diet and digesta is taken as a measure of EL.

The ^{15}N technique gives a good feed specific measure of endogenous lysine flow (Schulze *et al.*, 1995). Furthermore, it gives the EPL corrected for the specific feed, not just the protein in the feed, and so accounts for all ANFs such as fibre. Schulze *et al.* (1995) showed that fibre induces EL using the ^{15}N technique.

On the negative side, it is open to debate as to what pool of N to use (Souffrant, 1991) and this technique does not account for recycling of ^{15}N in the small intestine. The choice of body pool will affect the final estimates of EL. Three potential pools are the plasma free, plasma bound and intestinal mucosa amino acid pools (Moughan *et al.*, 1992b). A further consideration is the time lag between the infusion of the body pool and the introduction of the diet. Moughan *et al.* (1992b) found an 8 day infusion period sufficient to achieve steady state of ^{15}N to ^{14}N ratio in rats. Acceptable agreement with digestibility of individual amino acids is lacking though (Schulze *et al.*, 1995). The high cost of ^{15}N substances hinders the applicability of this technique (Souffrant, 1991).

Moughan *et al.* (1992b) adopted an approach with ^{15}N whereby the protein-free diet fed to labelled rats was unlabelled, and the rat was labelled instead. Therefore any endogenous protein at the ileum was labelled, and the ratio of ^{15}N to ^{14}N in the digesta was identical to the body pool ratio in principle. Due to the results being other than that expected in principle, the conclusion was that the technique in its present state was unsatisfactory as a means for determining EL, due mainly to the problem of identifying a suitable reference pool of precursor amino acids. However Schulze *et al.* (1995) conclude that it may be useful for ranking relative differences in endogenous ileal N flow.

The ^{15}N technique gives lower results than the EHC method with 10 kg piglets fed cornstarch (Schulze *et al.*, 1995). A shortcoming of this technique is that it can only be used to measure EL not specific EAAL (de Lange *et al*, 1989a).

Empirical estimates of EL

Boisen and Eggum (1991) proposed that the difference between their *in vitro* determined digestibilities and apparent digestibilities determined *in vivo* should

correspond to EL. This is expected to be true in theory, because there is no endogenous loss from glassware, the *in vitro* digestibility should therefore correspond to true digestibility Assuming this to be an accurate representation of the state of nature, then the true (*in vitro*) digestibility less the apparent (*in vivo*) digestibility will indeed correspond to EPL from the test animal.

However further work comparing *in vitro* and true digestibility is required. The *in vitro* situation gives no feed x gut interaction effects.

Lysine deficient feedstuffs

A final possibility is the use of lysine deficient feedstuffs. This is not common as there are few feedstuffs devoid of any specific AA. Butts *et al.* (1993) used Zein with growing pigs, a protein from maize (*Zea mays* L. Graminae) that is very low in lysine (<0.25%) and tryptophan (<0.15%). It was assumed then that the ileal flow of lysine was endogenous. Butts *et al.* (1993a) found 389 mg lysine / kg DM intake in the ileal digesta with pigs fed Zein and dietary lysine was expected to contribute at most 3.8% of this lysine. The Zein treatment gave a higher EL value than N-free and synthetic amino acid diets,

Composition of EPL

Boisen and Moughan (1996) reviewed each of the techniques for measuring EPL, and concluded no difference in AA composition of the EPL. They conclude that AA composition of the EPL is also relatively constant across diets. After a review of several experiments, Boisen and Moughan (1996) suggest the following means for general digestibility correction for EPL, (g/160 g): lysine 30, methionine 10, isoleucine 25, leucine 40, histidine 15, phenylalanine 30, tyrosine 20, valine 35. They compare this to "ideal" protein and observe that the proposed general composition is low in essential AAs except for threonine, cystine, and tryptophan.

This homogeneity of EAAL is advantageous. When combined with techniques such as the HA technique which allows measurement of the absolute level of endogenous lysine secretion, it is then a simple procedure to estimate other AA losses.

Taverner *et al.* (1981a) found the most abundant AAs in EL were proline, glycine, glutamic acid and aspartic acid (proline>glycine>glutamic acid). This is in concurrence with other studies (for example Sauer *et al.*, 1977). Glycine was the major AA in the EL (427 μg/g freeze dried matter) in the work of Butts *et al.* (1992a) with rats fed a protein free diet. Other amino acids that were highly abundant included glycine, proline and glutamic acid (427, 446 and 586 μg/g freeze dry matter intake respectively), which is in accordance with the observations of Taverner *et al.* (1981a). Also present in high quantities were aspartic acid and threonine, (641 and 388 μg/g freeze dry matter intake respectively). Tanksley and Knabe (1984) reviewed several studies which also found threonine to be at a relatively high concentration in EAAL from protein concentrates.

Moughan and Schuttert (1991) attempted to estimate the magnitude of the various fractions in the pig ileal digesta, under protein-free feeding. Their findings indicated that 86% of N flow at the terminal ileum was N in molecules >10,000 Da, of which 78% was protein N, indicating the majority of terminal ileum N flow was in the form of large (MW >10,000 Da) proteins. Less than 15% was, therefore, free amino acid and peptide N.

This is in contrast to rats (Butts *et al.*, 1992a) where 21% of total digesta N was in the form of free AA and small peptides. The majority of the amino acid N remaining was in the form of large proteins.

The findings of Moughan and Schuttert (1991) must be considered with discretion, however, as all work done with protein free diets is called into question.

Summary

It is necessary to correct apparent digestibility for endogenous losses in order to accurately meet the animal's true requirements. Generally speaking, EPL has much the same composition of AA, but will vary in quantity depending upon the method used for its determination and various dietary factors.

1.4 The rat as a model for the pig

The considerable expense of pig assays has led to the search for a less expensive alternative model animal. The laboratory rat has been evaluated as a model animal, and shows considerable promise and several workers have used the rat assay to determine amino acid digestibilities (Donkoh *et al.*, 1995, Skilton *et al.*, 1991). The rat is also used to allow evaluation of basic aspects of digestive physiology (Green and Nasset, 1983).

Aspects of protein digestion have been shown to be very similar in the pig and rat. For example, protein is absorbed mainly in the last two thirds of the rat small intestine, within 1-2 hours after feeding (Curtis *et al.*, 1978). Aspects of digestive physiology and anatomy have also been shown to be similar in the pig and rat (Church and Pond, 1988).

Using the rat has the advantage that large numbers of animals can be run at the same time, relatively inexpensively. It is also easier to sample digesta by the slaughter method. This means that the expense of surgery is not inhibitive to the trial and it is perhaps ethically more sound than using surgically modified animals. Furthermore, when feeding high priced feeds such as guanidinated feed, a rat requires far less food than a pig. Its ease of handling and low cost mean that this animal lends itself more readily to a routine feed evaluation programme.

Sampling from the rat

Donkoh (1993), and Donkoh *et al.* (1994c) report that sampling site and time had a significant ($p<0.05$) effect on the amount of digesta collected, but not on the apparent ileal N digestibility for a semi synthetic diet where MBM was the sole protein source.

Inter-species relationships with respect to protein digestibility

For an inter-species comparison, the physiological conditions must be kept as similar as possible, (Donkoh *et al.*, 1994c). A consideration is the liveweight and physiological age of the animal, and if the animal is in a positive linear growth phase. Although the live weights should be the same (as a % of mature bodyweight) when comparing species, if the growth stage is linear and positive this should not be essential (Donkoh *et al.*, 1994c). The issue here lies with the stage of development of the digestive tract. The relative food intake between species should also be comparable (Donkoh *et al.*, 1994c).

Moughan *et al.* (1987) investigated the relationship between pigs and rats in the determination of ileal AA digestibility for barleys. They found that there was generally good agreement for the essential AAs except for methionine and phenylalanine in terms of apparent digestibility and for methionine in terms of true digestibility. For the non-essential AAs there was less agreement for apparent digestibilities, with all AA being significantly different ($p<0.001$) between the pig and rat. For the true digestibilities there were significant differences in some essential AAs (lysine, valine, methionine and leucine). Non essential AAs showed agreement only for glutamic acid in terms of true digestibility for the rat and pig. The question remaining from this research is whether or not the variation is attributable to EL.

Donkoh *et al.* (1994c) demonstrated a net disappearance of amino acids in the large intestine of the rat and the pig, just as had been demonstrated in the pig by other workers (Chapter 2.2). Faecal digestibilities in the pig and rat were both higher in general ($p<0.05$) than the ileal digestibilities.

In their work comparing ileal digestibilities of MBM in the rat and pig, Donkoh *et al.* (1994c) found no species x type (ileal or faecal) interaction for N or AA digestibility except for glutamic acid in each of two MBMs (high quality vs low quality). This is very positive evidence in support of the rat as a model animal for the evaluation of animal proteins.

For plant protein sources though, there may be a species effect. There has been shown to be a species effect for protein in peas (Moughan *et al.*, 1984). Huisman *et al.* (1991) demonstrated different responses in rats and pigs to ANFs in beans and field peas, such as a hypertrophy of the pancreas in rats but not piglets. Furthermore, weight

gain was much more negatively effected in pigs than rats. This is significant, as ideally model animals should display similar responses to ANFs, to accurately predict the response to a feed of the target animal.

Shortcomings:

One question that remains is whether or not differences in the relative growth rates between species will affect digestibilities. Another area for further work is whether or not there is a difference in EPL between rats and pigs.

Donkoh *et al.* (1994b) found significant differences in the digestibility of glutamic acid between pigs and rats. Glutamic acid is usually a high component in EPL. This could indicate EL differences between the species, however, no other differences in amino acid digestibility were found in the work of these authors. Nonetheless, there is more work to be done on this aspect of inter-species comparison.

Summary

The rat has been demonstrated as a suitable animal model for studying aspects of protein digestion in the pig. The rat is suitable because of its low cost, and similarity of digestive tract. Use of the rat has the advantage that large numbers of animals can be run at the same time. It is also easier to sample digesta by the slaughter method which is commonly used in small animals such as the rat. Its ease of handling and low cost mean that this animal lends itself more readily to a routine feed evaluation programme.

