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**THE EFFECT OF ENZYMATIC HYDROLYSIS OF A  
DIETARY PROTEIN ON THE EXCRETION OF  
URINARY NITROGEN METABOLITES**

**A thesis submitted in partial fulfilment of the requirements for the  
degree of Master of Science in Nutritional Science at Massey  
University, Palmerston North, New Zealand.**

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**2000**

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**TO TERESA CINTORA  
AND  
ANTONIO QUINTINO,  
  
MY PARENTS**

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## ABSTRACT

Hydrolysed milk proteins are used for many purposes in human nutrition. Although it is assumed that the nutritive value of a protein hydrolysate is the same, or even superior to the corresponding intact protein, there is limited research available to support this assumption.

The aim of this study was to compare amino acid utilisation and the pattern of excretion in the urine of the nitrogenous metabolites (urea, ammonia and creatinine) as an immediate response to the ingestion of a meal containing an intact protein or its enzymatic hydrolysate. This involved a novel technique, 'acute urine collection' (AUC), in which urine was drained from the bladder at short time periods (30 min to 2 hr) through a catheter.

The performance and nitrogen balance results indicated that the two sources of amino acid were equally effective in supporting nitrogen retention and growth of the pigs. Nevertheless, the pattern of excretion of the metabolites of nitrogen digestion suggested important differences in the metabolism of the pigs on the two diets.

Both groups of pig excreted creatinine nitrogen, at constant and comparable rates over the sampling period indicating similar rates of catabolism in the muscle. The total excretion of nitrogen by AUC by the two groups was similar but the pattern of excretion over the day differed which indicated a difference in the metabolism of the amino acids in the diets. This may have been in part due to a more rapid absorption of amino acids from the hydrolysed diet and in part due to a higher rate of glutamine and asparagine breakdown in the gut of pigs fed the hydrolysate.

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Excretion of nitrogen as urea and ammonia was similar for the two groups but there were differences between the groups in the pattern of excretion of these metabolites. In addition, the excretion of ammonia was significantly lower ( $P < 0.0001$ ) in the pigs fed the hydrolysate. This was due to a higher content of fixed cations in the diet containing the hydrolysate that led to a compensatory reduction in ammonia excretion. There was a proportional increase in the excretion of urea in the pigs on the hydrolysed diet as a result of the reduction in ammonia excretion but the differences were small relative to the total urea excretion and not significant.

AUC not only gives comparable information to the nitrogen balance if it is carried out over a 24 hr period but it also provides detailed information about the protein utilisation during the immediate postprandial period. In particular, AUC can indicate differences and/or similarities in protein absorption by allowing the observation of the pattern of production of urea directly related to the catabolism of dietary amino acids. In addition, it may be possible to use this technique to estimate the optimum time between meals.

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## LIST OF ABBREVIATIONS

ADG	Average Daily Gain
AT/TN	Amino acid nitrogen in the hydrolysate relative to the total amount of nitrogen in the substrate
AOAC	Association of Official Analytical Chemistry
APU	Animal Physiology Unit
AUC	Acute Urine Collection
D	Daltons
DEB	Dietary Electrolyte Balance
DH	Degree of Hydrolysis
GLM	the general linear method procedure
HP	Diet containing hydrolysed protein
IFNHH	Institute of Food Nutrition and Human Health
IP	Diet containing intact protein
MBW	Metabolic Body Weight
MW	Molecular Weight
N	Nitrogen
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NB	Nitrogen Balance
PDR	Protein Deposition Ratio
UTI	Urinary Tract Infections

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## INTRODUCTION

Proteins are vital molecules for life. Their importance has been recognised since the 19th century, when they were called the 'primary material of life'. Even though protein structure is built using only 20 different amino acids, the different arrangement and repetition in the chain make it possible to have thousands of different proteins performing unique and specific functions.