1.5 *In vitro* techniques for determination of protein digestibility

The cost and labour requirement for *in vivo* digestibility assays are high and this has motivated the search for an alternative *in vitro* assay. Further, if the true amino acid digestibilities can be accurately determined *in vitro*, this is considerably easier than trying to establish EPL by biological means. For example, Löwgren *et al.*

(1989) report the capacity to run 250 inoculations concurrently in one laboratory. A great deal of interest has been shown in *in vitro* procedures, especially in recent decades although *in vitro* assays have been experimented with for close to 200 years (Smith, 1980).

An *in vitro* digestibility assay must be repeatable and transferable between laboratories. It should be calibrated in the initial establishment period, and checked regularly against samples of known *in vivo* digestibility.

The key Factor: enzyme specificity

Enzymes are very specific in their action, determining which bonds in the substrate are cleaved. Therefore enzyme selection is paramount to the success or failure of an *in vitro* method. Some enzymes, however, may require additional enzymes to modify the substrate for access. This is why enzyme mixes should closely match those found *in vivo*.

The importance of enzyme specificity is shown in the work of Büchmann (1979) who worked on developing an *in vitro* assay. Büchmann found that the ranking of cereals for the digestibility of protein depended on both the type of enzyme and the enzyme to substrate ratio.

Löwgren *et al.* (1989) used inoculum from the ileum, duodenum and faeces in an investigation to simulate intestinal digestion. Rapid solubility of starch and proteins occurred in all three innocula, but was attributed to bacteria in the last two, and pancreatic enzymes in the first. There was an indication that the faecal innocula digested protein more slowly, this could be due to bacterial binding to undegraded protein.

Particle size

Normally a smaller particle size is used *in vitro* than in *in vivo* assays, but particle size should not be less than 0.5mm for *in vitro* digestion or problems may occur with sample loss (Boisen and Eggum, 1991). Löwgren *et al.* (1989) reported

that decreasing the particle size for their *in vitro* incubations led to increased buffering capacity.

The various *in vitro* methods for measuring protein and amino acid digestibility

Boisen and Eggum (1991) provide an excellent review of the current state of most techniques for *in vitro* digestion. The following methods are reviewed in detail: the pH drop, the pH stat, dialysis and filtration methods.

Filtration

This filtration type of assay is the key *in vitro* method addressed in this dissertation and follows on from some exciting developments by Boisen and Fernandez (1995). Filtration methods rely on an *in vitro* digestion with enzymes, and then separation of the digested and indigestible fractions by filtration. An alternative to filtration is centrifugation.

There are one, two and three step systems. The results and methodology of many workers in these areas are reviewed by Boisen and Eggum (1991). The one step system involves digestion by incubation with pepsin, papain, trypsin, pronase or renin. Pronase *in vitro* assays are the most widely used in commercial practice (Moughan *et al.*, 1989). Two step methods include subsequent incubations with pepsin and jejunal fluid, or pepsin then pancreatin, each step at the appropriate pH. Three step systems are more for the determination of digestible energy contents, as energy estimates are acceptably measured at the faecal level only. Therefore three step systems involves a third step simulating the large intestine. The appropriate enzymes for this stage are either a combination of fibre degrading enzymes or rumen fluid. The third enzyme step by Boisen and Fernandez, (1991) included a commercial preparation, Viscozyme (Novo, Denmark) made from enzymes of the *Aspergillus* group.

A technique using pepsin, pancreatin and amylase (Babinszky *et al.*, 1990) gave correlation coefficients of 0.99 and 0.95 for feedstuffs and mixed diets respectively, between digestible CP *in vitro* and digestible CP *in vivo*. Again, the *in vitro* values were higher than *in vivo* values for digestible CP, but the feeds were ranked similarly. The fat extracted feedstuffs analysed were groundnut meal, soya

bean meal solvent extract, linseed expeller meal, lupin, rapeseed meal, corn feed meal and tapioca. The high value for correlation is partly attributed to the wide range in CP (40-474 g/kg DM). The lower correlation of r = 0.95 for diet mixes was coupled with a lower CP range (170-224 g/kg DM), (Babinszky *et al.*, 1990).

pH methods

The principle this technique relies upon is measuring the release of protons during the enzyme hydrolysis of proteins. The pH methods determine the digestibility by either measuring the resulting drop in pH under incubation with enzyme, or by measuring the amount of NaOH required to maintain a constant pH under incubation with enzyme. These are termed the pH drop and the pH stat methods, respectively. They assume a close correlation between peptide hydrolysis, hence H^+ release, and digestibility.

In the review of Boisen and Eggum (1991), the correlations with *in vivo* determinations as found by several workers are reviewed. The pH drop method is found to correlate well with faecal digestibilities in rats for various plant proteins, however, as previously discussed the faecal digestibilities of protein are quite meaningless for accurate diet formulation. Both the pH drop and pH stat methods suffer in that they have been reported as giving poor correlation with *in vivo* digestibility values in many feedstuffs in reports reviewed by Boisen and Eggum (1991), although the pH stat method was far more accurate than the pH drop method in predicting protein digestibilities. *In vivo* work with rats determining true faecal digestibility has shown good correlation with pH stat data (Boisen and Eggum, 1991). The question remains as to the usefulness of these assays without validation against ileal digestibility.

The dialysis cell technique

Samples are diluted in HCl at pH 1.9 with pepsin for 30 minutes, and then adjusted to pH 7.5. Dialysis is then performed through a dialysis tube (MWCO 1000) while incubating with pancreatin solution. The method is good for direct measurement of protein susceptibility to proteolytic enzymes (Boisen and Eggum, 1991), and it

appears to predict availability of AAs. It relies upon choosing the right enzymes for each substrate (Boisen and Eggum, 1991).

Microbiological methods

These are particularly useful for the sulphur containing amino acids (methionine and cysteine). An advantage of these techniques is that they can rank feeds according to nutritional quality. A disadvantage is that antagonists of methionine may confound the results. Protozoan are grown in a proteaceous medium. Such protozoan as *Tetrahymena pyriformis*, *Streptomyces zymogenes* and *Leuconostoc mesenteroides* (now classified as *Pediococcus acidilactici*) are the main strains used for sulphur amino acid assays. Many feedstuffs have been analysed with *Streptomyces zymogenes* (reviewed by Mackenzie, 1985).

Comparison of techniques

Moughan *et al.* (1989) compared various *in vitro* techniques (pH drop, a modified pepsin-pancreatin and a pronase method) with rat ileal digestibility data for CP in MBM. Pepsin-pancreatin digestion gave precise figures, but did not rank the MBMs as with the *in vitro* method. It was concluded these assays were not suitable for MBM in pig diets. The pH drop method did not show linearity between the digestibility *in vivo* and the rate of pH drop. This was thought to be due to a large buffering capacity of MBM, due to its high ash content.

Shortfalls of *in vitro* work

Snook (1973) reviews several criticisms of *in vitro* procedures that all warrant further investigation, especially if *in vitro* technologies are to be adopted in the future on a large scale. One major criticism as reviewed by Snook (1973) is that hormonal, neural and nutritional factors are not accounted for in the *in vitro* studies. Furthermore the rates of digestion *in vivo* compared to in vitro are often found to be different.

Büchmann (1977) cites several workers who warn against amino acid accumulation during *in vitro* proteolysis. Perfect *in vivo* imitation is impossible, given an incomplete understanding of all the biological factors (Büchmann, 1977).

Inter-laboratory repeatability

Inter-laboratory studies are required especially if the results are going to an international databank. An inter-laboratory study was conducted between six laboratories in four countries with a pH stat *in vitro* technique (McDonough *et al.*, 1990). Agreement was excellent within each laboratory, and between labs the mean relative standard deviations for repeatability were all less than 1.4% and mean relative standard deviations for reproducibility were 0.8-5.0%. This is the level of repeatability that is necessary in an *in vitro* technique.

Of great importance was the fact that all six labs ranked the same feed, casein, as the most digestible and non-fat dried milk ranked second in five labs.

Summary

Given the current technologies, the best that an *in vitro* procedure can be reliably used for, is perhaps, the rapid assessment of the relative ranking of feedstuffs. Many *in vitro* procedures exist but few have been widely accepted by the scientific community. *In vitro* digestion does not account for the effects of ANFs.

Further work is required on optimal enzyme to substrate ratios, and the balance of pepsin to pancreatin proteolysis, as well as optimal digestion times.

Overall conclusion

The preceding review outlines the current level of understanding on protein digestion in the pig and on *in vitro* digestion studies. It also demonstrates the use of the rat as a suitable model animal for studying certain aspects of protein digestion in the pig. The following dissertation uses this model animal technology to provide biological data for the evaluation of a new *in vitro* digestion technique (Boisen and Fernandez, 1995). Having shown in the preceding review that true digestibilities are desirable, the present work also uses the homoarginine technique to generate true digestibilities of protein and lysine.

Chapter 2

A DESCRIPTION OF NEW ZEALAND BARLEYS: CHEMICAL AND PHYSICAL MEASUREMENTS AND APPLICATION OF A MULTI-ENZYME *IN VITRO* PROTEIN DIGESTIBILITY ASSAY

2.1 Introduction

Barley is a feedstuff of major importance to the New Zealand pig industry. The determination of digestible protein and amino acids is necessary to allow utilisation of barley to its fullest potential in meeting the amino acid requirements of monogastric farm animals. Because of the expensive and time consuming nature of *in vivo* protein digestibility studies it would be of considerable advantage to have the ability to accurately predict *in vivo* digestibility from either the chemical composition of the barley, or based on *in vitro* digestibility. Boisen and Fernandez (1995) have reported success with a new *in vitro* digestibility assay when applied to a wide range of feedstuffs. The presently reported work was designed to evaluate the suitability of this technique to allow prediction (reported in a subsequent chapter) of *in vivo* digestibilities of protein and amino acids in New Zealand barleys. Chemical and physical measurements were also determined on a range of barleys to allow subsequent investigation of possible relationships between chemical and physical characteristics and *in vivo* digestibilities of protein and amino acids. To date, there has been no such comprehensive study conducted on New Zealand barleys.

2.2 Materials and methods

2.2.1 Sample collection

Seventeen samples of barley were collected from around New Zealand, representing six growing regions (Manawatu, Hawkes Bay, Rangitikei, Mid-Canterbury, South-Canterbury, Wairarapa), and nine varieties (Table 2.1). A random sample (250kg) of each harvest was collected and stored at room temperature for 2 months.

Data on the sowing rate, days to harvest from planting and yield were recorded (Table 2.2). The seventeen barley samples were designated B1 through to B17. All seventeen barleys were submitted to a range of physical and chemical measurements. Ten samples were selected (to give a range in crude protein, CP, contents), for *in vitro* digestibility analysis.

2.2.2 Physical measures

Physical measures included grain bulk density (kg/hectolitre) and moisture content (%), measured using a portable moisture meter (Dicky John, USA), screenings (%) measured on a Rotoscreen (D.H. Brown and Son, Ltd. Christchurch, New Zealand) and 1000 seed weight (g).

2.2.3 Chemical analysis

Dry matter (DM) was determined by weighing samples, drying the samples overnight at 105°C and then re-weighing.

The N content of each barley sample was determined using a Kjeltech 1030 auto analyser (Tecator, Sweden) following the standard Kjeldahl procedure. (AOAC, 1980). Duplicate determinations were on average within 2% of each other and complete recovery of a known standard was accomplished. CP in the barley samples was determined as N X 5.83 (Breese Jones, 1931). AA compositions of the barley samples were determined on a Beckman 119 BL amino acid analyser (Beckman Instruments, Palo Alto, CA, USA). Five mg samples were hydrolysed in 1 ml of 6 M

glass distilled HCl containing 0.1% phenol for 24 h at 110 ± 2°C in glass tubes sealed under vacuum. Cystine, Methionine and Tryptophan were not determined as these are destroyed by acid hydrolysis.

Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were all determined using the method of Robertson and Van Soest (1981). Agreement between duplicates was within 3%, 5% and 8% for NDF, ADF and lignin respectively.

Table 2.1: Variety, location and harvest date for seventeen barley samples harvested in New Zealand in 1995.

Code	Variety	Location	Harvest Date
B1	Fleet	Manawatu	7 Feb
B2	Fleet	Hawkes Bay	18 Dec†
B3	Nugget	Hawkes Bay	7 Mar
B4	Corniche	Rangitikei	7 Mar
B5	Valetta	Rangitikei	10 Mar
B6	Magnum	Mid-Canterbury	2 Feb
B7	Regatta	Mid-Canterbury	10 Feb
B8	Nugget	Mid-Canterbury	10 Feb
B9	Fleet	Rangitikei	16 Feb
B10	Fleet	Rangitikei	22 Mar
B11	Opiki	Manawatu	13 Mar
B12	Fleet	South-Canterbury	20 Jan
B13	Valetta	South-Canterbury	10 Jan
B14	Magnum	South-Canterbury	15 Jan
B15	Liberty	South-Canterbury	20 Jan
B16	Triumph	South-Canterbury	20 Jan
B17	Fleet	Wairarapa	20 Jan

† 1994

Table 2.2: Production data for seventeen New Zealand barleys including yield, interval from sowing to harvest and sowing rate.

Code	Yield (t/hectare)	Interval Sowing-harvest (days)	Sowing Rate (kg/hectare)
B1	1.6	112	140
B2	-	74	135
B3	3.75	135	160
B4	3.93	100	143
B5	3.85	103	150
B6	6.00	146	100
B7	7.50	130	125
B8	7.50	129	125
B9	4.90	97	150
B10	5.90	112	150
B11	4.40	113	165
B12	5.00	122	115
B13	5.60	112	115
B14	5.50	148	105
B15	6.00	132	110
B16	4.50	122	105
B17	5.20	127	111
Average	5.53	118	132

The levels of (1-3)-(1-4)-β-D-glucan (hereafter referred to as β-Glucan) in ten of the 17 barleys were determined following the procedures of Jørgensen and Aastrup (1988). Total β-glucan levels were determined by suspending 50 mg of ground barley in 10 ml H_2O and incubating in a boiling waterbath for 1.0 hour. Ten ml 0.075 M H_2SO_4 were added and incubation continued for 10 minutes. Samples were centrifuged and supernatants were chilled until their analysis by flow injection analysis (FIA).

Gastro intestinal (GI) extracted β-glucans were determined by suspending 4.0 g of barley in 20.0 ml HCl buffer (pH 1.5) and incubating for 2.0 hours at 37°C. The material was neutralised by adding 0.8 ml 10% NaOH to the suspension. This was incubated a further 3.5 hours at 37°C, before analysing supernatants using FIA. GI extraction may provide a more nutritionally appropriate measure, as it simulates natural digestion in the gastro-intestinal tract.

2.2.4 *In vitro* protein digestion

Ten of the barley samples were subjected to an *in vitro* N digestibility assay. The method used was that of Boisen and Fernandez, (1995). A full list of reagents is given in appendix 1 and the steps in the assay were as follows:

Steps:

1. Approximately 0.5 g of finely ground barley (<0.5mm) were accurately weighed (±0.0005g) and suspended in 25 ml phosphate buffer A in a 100 ml conical flask, with magnetic stirring on a Variomag stirrer.
2. 10 ml 0.2 M HCl was added and this was followed by 1 ml pepsin solution. The pH was then adjusted to 2.0. All pH adjustments were made with 1 M NaOH, or 1 M HCl.
3. 0.5 ml chloramphenicol solution was added as a bacteriostat. Samples were sealed with plastic wrap, and incubated at 40°C (±2°C) for 6.0 hours, under constant magnetic stirring.

4. 5 ml 0.6 M NaOH were added along with 10 ml phosphate buffer B. One ml pancreatin solution was added and the samples closed sealed with plastic wrap and incubated for a further 16.0 hours.
5. 5 mls of 20% sulphosalicylic acid were added and the samples stirred at room temperature for 30 minutes to precipitate undigested protein.
6. Samples were filtered on a fibretech filtration apparatus (Tecator, Sweden). Glass filter crucibles (pore size 2) containing approximately 0.5g celite had been previously dried at 80°C, then cooled in a desiccator, and weighed. The samples were transferred to the crucibles with rinsing of the flasks with 1% sulphosalicylic acid, and then samples were rinsed with 20 ml ethanol (96%) under vacuum filtration.
7. The samples were then rinsed twice in 10 ml acetone, and left in each rinse for approximately three minutes, before vacuum filtration.
8. Samples were dried overnight at 105°C, cooled in a desiccator and weighed. Samples were transferred quantitatively to Kjeldahl tubes for N analysis.

The procedure was repeated, for each sample with a blank included in each run. This blank contained the digested enzyme N, which was used to correct the residual N in the barley samples that were digested *in vitro*. Unlike in the specified protocol (S. Boisen, unpublished) N digestibility was calculated in terms of moles of N rather than on a weight basis. N digestibility was calculated according to the following equation:

Nd_{vitro} (%) = (1-(RMN-Blank MN) / Sample MN)) X 100

Where RMN is moles of undigested N in undegraded dry matter (UDM) and MN is moles of N. Furthermore, the ten Barley samples were analysed in Denmark (S. Boisen Pers. Comm.) to allow a comparison of the same sample between laboratories.

2.3 Results

The physical parameters for the barleys are presented in Table 2.3. Coefficients of variation (CV) were rather low for all of the physical characteristics determined. The 1000 seed weight was the most variable characteristic measured.

The CP contents of the barleys are presented in Table 2.4. The range in CP was quite narrow (only 4.75 % units) and the mean value was 11%. Neutral detergent fibre and acid detergent fibre contents and lignin contents are all presented in Table 2.5. The NDF had the widest range at 6.7 % units. ADF and lignin were much narrower in range at 1.8 and 1.0 % units, respectively.

The total and gastro intestinal (GI)-extracted β-glucan contents are given in Table 2.6. GI-extracted β-glucans were consistently lower than the total β-glucan levels (3.49-5.96% cf 0.55-2.56%) and the ranking of the barleys also changed. The mean of the GI-extracted β-glucans was 1.7 % and the mean for the total β-glucan was 5.2%.

Table 2.7 provides a summary of the gross amino acid compositions (g/100gDM) for the 17 New Zealand barleys. On average, histidine was found at the lowest concentration and glutamic acid at the highest. Individual values are given in appendix 2.

The *in vitro* digestibilities (Nd_{vitro} %) determined at Massey University (means of two duplicates) are given in Table 2.8. Individual values for each replicate are given in appendix 3. *In vitro* digestibility ranged from 86.8% to 94.4%. The assay was reasonably precise with mean duplicate agreement being within 3% (difference expressed as a proportion of the lower duplicate value). The undigested dry matter values (g/kg DM, means of two duplicates) are also given in Table 2.8. Individual values for each replicate are given in appendix 4. The undigested dry matter values ranged from 161-229 g/kg DM.

Table 2.3: Physical data on the seventeen New Zealand barley samples, screenings and moisture.

Code	Grain Bulk Density (kg/hectolitre)	Screenings (%)	Moisture (%)	1000 seed weight (g)
B1	66.1	7.6	13.4	39.0
B2	64.7	11.6	12.0	37.9
B3	65.5	2.2	12.8	42.8
B4	62.4	1.2	12.8	49.8
B5	65.0	1.0	13.7	47.2
B6	68.3	3.0	14.1	49.6
B7	66.9	3.4	12.9	47.8
B8	69.0	1.6	13.7	49.2
B9	66.3	5.8	14.5	44.0
B10	62.9	4.6	16.2	47.6
B11	65.9	5.0	14.5	35.6
B12	65.0	8.0	12.3	39.2
B13	63.5	8.0	12.7	40.2
B14	65.5	7.8	12.5	42.6
B15	67.2	5.4	13.4	40.8
B16	64.8	5.8	12.0	42.8
B17	69.6	1.6	13.7	47.4
Ave	65.8	4.92	13.37	43.74
Max	69.6	11.6	16.3	49.8
Min	62.4	1.0	12.0	35.6
SE	0.48	0.73	0.26	1.09
CV (%)	2.7	6.1	8.15	10.3

Table 2.4: Crude Protein (CP, % dry matter) for the seventeen New Zealand barleys.