The human being requires a constant supply of protein in the diet for maintaining protein homeostasis in the body. The body of a normal 70 kg man contains about 11 kg of protein (Forbes, 1987). This protein mass contains thousands of different proteins each of different weight and chemical composition and each one with a definite function. Some provide the structure for cells and organs; others control the speed of biochemical reactions, and others control intracellular and intercellular communication. Although the total mass of protein in the body is relatively stable in the adult, proteins are continually degraded into their constituent amino acids. In order to keep the protein mass constant, new proteins must be synthesised to replace the degraded ones. About 0.3 kg of protein are degraded and replaced each day in a normal 70 kg man (Morais *et al.*, 1997). Synchronised degradation and synthesis of protein, referred to as 'protein turnover', consumes approximately 20% of the energy consumption of a person at rest after an overnight fast (Welle and Nair, 1990). Most of this energy is used for protein synthesis rather than degradation (Welle, 1999). The rapid alteration of the concentration of certain enzymes, such as occurs after ingestion of a meal, alcohol or stress and the replacement of altered proteins from oxidation,

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glycation, racemisation and isomerisation consumes much more energy than cell replication and growth (Berneis, 1997; Boirie *et al.*, 1997; Welle, 1999).

Under normal conditions, an individual can maintain the turnover of his/her body protein by eating a diet containing an adequate quantity and balance of proteins. The normal physiological processes of digestion and absorption will ensure the dietary amino acids reach the sites of protein metabolism. Under some circumstances, however, dietary proteins are unable to meet the amino acid requirements of the individual or whole dietary proteins are not tolerated. For example, an insufficient gut absorptive surface, inefficient function of some gastrointestinal organs, degenerated digestive and absorptive function, may all lead to insufficient absorption of amino acids from the gastrointestinal tract and therefore malnutrition. Alternatively, activation of allergic reactions to dietary proteins can induce life threatening anaphylactic reactions. Many of these problems may be avoided by replacing the protein in the diet with partially digested proteins that are readily digested and have low allergenicity.

Given the nutritional dependence that consumers, such as allergenic infants, and patients with pancreatic and Crohn's disease, have on pre-digested proteins, it is crucial to assess their nutritional value in comparison with the intact protein. Even though several studies have compared the nutritional value of intact proteins and their hydrolysates, there is still much controversy arising from the methods used in the comparisons. The objective of the present study was to compare both the immediate and long-term responses of pigs to a milk protein that was fed intact or hydrolysed. The response variables measured included the weight gain of the pigs, nitrogen balance and the pattern of excretion of nitrogenous compounds in the urine.

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# CHAPTER 1

## LITERATURE REVIEW

The present review is concerned with protein hydrolysates obtained enzymatically as a technological approach to fulfil consumers' nutritive needs. The review provides background information on the manufacture, properties and uses of protein hydrolysates. It focuses on assessment of the nutritional value of protein hydrolysates and the use of the pig as an animal model for human nutritional studies.

### 1.1 Protein Hydrolysates; Properties and Processing

Protein hydrolysates are mixtures of polypeptides, peptides and amino acids obtained by hydrolysis of the peptide bonds in the original protein. The extent of the hydrolytic process characterises hydrolysates as extensively or partially hydrolysed products. The former usually consist of free amino acids and very short peptides (di- and tri-peptides) while the latter usually have a wide range of molecular weights. Partially hydrolysed proteins contain small amounts of free amino acids and short peptides, a broad spectrum of medium sized peptides, and considerable amounts of high molecular-weight fractions (Mahmoud, 1994).

Hydrolysis confers new functional characteristics on the protein product that are commercially attractive (Adler-Nissen, 1986). Hydrolysis changes physicochemical characteristics such as solubility and emulsifying capacity (Turgeon *et al.*, 1992; Gauthier *et al.*, 1993). Furthermore it can improve the sensorial properties of the protein, imparting texture and removing off-flavours and odours. Hydrolysis may also

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improve the safety of some proteins by removing toxic or inhibitory constituents (Lahl and Grindstaff, 1989; Feeney, 1986; both cited by Lahl and Braun, 1994). By modifying the characteristics of the intact protein through hydrolysis, it is possible to fulfil the needs of specific consumers. Perhaps one of the most specific reasons to hydrolyse dietary proteins, especially those from cow's milk, is to decrease their allergenicity (Lee, 1992; Blecker, 1997).