Code	CP
B1	11.03
B2	9.65
B3	10.82
B4	11.45
B5	10.53
B6	10.84
B7	8.48
B8	9.37
B9	8.87
B10	11.61
B11	10.92
B12	10.89
B13	13.28
B14	11.63
B15	12.70
B16	12.78
B17	12.21
Ave	11.0
Max	13.23
Min	8.48
SE	0.33
CV (%)	12.33

Table 2.5: Neutral Detergent fibre (NDF), Acid Detergent fibre (ADF) and lignin contents for seventeen New Zealand barleys.

Code	NDF (%)†	ADF (%)†	Lignin (%)†
B1	21.6	5.0	1.3
B2	22.7	5.7	1.7
B3	20.8	5.0	1.7
B4	20.6	4.6	1.8
B5	19.7	3.8	2.0
B6	17.9	4.6	1.0
B7	17.6	5.1	1.1
B8	18.3	5.4	1.4
B9	18.7	5.3	1.3
B10	18.8	5.5	1.3
B11	17.2	5.2	1.2
B12	17.8	5.0	1.2
B13	16.7	4.7	1.0
B14	17.1	4.1	1.0
B15	17.2	5.1	1.3
B16	16.1	4.0	1.0
B17	20.2	4.2	1.4
Average	18.8	4.8	1.3
Max	22.7	5.7	2.0
Min	16.1	3.8	1.0
SE	0.45	0.13	0.07
CV (%)	10.0	11.2	22.3

† dry matter basis.

Table 2.6: Total and gastro intestinal (GI)-extracted β-Glucan for ten New Zealand barleys.

Barley ID	Total β-glucan %†	GI β-glucan %†
B2	5.25	1.38
B4	5.2	0.63
B5	5.06	1.17
B7	4.56	1.96
B8	4.58	1.40
B10	5.15	2.12
B11	6.56	1.45
B14	3.93	1.42
B15	5.65	3.03
B17	6.31	2.14
Average	5.23	1.67
Max	6.56	3.03
Min	3.93	0.63
SE	0.25	0.21
CV	15.1	23.3

† dry matter basis.

Table 2.7: Mean (n=17) gross amino acid (AA) compositions (g/100g DM) for the seventeen New Zealand barleys.

AA	Mean	max	min	SD	CV (%)
asp	0.75	1.06	0.56	0.159	21.19
thr	0.42	0.60	0.32	0.093	21.91
ser	0.49	0.72	0.36	0.105	21.26
glu	2.97	5.22	1.95	0.830	27.97
pro	1.25	2.33	0.85	0.361	28.78
gly	0.54	0.77	0.40	0.114	21.27
ala	0.53	0.78	0.39	0.119	22.31
val	0.66	0.99	0.49	0.152	23.10
iso	0.45	0.71	0.32	0.111	24.64
leu	0.88	1.3	0.64	0.205	23.48
tyr	0.39	0.60	0.29	0.088	22.55
phe	0.65	1.12	0.45	0.177	27.21
his	0.29	0.45	0.20	0.077	26.71
lys	0.46	0.68	0.34	0.101	21.86
arg	0.65	0.95	0.49	0.153	23.63

Table 2.8: Mean† *in vitro* digestibilities (Nd_{vitro} %) for ten New Zealand barleys and undigested dry matter (UDM, g/kg DM).

Code	Nd_{vitro}%	UDM
B2	90.4	210
B4	92.1	181
B5	94.4	161
B7	90.3	180
B8	90.4	190
B10	86.8	210
B11	91.1	229
B12	90.8	210
B15	90.4	216
B17	90.4	199
Average	90.7	198.5
Maximum	94.4	229
Minimum	86.8	161
SE	0.59	6.46
CV (%)	2.06	11.3

† Means of two duplicates.

2.4 Discussion

The present study provides original data on the physical and chemical composition of barley grown and harvested in New Zealand. It also includes the application of a potentially valuable tool (the *in vitro* digestibility assay) for evaluating barleys.

The chemical analysis showed that the range in CP (8.5-13.3% DM) for the seventeen New Zealand barleys harvested in 1995 was not very wide. In general the mean determined AA concentrations were very close to values for barleys reported in the literature (CSIRO, 1987). The range in amino acid content of the barleys was greater than for crude protein. All coefficients of variation for AAs were in the order of 21-28% whereas the coefficient of variation for the CP was in the order of 12%. This implies that the composition of the protein and probably the protein fractions differed between barleys.

The levels of β-glucans in the New Zealand barleys were consistent with other values reported for barley. Fincher and Stone, (1986) reviewed the total β-glucan levels in malting barleys and reported quite a wide range (2.00-10.7%) in total β-glucans. Total β-glucan contents are known to be strongly influenced by the growing climate (Hesselman and Thomake, 1982), which explains such a range in concentrations. GI extracted β-Glucans showed a similar range to total β-Glucans (2.4% units and 2.63% units, respectively) but ranked the barleys differently. Given that the process of extraction of GI β-Glucans more accurately simulates the digestive process in a monogastric animal, it is expected that these measurements may be more useful in evaluating barleys.

The *in vitro* digestion was performed in duplicate and a reasonable level of agreement between duplicates was observed, although the level of precision in the present study was considerably lower than that reported by other workers (S. Boisen, Pers. Com.). Moreover , in the present study the opportunity was taken to have the same set of samples analysed in another laboratory (Foulum Research Institute, Denmark) by another operator. When this was done (S. Boisen, Pers. Com.) it was found that the Danish data were always lower than those observed at Massey University and ranked the barley samples differently. For the Massey University data set, the range for *in vitro* crude protein digestibility was 86.8% to 94.4% while the

corresponding range for the Foulum laboratory was 85.0% to 91.0% (refer appendix 3 for the full data sets). The mean Foulum value was 87.8% which was 2.9% lower than the mean Massey University value. That the two laboratories generated different absolute digestibility values was disconcerting and indicates that further development work is required to ensure assay robustness. Nevertheless, the assay was reasonably repeatable and is applied here (refer Chapter 4) to investigate its suitability for predicting crude protein digestion *in vivo*. The in vitro digestibility assay is relatively straightforward requiring no specialised laboratory equipment. From the start of the process until the end of filtration, one operator can easily process 10 samples in duplicate per run and there is opportunity for increasing the number of samples run concurrently. The technology is relatively inexpensive.

The present work has demonstrated that New Zealand barleys exhibit a wide degree of variation in physical and chemical attributes and that a recently developed *in vitro* digestibility assay (Boisen and Fernandez, 1995) may have promise for the rapid prediction of *in vivo* crude protein digestibility.

Chapter 3

A DESCRIPTION OF NEW ZEALAND BARLEYS: THE ILEAL DIGESTIBILITY OF PROTEIN AND AMINO ACIDS AS MEASURED USING AN *IN VIVO* LABORATORY RAT ASSAY

3.1 Introduction

The previous chapter provided a detailed description of the chemical and physical characteristics of New Zealand barleys (refer Chapter 2). However, it is not sufficient to simply measure the gross nutrient composition of a feed. Modern diet formulation instead requires digestibility values of the component nutrients. The aim of the presently reported study was to determine apparent and true ileal digestibility values for protein and amino acids in a range of barleys. Ileal digestibility values are preferable to faecal values to avoid the confounding effect of the microbial action in the hind gut, where the breakdown of AAs to non-utilisable products means that digestibility measured on the faeces will overestimate AA digestibility (Moughan *et al.*, 1987).

There are considerable quantities of endogenous amino acids in ileal digesta and these must be quantified to allow calculation of true AA digestibility. There are several methods available to allow measurement of endogenous amino acid flow (Boisen and Moughan, 1996). In the present work the homoarginine method (Hagemeister and Erbersdobler, 1985) was used to determine the endogenous ileal loss of lysine. Data are given for the true ileal digestibility of lysine.

Several studies have reported on the apparent ileal digestibility of barley protein (Sauer and Ozimek, 1986), however, no comprehensive study has been conducted on New Zealand barleys and data on true digestibility are sparse. In the present work the growing rat was used as a model animal for the pig for the digestibility determinations. It has been established previously (Moughan *et al.*, 1987) that the rat is a suitable model animal for determining ileal amino acid digestibility in barley for the growing pig. With the rat, ileal digesta can be readily obtained by sampling at slaughter, which precludes the need for surgical implantation of indwelling cannulas.

3.2 Materials and methods

3.2.1 Barley samples and the experimental diets

The barley samples used were those described in Chapter 2. Ten samples were selected as also described previously (refer Chapter 2). Each diet consisted of barley ground to less than 0.5 mm, an indigestible marker (Cr_2O_3) included at 0.5% (w/w), a vitamin premix (Technik Products, Auckland, 0.05%), a mineral premix (Technik Products, Auckland, 0.15%) and $CaPO_4$ (2.4%). Six of the ten barleys were treated with O-methylisourea (OMIU) to convert the lysine to homoarginine (refer 3.2.2). In the case of the guanidinated barleys, composite test diets were formulated. Each diet consisted of 2 parts of untreated barley, 1 part of treated barley, and 2 parts of a protein-free mix. The protein-free mix consisted of 40% cornstarch, 10% olive oil and 50% sucrose (w/w). Each composite diet also contained Cr_2O_3, vitamins, minerals and $CaPO_4$ as described above for the barley diets.

3.2.2 Guanidination of barley

Six of the finely ground (<0.5 mm) barley samples (B2, B4, B5, B7, B8 and B11) were subjected to guanidination. Approximately 1 litre of nanopure filtered H_2O was brought to 100°C and allowed to boil for 15 minutes to degas the water. BaOH (BDH 10048, 250g) was added and dissolved and the solution then added to 125 grams O-methylisourea (OMIU, Sigma No. M-8893) and mixed thoroughly. When cooled, the total solution was centrifuged to separate the $BaSO_4$ from the OMIU solution at 3,500 rpm for 15 minutes in 250 ml flasks in a Sorval RC-2B centrifuge, fitted with a GSA rotor. The OMIU solution was then adjusted to pH 10.6 with NaOH, and made up to exactly 1200 ml. Eighty grams of barley were added to the solution which was then stirred magnetically for at least four days, with constant adjustment to pH 10.6 with NaOH. As the incubation continued, the pH dropped rapidly at first, then more slowly demonstrating that the rate of conversion decreased with increasing incubation time.

The whole solution was then put in dialysis tubing with a molecular weight cut-off of 8,000 daltons (Spectra/Por® 1, Spectrum, USA). It was left to dialyse

against distilled H_20 for 8 days with regular changing of the distilled H_2O. The material was then freeze dried and reground (<0.5 mm).

3.2.3 Ileal amino acid digestibility for barley

Sixty Sprague-Dawley male rats were randomly assigned to ten treatment groups and to cages so as to negate any effect of position in the room. Rats were housed individually in wire bottomed cages with *ad libitum* access to fresh water. The air temperature was 22°C (± 2°C). Artificial light was provided in a twelve hour light/twelve hour dark cycle.

Rats were given free access to their barley diet from stainless steel feeders on an hourly basis between 0830 and 1630 hours. The rats had access to the food for ten minutes at each meal. The frequent feeding was to establish a constant flow of digesta in the gut. Rats were fed the diet for 15 days to accustom them to eating in the hourly pattern.

Rats were slaughtered on the 15th day by asphyxiation with CO_2 gas, and then decapitation. The animal's body cavity was opened and the final 15-20 cm of ileum was carefully removed. The ileum was flushed out with distilled H_20 (Butts *et al.*, 1992b) and the digesta were frozen before freeze drying. Digesta were analysed for chromium, nitrogen and amino acids. Apparent ileal digestibility of N (Nd_{app} %) was determined using the following equation:

$$Nd_{app}\ \% = \frac{\dfrac{\text{N in feed}}{\text{Cr in Feed}} - \dfrac{\text{N in digesta}}{\text{Cr in digesta}}}{\dfrac{\text{\% N in feed}}{\text{Cr in Feed}}} \times \frac{100}{1}$$

Where Cr and N are in % DM

Digestible protein (DP, g/kgDM) was calculated as:

$$DP\ \% = CP\ (g/kgDM)\ \text{in the feed}\ X\ \frac{Nd_{app}\%}{100}.$$

Similarly, the apparent ileal digestibility of specific AA's (AAd_{app} %) was calculated as:

$$AAd_{app}\ \% = \frac{\dfrac{\text{AA in feed}}{\text{Cr in Feed}} - \dfrac{\text{AA in digesta}}{\text{Cr in digesta}}}{\dfrac{\text{AA in feed}}{\text{Cr in Feed}}} \times \frac{100}{1}$$

where AA and Cr are in g/100g DM

3.2.4 Endogenous lysine flow

Endogenous ileal lysine flow was determined in rats using the guanidination method. It was planned that the rats (six groups each of six rats) receive a normal barley based diet for 14 days followed by one of the six guanidinated barleys (refer 3.2.2) on the final day (day 15). The feeding and digesta sampling procedures were as described in 3.2.3. However when presented the guanidinated barley the rats did not eat the material. It was necessary to dilute the guanidinated barley in order to entice the rats to eat. A serial dilution was practised with successively diluted diets being presented on consecutive days until the majority of rats were eating sufficient quantities of the guanidinated diet. The result of this process was that a number of rats were consuming guanidinated barley for an extended period of time, approaching ten days.

In the groups fed the homoarginine containing material, even after dilution around 40% of the rats still ate little or no food, and these were then fed the diet after it had been mixed with a small amount of peanut oil. The quantity of peanut oil was considered negligible, and not to materially change the chromium concentration. After

addition of peanut oil, most of the rats were consuming adequate amounts of food for successful sampling from the ileum. However some rats produced very little digesta, hence digestibilities in some cases were calculated on fewer than six rats.

Endogenous lysine flow ($ELF_{determined}$) at the terminal ileum was calculated using the following equations (Moughan and Rutherfurd, 1990):

$$ELF_{determined} = TLD\ (\%)\ X\ \frac{Diet\ L_{conc}}{100} - Diet\ L_{conc} + ileal\ lysine\ flow$$

Where:

$ELF_{determined}$	=	endogenous lysine flow (μg/g DMI)
TLD	=	true lysine digestibility (%)
Diet L_{conc}	=	Dietary lysine concentration (μg/g DM)
ileal lysine flow	=	μg/g DM

Lysine flow is given as:

$$Lysine\ flow = Lysine_{conc}\ digesta\ X\ \frac{Diet\ Cr_{conc}\ (mg/g\ DMI)}{Digesta\ Cr_{conc}\ (mg/g\ DMI)}$$

where lysine flow is in μg/g DMI, and $lysine_{conc}$ digesta is in μg/g DM.

Assuming *a priori* that HA and lysine are absorbed identically, then true lysine digestibility (TLD) was calculated using the following equation:

$$TLD\ (\%) = \frac{Diet\ HA\ conc - Ileal\ HA\ conc}{Diet\ HA\ conc}\ X\ \frac{100}{1}$$

where diet and ileal concentrations (conc) of HA are in μg/g DM.

Endogenous protein flow was determined from the calculated endogenous lysine flow using an estimate (Donkoh, 1993) of the ratio of protein to lysine in endogenous ileal digesta.

In theory, as there is no endogenous loss from glassware, *in vitro* protein digestibility should correspond to real protein digestibility. Therefore *in vitro* ("real") protein digestibility less the *in vivo* ("apparent") digestibility of protein should correspond to EPL. This was calculated for the six New Zealand barleys (using the *in vitro* digestibility results reported in the previous chapter) which had been guanidinated, as shown below:

$$EPL\ (g/kg\ DMI) = CP\ X\ (Nd_{vitro} - Nd_{vivo}) / 100$$

Where CP is the crude protein in the feed g/kgDM

Nd_{vitro} is the digestibility coefficient of protein *in vitro*

Nd_{vivo} is the apparent digestibility coefficient of protein *in vivo*

3.3Results

3.3.1 Apparent *in vivo* ileal amino acid digestibility

The apparent ileal digestibility of nitrogen (Nd_{app}%) results are summarised in Table 3.1 and apparent ileal amino acid digestibilities in Table 3.2. The range for N digestibility was quite low while the amino acid digestibilities had a wider range. The apparent AA digestibilities were highest for Proline and Glutamic acid.

3.3.2 True *in vivo* ileal lysine digestibility

The true (TLD %) and apparent ileal lysine digestibility (ApLD %) data for the six barleys which were subjected to guanidination are given in table 3.3. The ApLD (%) was considerably more variable in comparison to Nd_{app}% (refer table 3.1). The determined endogenous lysine losses at the terminal ileum of the rat are given in table 3.4.The range was very wide (152-1052 µg/g DMI).

An estimate of ileal endogenous total N excretion was made by multiplying the mean endogenous lysine losses (Table 3.4) by the ratio of N/lysine in endogenous ileal digesta (Donkoh, 1993). The latter estimates were used to calculate the true digestible total N contents for the barleys (Table 3.5).

An estimate of ileal endogenous protein excretion (Table 3.6) was made by multiplying the mean endogenous lysine losses (Table 3.4) by the ratio of N/lysine in endogenous ileal digesta (Donkoh, 1993) and then multiplying by 6.25.

Table 3.1: Apparent ileal digestibility of N in the growing rat given ten barley samples.

Code	Apparent digestibility (%)	Digestible protein (g/kg DM)
B2	78.15	75.41
B4	80.31	91.95
B5	79.25	83.45
B7	77.03	65.32
B8	75.49	70.73
B10	78.51	91.15
B11	77.80	84.96
B12	76.52	88.99
B15	71.37	90.64
B17	75.54	92.25
Ave	77.00	83.49
Max	80.31	91.95
Min	71.37	65.32
Overall SE of mean	0.624	

Table 3.2: Mean apparent ileal amino acid digestibility (%) for ten New Zealand barleys as determined with the growing rat.

	Mean	Max	Min	SE	CV(%)
Asp	67.2	84.0	56.2	2.52	11.9
Thr	66.9	83.4	51.4	2.63	12.4
Ser	72.3	86.8	59.4	2.32	10.3
Glu	87.4	93.8	73.4	1.82	6.78
Pro	84.3	93.1	71.8	1.93	7.06
Gly	49.7	70.9	20.9	4.28	27.2
Ala	74.6	86.0	61.4	2.35	10.1
Val	77.8	87.2	66.2	1.98	8.21
Iso	77.6	88.1	67.0	1.80	7.40
Leu	81.1	89.9	69.4	1.97	7.78
Tyr	74.9	87.1	65.1	1.88	7.87
Phe	80.63	89.4	67.0	1.86	11.6
His	74.7	90.2	57.7	2.77	8.27
Lys	75.1	86.0	63.9	2.10	8.67
Arg	80.1	89.5	69.4	2.01	7.34

Complete data are given in appendix 4.

Table 3.3: Mean apparent (ApLD, %) and true (TLD, %) ileal lysine digestibility determined using the homoarginine technique for six New Zealand barleys.

Diet	n	ApLD (%) (SE)	TLD (%) (SE)
B2	5	76.5 (3.12)	98.6 (1.41)
B4	6	66.2 (5.45)	88.4 (6.01)
B5	3	76.1 (1.99)	83.9 (3.56)
B7	3	24.0 (11.56)	100 (0)†
B8	3	23.8 (2.31)	100 (0)†
B11	4	45.1 (11.52)	73.2 (8.27)

† No homoarginine was detected in the ileal digesta.

Table 3.4: Determined mean (± SE) endogenous lysine losses (EL, µg/g DMI) in the growing rat fed barley based diets.

Code	EL (µg/g DMI)	n
B2	323 (45.6)	5
B4	389 (120.0)	6
B5	152 (80.3)	3
B7	936 (142.0)	3
B8	1052 (31.8)	3
B11	407 (122.5)	4

Table 3.5: Estimates of true digestible nitrogen (g/kg DM) contents for six New Zealand barleys.

Diet	Apparent digestible N (g/kg DM)†	True digestible N (g/kg DM)‡
B2	12.94	14.97
B4	15.77	18.23
B5	14.31	15.24
B7	11.20	17.81
B8	12.13	18.71
B11	14.57	17.14

† As determined *in vivo*.

‡ Calculated based on a correction factor for endogenous ileal N loss relative to endogenous lysine loss (Donkoh, 1993).

3.4 Discussion

Modern diet formulation requires accurate determination of digestible protein and amino acids. Information on these aspects of New Zealand barleys, a major feed ingredient for pigs in New Zealand, is lacking. It is now well established that diets should be characterised using "ileal" as opposed to the more traditional "faecal" amino acid digestibility. Furthermore, it is desirable to characterise diets and feed ingredients using "true" digestibility estimates, (Boisen and Moughan, 1996). To obtain true estimates of digestibility, however, requires that endogenous amino acid loss at the terminal ileum be measured.