Milk protein hydrolysates have also been used for the nutrition of persons with special protein needs such as those under medical care, the elderly and athletes. The specific use depends on the characteristics acquired during processing of the hydrolysate. A brief description of the processes used in the production of hydrolysed milk proteins follows.

### **1.1.1 Manufacture of Milk Protein Hydrolysates**

Concentrates and isolated milk proteins are the base materials for manufacturing milk protein hydrolysates (MPH). Milk protein hydrolysates are produced mainly from protein mixtures, such as whey protein concentrates (WPC), whey protein isolates (WPI), total milk protein concentrates (TMPC), and casein concentrates (CC) (Giese, 1994).

Whey protein concentrates are extracted from cheese and casein wheys and contain 35% to 85% protein on a dry matter basis (Bylund, 1995). Whey protein isolates contain 90% (or higher) protein (Giese, 1994). Casein concentrates are protein extracts made from skim milk by isoelectric or enzymatic destabilisation of the casein (Swaisgood, 1996). Casein concentrates are usually referred to as 'acid casein, lactic casein or rennet casein', depending on the agent used during extraction (Giese, 1994; Swaisgood, 1996). Total milk protein concentrates are manufactured by

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precipitation of both casein and whey proteins from skim milk. These concentrates are the raw material for the manufacture of total milk protein hydrolysates (Giese, 1994).

#### 1.1.1.1 Enzymatic hydrolysis

Peptide bonds between the amino acids in proteins can be broken either by physicochemical or biological procedures. Acids, alkalis or enzymes can reduce proteins to peptides or amino acids. The specificity and efficiency of the hydrolysis determine the nutritional quality of the hydrolysate (Keohane *et al.*, 1985). Procedures based on enzymes are most often used to yield hydrolysates for nutritional purposes (Adler-Nissen, 1986; Lahl and Braun, 1994).

Peptide hydrolases are functional proteins, or enzymes, that act on specific peptide bonds causing the release of proteoses, peptones, peptides and or amino acids from the protein chain. Proteases are peptide hydrolases that have endo-peptidolytic activity, splitting peptide bonds inside a polypeptide protein chain. Peptide hydrolases having exo-peptidolytic activity cleave peptide bonds either at the N-terminal (amino peptidases) of the polypeptide or at the C-terminal end (carboxypeptidases) (Stauffer, 1989). A mixture of enzymes with exo- and endo-peptidolytic activity is often used for the manufacture of casein hydrolysates for nutritional purposes (Lahl and Braun, 1994). Table 1 lists sources of proteolytic enzymes available, along with their common names and the pH range at which they are normally active.

Commercial proteases differ in their specificity. For instance pancreatin is a mixture of trypsin and chymotrypsin in which the former enzyme specifically acts on the carboxyl side of lysine and arginine residues, and the latter enzyme requires an aromatic amino acid (tyrosine, tryptophan or phenylalanine) or bulky non-polar side chain with an amino acid such as methionine (Stryer, 1988). Papain, ficin and

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bromelain are sulfhydryl proteases with hydrolytic function similar to each other but lower specificity than pancreatin (Lahl and Braun, 1994). Papain can split a variety of bonds because of its seven active subsites, which can attack several amino acid arrangements in the substrate. For this reason, papain specificity is determined by the adjacent amino acids rather than by the nature of the side chain of the amino acid (DeMan, 1999). Even though their specificity is broad (DeMan, 1999), microbial enzymes such as those obtained from *Bacillus licheniformis* are more specific than those from papain, ficin and bromelain (Lahl and Braun, 1994) and have some preference for terminal hydrophobic amino acids (DeMan, 1999).

Table 1. Enzymes available for food protein hydrolysis<sup>1</sup>

Source	Type	Common name	pH range
Ox, pig	Aspartic protease	Pepsin	1-4
	Serine protease	Trypsin	7-9
	Serine protease	Pancreatin	7-9
Papaya fruit	Cysteine protease	Papain	5-7
Pineapple fruit and stems	Cysteine protease	Bromelain	5-8
Kiwifruit	Cysteine protease	Actinidin	6-9
<i>Bacillus subtilis</i>	Metalloprotease	Neutrase	6-8
	Serine protease		
<i>Bacillus licheniformis</i>	Serine protease	Subtilisin Carlsberg Alcalase	6-10

<sup>1</sup> From Adler-Nissen, 1986

The formation of compounds of lower molecular weight, such as proteoses, peptones, peptide/amino acid mixtures increases with time of hydrolysis. The extent of hydrolysis in a hydrolysate is measured by the degree of hydrolysis (DH) (Adler-Nissen, 1986). The DH is usually expressed as the percentage of the peptide bonds cleaved or as the ratio of the amount of amino nitrogen present in the hydrolysate (AT) relative to the total amount of nitrogen present in the substrate (TN). Highly hydrolysed milk protein hydrolysates have an AT/TN ratio of 0.50 or greater (Lahl and Braun, 1994). An AN/TN ratio of 0.35 or lower is characteristic of those hydrolysates produced with pure enzymes (Lahl and Braun, 1994). Partially hydrolysed proteins (PHPs) were defined in the Food Chemical Codex as those having AN/TN ratios from 0.02 to 0.67

(National Academy of Sciences, Committee on Food Chemicals Food Codex, 1996). Table 2 shows the molecular weight of milk protein and its hydrolysates at distinct AN/TN ratios.

Table 2. Hydrolysates from casein and whey protein concentrate (WPC)<sup>1</sup>

Hydrolysate <sup>2</sup>	Protein source	Average M.W. <sup>3</sup> (Daltons)	AN/TN <sup>3,4</sup>
Intact protein	Casein	28,500	0.07
	WPC	25,000	0.06
Proteose	Casein	6,000	0.13
	WPC	6,800	0.11
Peptone	Casein	2,000	0.24
	WPC	1,400	0.24
Peptides	Casein	400	0.48
	WPC	375	0.43
Peptides and free amino acids	Casein	260	0.55
	WPC	275	0.58

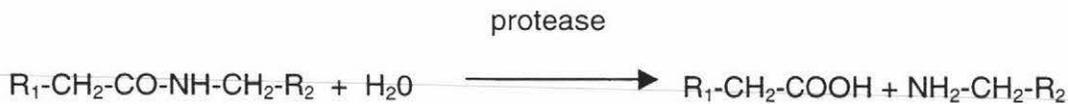
<sup>1</sup> From Lahl and Braun, 1994

<sup>2</sup> Commercial hydrolysates produced by Deltown Specialties, Fraser, NY.

<sup>3</sup> Molecular weight determined by reverse phase HPLC.

<sup>4</sup> AN/TN = ratio of amino nitrogen present in the hydrolysate relative to the total amount of nitrogen present in the substrate.

When a protein is hydrolysed enzymatically, the peptide bonds are cleaved and water is incorporated into the resulting peptides (see equation below). Hydrolysis is favoured, therefore, by a high concentration of water (Adler-Nissen, 1986).



During hydrolysis, the degree of ionisation of the carboxyl and amino groups will depend on the pH of the reaction mixture (Adler-Nissen, 1986). Below pH 2 carboxyl groups are undissociated. As the pH increases, however, they start to dissociate so that at pH 5, carboxyl groups are completely dissociated. Amino groups are completely protonated below pH 6. As the pH increases amino groups start to lose protons: they are completely dissociated above pH 9.5 (Adler-Nissen, 1986).