Endogenous lysine flows (ELF) were determined in the present study by feeding rats diets containing guanidinated barley. The determined digestibility of homoarginine was used as an indicator for true lysine digestibility (TLD). Comparison of true and apparent digestibilities of lysine effectively allows determination of ELF.

There are several assumptions inherent in the homoarginine technique. It is assumed that the homoarginine labels protein evenly. However less than complete accessibility to lysine moieties by the OMIU may lead to the homoarginine not being evenly distributed. Secondly, it is assumed that the homoarginine is absorbed at the same rate as lysine. Schmitz *et al.* (1991) demonstrated this to be an accurate assumption. Thirdly it is assumed that homoarginine is not re-excreted into the gut lumen. This seems reasonable, given that Schmitz *et al.* (1991) demonstrated strong evidence in support of the non-returning nature of homoarginine (chapter 1.3). It appears therefore that homoarginine, although not universally accepted, is not an unreasonable choice of protein label. A further assumption is that homoarginine does not nullify or enhance the effects of ANFs. The latter effect should not be a major consideration for barley.

A major limitation with the approach is that it is difficult to entice animals to consume guanidinated material. Furthermore, it is an expensive process to guanidinate proteins, and high or complete conversion of lysine to homoarginine is difficult to achieve.

In the present study difficulty was experienced encouraging rats to eat the guanidinated material. Homoarginine has been shown to be toxic to animals (chapter 1.3) but there may be reasons other than toxicity for the feed refusals when a

guanidinated barley is fed as a sole ingredient. Such factors remain unclear, but presumably taste is important. The use of peanut oil in the present study greatly assisted inducing food intake. Low palatability, amino acid imbalance, or metabolic interference of lysine analogues could all contribute to low intakes (Moughan and Rutherfurd, 1991).

The apparent ileal digestibility coefficients for lysine in the guanidinated barleys were very low and generally much lower than their native barley counterparts. This may have been due to the rather low food intakes (refer Appendix 6) of the guanidinated barley-fed rats.

It has been suggested (Boisen and Fernandez, 1995) that the *in vitro* digestibility of protein less the *in vivo* apparent digestibility of protein corresponds to endogenous protein loss (EPL). The EPL calculated in this way using the data from the presently reported study did not show the same trends in EPL as those determined in the rats fed guanidinated barley (Table 3.6). The lack of agreement is possibly due to a shortfall in the *in vitro* procedure. The *in vitro* technique does not account for feed/gut interactions, and these are the most likely causes of the determined losses differing from the calculated losses.

Table 3.6: Endogenous ileal protein loss (EPL, g/kgDMI) determined using guanidination and that calculated based on *in vitro*/*in vivo* comparison.

Code	EPL †	$EPL_{determined}$ ‡ (g/kg DMI)
B2	11.82	13.69
B4	13.50	16.5
B5	15.95	6.44
B7	11.25	44.75
B8	13.97	44.63
B10	9.63	26.06

† Refer section 3.2.4

‡ Refer section 3.3.2

In spite of the low food intake and in some cases low recoveries of ileal digesta, the apparent and true digestibility coefficients for lysine determined in the present study appear sensible The calculated endogenous ileal lysine and endogenous ileal total N flows were similar to other mean values reported in the literature for growing rats receiving protein containing diets (Donkoh, 1993).

The apparent ileal digestibilities of amino acids for barley determined in the present study generally agree well with published values (Rhône Poulenc, 1989).

The *in vivo* technique employed in this chapter provides useful data on the apparent and true digestibility of protein and amino acids in New Zealand barley. The present *in vivo* data are used in the following chapter to develop mathematical relationships.

Chapter 4

A DESCRIPTION OF NEW ZEALAND BARLEYS: THE PREDICTION OF PROTEIN AND AMINO ACID DIGESTIBILITIES

4.1 Introduction

It is important to be able to accurately and routinely predict the *in vivo* apparent and true ileal digestibilities of protein and amino acids in feedstuffs for pigs. This offers the potential to minimise the need for expensive and time consuming animal digestibility trials. There are several possible approaches. Potential predictors of digestibility include various physical and chemical attributes of the feed (Chapter 2). Many physical and chemical factors are known to influence the apparent and true digestibility of protein and amino acids in feedstuffs for pigs. For example, a widely documented influence of a chemical characteristic is that of fibre content in the feed (Sauer and Ozimek, 1986). A further possibility in the prediction of *in vivo* apparent and true digestibilities of protein and amino acids in feedstuffs is the use of an *in vitro* digestibility technology. Yet another possibility is near infrared spectroscopy (NIRA).

One objective of the present study was to investigate the prediction of apparent and true ileal N digestibility based upon physical and chemical characteristics of barley. The physical measures included grain bulk density (kg/hectolitre), moisture content (%), screenings (%) and 1000 seed weight (g), all measured as described in Chapter 2.

A second objective was to investigate the predictive accuracy of an *in vitro* digestibility method. Many attempts to create an *in vitro* digestibility assay for protein and, or amino acids have been reported in the literature. (Boisen and Eggum 1991), covering a wide range of approaches. The *in vitro* protein digestibility assay used in the present work (Boisen and Fernandez, 1995, refer Chapter 2) was designed to allow an accurate prediction of protein and amino acid digestibilities in cereals, thus providing a relatively inexpensive and accurate way to grade and evaluate barleys based on their nutritive value. A rapid, inexpensive yet accurate method for predicting

ileal AA digestibility would be of considerable advantage to nutritionists, and others working in the monogastric livestock industry.

4.2 Materials and methods

4.2.1 Raw data

The *in vitro* protein digestibility data (mean of duplicate determinations) used herein are those reported in Chapter 2 of this dissertation. *In vivo* digestibility data for protein and amino acids used here are those presented and discussed in Chapter 3. Data on a range of physical and chemical characteristics are used in the present work and these are reported in Chapter 2.

4.2.2 Data analysis

1. Physical characteristics, and their relationships with the *in vivo* digestibility of N and the true digestibility of lysine.

Correlation analysis (Snedecor and Cochran, 1989) was performed to determine the degree of relationship between the *in vivo* digestibilities of N and true ileal lysine digestibility in barley and several physical characteristics.

2. Chemical characteristics, and their relationships with the *in vivo* digestibility of N and the true digestibility of lysine.

Correlation analysis (Snedecor and Cochran, 1989) was performed to determine the degree of relationship between the *in vivo* ileal digestibility of N and true ileal lysine digestibility and several chemical components in barley. Any characteristics showing a high degree of relationship with the digestibility of N or lysine were used in subsequent simple linear regression analysis to describe the form of the relationship.

3. *In vitro* nitrogen digestibility and its relationship with the *in vivo* digestibility of N and lysine.

Correlation (Snedecor and Cochran, 1989) was determined between *in vitro* estimates of protein digestibility and *in vivo* protein digestibility

The published methodology of Boisen and Fernandez (1995) to allow prediction of apparent ileal N digestibility included correcting *in vitro* N digestibility values for an endogenous loss estimate, based upon the *in vitro* undigested dry matter (UDM). In their original study (Boisen and Fernandez, 1995) endogenous protein loss ($EPL_{calculated}$) was found to be related to UDM (r^2 = 0.61, RSD = 4.5) for 17 single feedstuffs. This relation was then used to correct *in vitro* (real) protein digestibility values and arrive at *in vitro* apparent digestibility values, which were found to be highly related to $dN_{vivo}(\%)$ determined in pigs (r^2 =0.92). The same method was used in the present work.

In the present study it was also attempted to find other regression relationships that might accurately predict $Nd_{app}(\%)$ *in vivo*. Several multiple regressions (Snedecor and Cochran, 1989) were developed, which included several other chemical and physical characteristics of barley, and the significance of each predictor was determined by analysis of variance.

4.3 Results

1. Physical characteristics, and their relationships with the *in vivo* apparent digestibility of N and amino acids and the true digestibility of lysine.

Correlations between several physical characteristics and the *in vivo* apparent ileal digestibility of N, true lysine digestibility (TLD, %) and apparent ileal digestibility of essential amino acids are given in Table 4.1. Generally the correlations were low and not ($p>0.05$) statistically significant.

2. Chemical characteristics, and their relationships with the *in vivo* apparent digestibility of N and amino acids and the true digestibility of lysine.

Correlations between several chemical components, the *in vivo* apparent ileal digestibility of N, true lysine digestibility (TLD, %) and apparent ileal digestibility of the essential amino acids are given in Table 4.2. Several of the correlations were statistically significant ($p<0.05$).

Using chemical components that demonstrated a significant relationship (Table 4.2) the following regression equations were generated:

1. $Nd_{app}(\%) = 82.1 - 3.08\ G$

$r^2 = 0.67$ RSD=1.539

where G = GI extracted β-Glucans (%DM)

and $Nd_{app}(\%)$ = apparent (*in vivo*) digestibility of N (%)

2. TLD (%) = 304 - 17.3 CP - 29.2 G

r^2= 0.97 RSD=2.520

where CP = crude protein (%)

G = GI extracted β-Glucans (%DM)

3. TLD (%) = 157 - 12.8 TotGluc

r^2= 0.74 RSD=6.228

where TotGluc = Total β-Glucans (%DM)

Table 4.1: Correlation coefficients† between various physical characteristics (% DM) of New Zealand barleys and apparent ileal digestibility of N (Ndapp,) or true digestibility of lysine (TLD) or the apparent ileal digestibilities of the essential amino acids.

	Nd_{app} (%)	TLD (%)†‡	Apparent amino acid digestibility (%)								
			Arg	His	Iso	Leu	Lys	Met	Phe	Thr	Val
Grain bulk density (kg/hl)	-0.692	0.353	-0.047	-0.055	-0.082	-0.106	-0.193	-0.188	-0.111	-0.126	-0.040
Screenings (%)	-0.107	0.188	-0.141	-0.228	-0.262	-0.178	-0.055	-0.170	-0.251	0.015	-0.185
1000 seed weight (g)	0.213	0.438	0.391	0.197	0.323	0.0	-0.033	0.336	-0.342	-0.054	0.014

† based on 10 observation, all correlations were non significant ($p>0.05$).
‡ based on 6 observations.