The hydrolysis of a protein releases  $H^+$  or consumes  $H^+$  ions, depending on the pH of the reaction mixture. The pH of the reaction is selected according to the enzyme activity. For example, if the reaction is performed at pH 8, as more peptide bonds are cleaved, more  $H^+$  will be released into the reaction mixture. As a consequence, pH will decrease. Because pH must be maintained for the optimum enzyme action, NaOH is added. The NaOH equivalents (or HCl in the case of  $H^+$  uptake) used to re-establish the pH are proportional to the number of peptide bonds cleaved, and are an indicator of DH. This is the principle behind the pH-stat technique for continuously monitoring the DH (Adler-Nissen, 1986).

#### 1.1.1.2 Control of enzymatic hydrolysis

Temperature, pH, substrate concentration, enzyme/substrate ratio, time of hydrolysis and AN/TN ratio are the most crucial parameters to monitor in the enzymatic process for producing hydrolysates. Adler-Nissen (1982) reported that DH is the most practical and convenient measure of the course of the enzymatic degradation of a protein. He demonstrated that except for pH, parameters such as substrate concentration, enzyme/substrate ratio, temperature and time could be replaced by DH.

Lanh and Braun (1994) recently reviewed the control of the hydrolysis of proteins. They proposed that not only the AN/TN ratio but also temperature, pH and time of hydrolysis are important parameters for controlling an enzymatic reaction. They added that by manipulating these parameters, it is possible to obtain hydrolysates with a specific amino acid distribution, molecular weight distribution, and residual intact protein.

Lanh and Braun (1994) also point out that in the production of hydrolysates, processing temperature and pH are usually set to maximise the rate of the hydrolytic

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reaction. A temperature of 60 °C and a pH between 8 - 10 are optimum for hydrolysing whey proteins with a mixture of bacterial and animal enzymes (Camacho *et al.*, 1998). From their experience, Lanh and Braun (1994) indicated that a pH around 7.0 is best when pancreatin is used for hydrolysis.

The time of hydrolysis and the AN/TN ratio are directly related. As the time of hydrolysis is increased, the proportion of amino nitrogen present increases relative to the total amount of nitrogen in the hydrolysate (Lanh and Braun, 1994).

It is important in the production of hydrolysates to control their bitterness. Bitterness has been attributed to peptides released by hydrolysis that contain hydrophobic amino acids (Ney, 1971 cited by Pedersen, 1994). Bitterness is a sensation attributed to chemical compounds that have hydrophobic and hydrophilic regions spaced 0.3 nm apart (Belitz *et al.*, 1979). Therefore, the hydrophobic L-amino acids and the peptides containing them are associated with this problem (Belitz *et al.*, 1979). In an intact globular protein, the hydrophobic side-chains are in the molecule interior, and therefore presumably unable to interact with the taste buds (Matoba and Hata, 1972, cited by Adler-Nissen, 1979). As the hydrolysis continues, hydrophobic residues are gradually exposed, and the bitter taste becomes detectable (Adler-Nissen, 1979). However, the complete hydrolysis of protein will yield a mixture of amino acids with lower bitterness than the hydrophobic peptides (Wieser and Belitz, 1976).

Because the enzyme determines the cleavage site, several commercial enzymes such as Alcalase® (Novo Laboratories, Inc., Danbury, Conn, USA), Papain W-40 (Amano Pharm. Co. Ltd., Nagoya, Japan) and Proleather® (Amano Intern. Enzymes Co., Troy, Va, USA) have been introduced to yield peptides with hydrophobic

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amino acids at the carboxylic end or amino end of the peptide to decrease the bitterness of the hydrolysates (Lahl and Braun, 1994; Pedersen, 1994).

Bitterness can also be controlled by restriction of the DH and hydrolysates with DH 3% - 5% have low bitterness (Adler-Nissen, 1984). Furthermore, when proteins need to be extensively hydrolysed, a product with reduced bitterness can be achieved by iso-electric precipitation of bitter peptides (Adler-Nissen, 1984).

The solubility of the hydrolysates determines their biological utilisation and commercial value. Because the solubility of the product increases with the degree of hydrolysis, AN/TN ratios are monitored during the process (Mahmoud, 1994). Undigested proteins and the hydrolytic enzymes, which reduce the solubility of the product are separated from the hydrolysate by filtration (Lahl and Braun, 1994).