Table 4.2: Correlation coefficients† between various chemical characteristics (% DM) of New Zealand barleys and apparent ileal digestibility of N (Ndapp,) or true digestibility of lysine (TLD) or the apparent ileal digestibilities of the essential amino acids.

	Nd_{app} (%)	TLD (%)‡	Apparent amino acid digestibility (%)								
			Arg	His	Iso	Leu	Lys	Met	Phe	Thr	Val
CP	-0.314	-0.765	0.267	0.246	0.085	0.047	0.209	0.134	-0.179	0.491	0.023
NDF	0.479	0.287	0.251	0.087	0.335	0.153	0.359	0.527	0.353	0.276	0.173
ADF	-0.104	0.409	-0.420	-0.037	-0.444	-0.270	-0.234	-0.226	-0.306	-0.361	-0.288
Lignin	0.482	-0.089	0.055	-0.121	0.094	-0.057	0.070	0.242	0.105	-0.005	0.058
Total β-Glucans	-0.104	-0.871*	0.009	0.342	0.199	0.417	0.506	0.253	0.111	0.479	-0.431
GI extracted β-Glucans	-0.815*	0.517	0.299	0.165	-0.236	-0.302	-0.149	-0.191	-0.378	0.111	-0.243

† based on 10 observations.=6

‡ based on 6 observations.

* $p<0.05$. Correlation coefficients without * were not significant ($p>0.05$).

3. *In vitro* total N digestibility and its relationship with the *in vivo* ileal digestibility of N, true ileal digestibility of lysine and endogenous protein loss.

The correlation (r = 0.23) between *in vitro* (real) protein digestibility and *in vivo* apparent ileal protein digestibility was not statistically significant. Further, the correlation between calculated endogenous ileal protein flow and undigested dry matter (*in vitro* assay) (r= -0.77) was not statistically significant. Therefore the correlation between *in vitro* apparent N digestibility (refer section 4.2) and *in vivo* apparent ileal N digestibility was not examined.

4.4 Discussion

Accurate, rapid and inexpensive prediction of protein digestibility and the digestibility of amino acids has been the aim of the feed industry for some time. The possibility of predicting these *in vivo* values from simple chemical or physical measurements offers exciting possibilities in the routine, cost effective evaluation of feedstuffs. In addition, *in vitro* prediction of *in vivo* digestibility of protein and amino acids may provide an ideal way to reduce the cost of feed evaluation and promote the wider practice of measuring and reporting digestible protein and amino acid values. The present work has investigated the mathematical relationships between physical, chemical and *in vitro* protein digestibility data on the one hand and *in vivo* ileal protein digestibility in the laboratory rat, on the other.

Correlation between *in vitro* (real) protein digestibility and *in vivo* apparent ileal protein digestibility was not high ($r = 0.233$). The lack of correlation may relate to differing levels of EPL from the various barley samples. Correction of *in vitro* (real) digestibilities to allow for EPL, would determine *in vitro* apparent digestibilities. Boisen and Fernandez, (1995) found $EPL_{calculated}$ to relate reasonably with UDM ($r^2 = 0.61$, RSD = 4.5) for 17 single feedstuffs. However, no such significant ($p<0.05$) relationship was found between UDM and $EPL_{calculated}$ (Table 3.6) was found in the present work. Full correlation analysis showed no other significant relationships between physical or chemical descriptors of the barley with $EPL_{calculated}$.

The prediction procedure of Boisen and Fernandez (1995) relies upon the assumption (*a priori*) that *in vitro* digestibility of protein accurately reflects *in vivo* true ileal protein digestibility values. Calculating expected EPL as *in vitro* less *in vivo* N digestibilities also relies upon the assumption that the *in vitro* figures accurately reflect real protein digestibility. However, this assumption is doubtful. The biological complexity of a gastro intestinal tract can not be fully replicated in a laboratory. The assumption that the true digestibility of protein is represented by the *in vitro* protein digestibility needs further testing. The results of the present work do not support the validity of this assumption.

Using the prediction procedure of Boisen and Fernandez (1995) did not result in accurate predictions of apparent ileal N or amino acid digestibility in the rat. Boisen and Fernandez (1995) found high prediction ability from equations generated with *in*

vitro data digestibilities. One possible reason that the predictive accuracy was not repeated in the present work is that Boisen and Fernandez used a wider range of *in vitro* digestible CP to generate their prediction equations.

It is concluded that the *in vitro* technique as applied in the present work is not suitable for prediction of apparent ileal digestibility of protein or amino acids within the single feedstuff, barley. However, given contradictory evidence between the present results and other findings (Boisen and Fernandez, 1995) further evaluation is required before any firm conclusions can be drawn.

Physical characteristics offered no predictive ability for apparent ileal digestibility of protein or amino acids. However, the β-Glucans offered some degree of prediction ability. The relationships between GI extracted β-Glucans and true lysine digestibility or apparent digestibility of N offer an advance in the search for fast accurate prediction of these parameters. Given that the *in vitro* protein digestibility assay investigated in the present work did not meet the objectives outlined earlier (refer Introduction), the relationships between GI extracted β-Glucans with true lysine digestibility or the apparent digestibility of N should be evaluated in more detail.

The results indicate that there is potential for the prediction of *in vivo* digestibility values from chemical measurements in some instances. Given the potential demonstrated in the present work, and the advantages that can be gained by industry, further investigation is warranted.

Chapter 5

GENERAL DISCUSSION

It is not enough to measure the gross nutrient composition of feedstuffs alone. Comprehensive analysis necessary for maximising utility of a given feedstuff should include a measure of the digestibility of nutrients. Protein digestibility in the growing pig is best determined at the terminal ileum, and ideally is expressed in terms of true digestibility, which accounts for endogenously excreted proteins. Due to the relatively high cost of protein digestibility studies *in vivo*, it is desirable to develop a more cost effective way of accurately determining protein digestibility in a feedstuff. Ideal methods might include prediction based upon physical or chemical measures, or the use of *in vitro* protein digestibility assays.

The primary purpose of the present work was the evaluation of a practical *in vitro* assay (Boisen and Fernandez, 1995) for the prediction of *in vivo* digestibilities of protein and amino acids in New Zealand barleys. The main objectives of an *in vitro* feed evaluation system are low cost, repeatability, and rapid, accurate determination of results. A secondary objective of the present study was the examination of physical or chemical measurements to predict *in vivo* digestibilities of protein and amino acids in New Zealand barleys.

In vivo ileal protein digestibility data were generated using the laboratory rat. This model animal has been demonstrated to furnish protein digestibility values that accurately reflect protein digestibility in the growing pig (Moughan *et al.*, 1984, Donkoh, 1993).

It was found that several physical measurements made on a range of barleys provided no significant ($p>0.05$) prediction ability for the apparent or true ileal digestibility of protein, apparent ileal amino acid digestibility, or ileal endogenous protein loss.

Chemical measurements furnished some strong relationships with these determinations, however. It was possible to predict the true lysine digestibility from the levels of gastro-intestinal extracted β-glucans and CP (r^2= 0.97, RSD=2.520). It

was also possible to predict the *in vivo* determined endogenous protein loss from the levels of total β-glucans ($r^2 = 0.77$, RSD =8.735).

With respect to the *in vitro* digestibility assay undertaken in the present work, the prediction of apparent ileal digestibility of N (%) and therefore, protein, was unsatisfactory in the present situation. This was probably due to a number of factors. One possible influencing factor is that the total undigested protein and large peptides in the *in vitro* situation were not accurately measured. Two possible sources of variation here are the precipitation and filtration effects. Other studies have highlighted the unreliability of some precipitating agents (Chapter 1). It is possible that sulphosalicylic acid is not a good precipitant. Comparative studies need to be undertaken under tightly controlled conditions in the context of the present *in vitro* method to establish the superiority or otherwise of sulphosalicylic acid as a precipitant, and furthermore appropriate concentration and reaction times of this acid. Until this question is resolved, this will always stand as an unknown source of variation and inaccuracy in the present methodology.

Secondly the filtration step may not be satisfactory. This seems to be the largest source of variation given that the undigested dry matter varies so widely after filtration. Other techniques such as dialysis or ultra-filtration may increase accuracy, but add to the complexity of the system.

Furthermore, the enzyme concentration is high, providing some 20% of the nitrogen in the *in vitro* preparation. The reduction of the enzyme concentration should be seriously investigated. If possible the enzyme concentration should be reduced, to minimise the confounding of errors associated with filtration of the blank.

The above reasons for concern are applicable not only to the estimation of apparent N digestibility, but also the true N digestibility (%). The digestibility range *in vitro* is tighter than the *in vivo* range, (6.3 % units, cf 8.94 % units) and the *in vitro* digestibilities were consistently higher than the *in vivo* digestibilities. This reflects the fact that the *in vitro* situation may provide optimum conditions, coupled with an enzyme concentration far in excess of normal biological levels. Hence most of the protein is digested It is assumed that the *in vitro* values also correspond to true digestibilities. The difference of *in vitro* and *in vivo* is assumed to correspond to endogenous protein loss from the animal, (Boisen and Fernandez, 1995). However these were not in accord with determined biological values in the rats. However, the

determined endogenous lysine losses in the rat using the homoarginine technique were highly correlated to predicted theoretical endogenous lysine losses (r = 0.998).

Of greater benefit yet would have been the accurate prediction ability of apparent amino acid digestibilities. Moreover, the accurate prediction of EAAL would have led to acceptable true digestibility prediction. Regrettably, neither was found in the present work. In the work of Boisen and Fernandez (1995) *in vitro* digestibility of amino acids was determined directly. In the present work however, it was not determined, rather it was attempted to relate *in vivo* digestibility of amino acids with in vitro digestibility of Nitrogen. This may account for the lack of correlation as the *in vivo* digestibilities of amino acids were apparent measures, and *in vitro* digestibility of nitrogen was a 'true' measure. It would be advantageous and informative to examine *in vitro* digestibility (%) of amino acids, but the experimental cost and effort is not justified without first resolving the above mentioned problem of poor apparent N digestibility (%) prediction ability, and the issue of repeatability.

Repeatability is a major issue with *in vitro* techniques and many will stand or fall on these grounds. In the present work, little correlation was demonstrated with the Denmark laboratory (r=0.114) although Massey between duplicate correlation was acceptable (r=0.728). Furthermore, there was no correlation between theoretical endogenous protein loss for the Massey analysis or the Denmark analysis (r= 0.087). This highlights the problems of repeatability outlined in Chapter 2.

Other predictors have been shown in the present work to be effective. For example, the most noteworthy is the ability of CP in barley to predict apparent digestible protein (r^2 = 0.915, RSD = 2.999).

The *in vitro* assay itself is very straightforward, and is reasonably precise with duplicate agreement being within 3% (expressed relative to the lowest duplicate value). It does not require any specialised equipment, other than that found in a well equipped nutrition laboratory. The relatively low cost is certainly more attractive than animal trials. However, in its present state, it is concluded that further developmental work is imperative before this particular *in vitro* protein digestibility assay can be applied routinely to New Zealand barleys.

The *in vitro* technique does not account for feed/gut interactions, and these are the most likely causes of the measured losses differing from the predicted losses encountered in the present work

Given that the range of apparent ileal protein digestibility in New Zealand barleys is low, and that the present *in vitro* protein digestibility values explain very little of the variation in the *in vivo* data, the present *in vitro* assay needs major developmental work for application to New Zealand barleys. In the mean time, chemical measures offer some degree of reliability that should be further evaluated.

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Appendix 1

Chemical reagents for the *in vitro* assay

All H_20 is distilled.

1 Acetone

2 Chloramphenicol (Sigma No. C-0378)

3 Ethanol 95%

4 Concentrated HCl (Merck no. 317)

5 $Na_2HPO_4.2H_20$ (Merck no. 6580)

6 $NaH_2PO_4.2H_20$ (Merck no. 6345)

7 Pancreatin (Porcine pancreas grade VI, Sigma no. p-1750)

8 Pepsin (2000 FIP U/g, Merck no. 1790)

9 Sulphosalicyl Acid (Merck no. 691)

10 Chloramphenicol solution (0.5%). 1 g chloramphenicol (2) is dissolved in 200 ml 95% ethanol (3).

11 Phosphate Buffer A, pH 6.0, (0.1M). 1.98 g $Na_2HPO_4.2H_20$ (5) and 29.44 $NaH_2PO_4.2H_20$ (6) are dissolved in 1.5 litres H_2O. pH is adjusted with 1 M HCl (4) or NaOH (13), then the solution is made up to 2.0 litres mls with H_2O.

12 Phosphate Buffer B, pH 6.8, (0.1M). 19.3 g $Na_2HPO_4.2H_20$ (5) and 45.48 $NaH_2PO_4.2H_20$ (6) are dissolved in 1.5 litres H_2O. pH is adjusted with 1 M HCl (4) (13) or NaOH, then the solution is made up to 2.0 litres mls with H_2O.

13 NaOH

14 NaOH solution (1.0M). 40 g of NaOH (13) is dissolved in H_20, and made up to 1 litre.

15 NaOH solution (0.6M). 24 g of NaOH (13) is dissolved in H_20, and made up to 1 litre.

16 Pancreatin Solution (0.05g/ml). 1.5 g pancreatin (7) are suspended in 30 mls of Phosphate buffer B (12), by 15 mins of magnetic stirring. The sediments are removed by centrifugation, (5 mins at 3000 rpm).

17 Pepsin Solution (0.01 g/ml). 0.3 g pepsin (8) is dissolved in 30 mls 0.2 M HCl (18).

18 HCl (0.2 M). 83.5 ml concentrated HCl (4) is made up to 5.0 litres with H_20.

19 HCl (1.0 M). 83.5 ml concentrated HCl (4) is made up to 1.0 litre with H_20.

20 Sulphosalicyl acid (20%). 200 g of sulphosalicyl acid (9) is diluted to 1.0 litre with H_20.

21 Sulphosalicyl acid (1%). 100mls of 20% sulphosalicyl acid is diluted to 2.0 litre with H_20.

Appendix 2

Table A.1:Apparent Digestibilities of AA in ten New Zealand Barleys.

	B2	B4	B5	B7	B8	B10	B11	B14	B15	B17
n		5	6	4	6					
Asp	0.65	0.66	0.66	0.67	0.56	0.73	0.73	0.62	0.59	0.84
Thr	0.67	0.66	0.68	0.63	0.51	0.74	0.69	0.68	0.61	0.83
Ser	0.71	0.73	0.74	0.73	0.59	0.78	0.69	0.73	0.65	0.87
Glu	0.87	0.88	0.90	0.89	0.83	0.90	0.92	0.88	0.73	0.94
Pro	0.83	0.86	0.88	0.88	0.79	0.88	0.84	0.85	0.71	0.93
Gly	0.42	0.53	0.50	0.58	0.21	0.54	0.62	0.46	0.42	0.71
Ala	0.72	0.74	0.73	0.71	0.61	0.78	0.83	0.72	0.65	0.86
Val	0.76	0.78	0.79	0.80	0.70	0.80	0.85	0.77	0.66	0.88
Iso	0.76	0.79	0.80	0.79	0.71	0.81	0.77	0.78	0.67	0.88
Leu	0.79	0.82	0.82	0.82	0.74	0.84	0.89	0.81	0.69	0.90
Tyr	0.75	0.74	0.77	0.71	0.69	0.79	0.75	0.76	0.65	0.87
Phe	0.80	0.82	0.83	0.84	0.76	0.84	0.81	0.80	0.67	0.89
His	0.69	0.75	0.76	0.82	0.58	0.81	0.75	0.73	0.68	0.90
Lys	0.76	0.76	0.76	0.74	0.64	0.80	0.80	0.73	0.65	0.86
Arg	0.79	0.82	0.82	0.86	0.69	0.83	0.70	0.80	0.81	0.90

Appendix 3

Table A.2: *In vitro* digestibilities (Nd_{vitro} %) of the two replicates for ten New Zealand barleys determined in both in the present work (*in vitro* 1 and *in vitro* 2), and the Denmark determination (S. Boisen, Pers. Com.) of the same material (*in vitro* Denmark 1 and *in vitro* Denmark 2).

Code	*In Vitro* 1 (Nd_{vitro}%)	*In Vitro* 2 (Nd_{vitro}%)	*In vitro* Denmark (Nd_{vitro}%)	*In vitro* Denmark 2 (Nd_{vitro}%)
B2	90.5	90.3	85.2	84.8
B4	90.2	94.0	88.3	87.5
B5	93.0	95.8	88.4	88.8
B7	89.7	90.9	87.2	87.5
B8	87.6	93.2	86.6	-
B10	-	86.8	88.1	87.4
B11	90.2	92.0	88.3	87.0
B12	90.0	91.5	87.1	87.0
B15	89.7	91.0	91.2	90.8
B17	89.5	91.3	89.1	89.2
Average	97.4	91.7	88.0	88.3
Maximum	93.0	95.8	91.2	90.8
Minimum	87.6	86.8	81.2	84.8
SD	4.60	2.40	1.60	1.68
CV	4.72	2.62	1.82	1.90

Appendix 4

Table A.3: UDM (g/kg DM) for the present work (InVit1, InVit2), and the two *in vitro* replicates in Denmark (IVD, S. Boisen, Pers. Com.). The two *in vitro* analyses in Denmark had the same UDM.

Barley ID	InVit1	InVit2	IVD
B2	214	206	215
B4	181	181	187
B5	167	155	181
B7	184	175	180
B8	218	162	199
B10		210	211
B11	213	245	217
B14	216	204	211
B15	221	210	212
B17	207	191	219
Ave	202.5	194	203.2
Max	221	246	219
Min	167	155	180
SD	19.68	26.91	15.24
CV	9.78	13.87	7.50

Appendix 5

Table A.4: Apparent Digestibilities of AA in ten New Zealand Barleys.

	B2	B4	B5	B7	B8	B10	B11	B14	B15	B17
n		5	6	4	6					
Asp	0.65	0.66	0.66	0.67	0.56	0.73	0.73	0.62	0.59	0.84
Thr	0.67	0.66	0.68	0.63	0.51	0.74	0.69	0.68	0.61	0.83
Ser	0.71	0.73	0.74	0.73	0.59	0.78	0.69	0.73	0.65	0.87
Glu	0.87	0.88	0.90	0.89	0.83	0.90	0.92	0.88	0.73	0.94
Pro	0.83	0.86	0.88	0.88	0.79	0.88	0.84	0.85	0.71	0.93
Gly	0.42	0.53	0.50	0.58	0.21	0.54	0.62	0.46	0.42	0.71
Ala	0.72	0.74	0.73	0.71	0.61	0.78	0.83	0.72	0.65	0.86
Val	0.76	0.78	0.79	0.80	0.70	0.80	0.85	0.77	0.66	0.88
Iso	0.76	0.79	0.80	0.79	0.71	0.81	0.77	0.78	0.67	0.88
Leu	0.79	0.82	0.82	0.82	0.74	0.84	0.89	0.81	0.69	0.90
Tyr	0.75	0.74	0.77	0.71	0.69	0.79	0.75	0.76	0.65	0.87
Phe	0.80	0.82	0.83	0.84	0.76	0.84	0.81	0.80	0.67	0.89
His	0.69	0.75	0.76	0.82	0.58	0.81	0.75	0.73	0.68	0.90
Lys	0.76	0.76	0.76	0.74	0.64	0.80	0.80	0.73	0.65	0.86
Arg	0.79	0.82	0.82	0.86	0.69	0.83	0.70	0.80	0.81	0.90

Appendix 6

Table A5: Voluntary feed intakes of the rats in the study to determine endogenous lysine loss (grams VFI, on day of slaughter).

Code	Average	SE
Native B2	6.0	1.08
Native B4	7.9	0.14
Native B5	7.8	1.67
Native B7	0.2	0.03
Native B11	7.9	1.60
Native B8	5.7	1.49
Guanidinated B2	3.8	0.88
Guanidinated B4	2.3	0.84
Guanidinated B5	8.7	0.62
Guanidinated B7	1.8	0.55
Guanidinated B11	2.4	0.97
Guanidinated B8	4.1	1.06