When the desired compositional characteristics have been achieved, the hydrolytic reaction must be stopped. The enzymatic reaction is usually ended by adjusting the pH of the reaction mixture and heating it (Adler-Nissen, 1986; Lahl and Braun, 1994). The pH and temperature are regulated simultaneously, so that the enzyme can be stopped with a small temperature increase. This procedure reduces the damage to the product (Lahl and Braun, 1994). An alternative method of inactivation is with acid alone but it is not often used in the commercial production of hydrolysates (Lahl and Braun, 1994). When a large amount of acid is added the subsequent neutralisation leads to formation of excessive amounts of salt in the hydrolysate that has to be removed (Adler-Nissen, 1986; Lahl and Braun, 1994). High temperature inactivation can diminish the nutritional value of the hydrolysate either by destroying nutritional compounds or decreasing their availability (Lahl and Braun, 1994). Temperature extremes can also cause the formation of Maillard compounds that decrease the nutritive value of the hydrolysate. Finally, hydrolysis can be

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terminated by the removal of the enzymes by filtration with either diatomaceous earth, fibreglass depth-type, micro-porous or ultra-filtration membranes (Jelen, 1991; Lahl and Braun, 1994).

Processing after enzymatic hydrolysis can change various characteristics of the hydrolysates including the flavour, colour, amino acid profile, molecular weight distribution and the nutritional value (Lahl and Braun, 1994).

Charcoal treatment is used to remove higher molecular weight peptides, undigested protein fragments (Alder-Nissen, 1986) and potential antigenic materials from the hydrolysate after hydrolysis (Lahl and Braun, 1994). This process also removes any off-colour material, slightly off-odours and slightly bitter tastes. Although the active carbon is a non-selective absorbent, no significant removal of desirable free amino acids has been reported (Lahl and Braun, 1994).

Filtration is used to clarify hydrolysates of insoluble substrate fragments and residual enzyme. Although an array of filtering systems have been used, ultrafiltration membranes are preferred for their high yields and separation capacities (Anonymous, 1981 cited by Adler-Nissen, 1986). Ultrafiltration is used in the production of nutritional protein hydrolysates (Lahl and Braun, 1994). Ultrafiltration membranes, in addition to the removal of the proteases, yield a product with a normal distribution of amino acids and short peptides from non-highly hydrolysed material without extensive hydrolysis of the protein (Halken *et al.*, 1993). Ultrafilters, by removing compounds with molecular weights in excess of 5,000 to 10,000 Daltons, also remove highly antigenic compounds (Halken *et al.*, 1993; Lahl and Braun, 1994). Indeed, the molecular weight distribution of the peptides provides an indication of the allergenicity of the hydrolysate (Bindels and Verwimp, 1990 cited by David, 1993).

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Ultrafiltration is also used after enzymatic hydrolysis to improve the emulsifying properties of high-weight hydrolysates. In this case the small peptides are removed from the hydrolysate by ultrafiltration to yield products with improved whipping and foaming stability (Olsen and Alder-Niesen, 1981; Ochiai *et al.*, 1982).

Drying the hydrolysate is the last step in its production. Technologies such as freeze drying, drum/roller drying or spray drying, can be used, the latter being the most economical and reliable method available commercially (Lahl and Braun, 1994). Control of the temperature is crucial during the drying to prevent caramelisation of residual carbohydrates, or formation of Maillard products. The drying is usually at pasteurisation temperature. Finally after drying, the hydrolysed powder is cooled. Figure 1 summaries the processing for manufacturing protein hydrolysates used for nutritional proposes.

The nutritional advantages of the final product have meant that the biological procedure for manufacturing hydrolysates is preferred to the chemical (Adler-Nissen, 1986). In comparison to the hydrolysates produced by acid or alkali processing, the hydrolysate produced by enzymatic treatment has a well-defined peptide profile, which is important in those cases where the size of the resulting peptides is crucial for their application (David, 1993). Enzymatic hydrolysis does not destroy L- amino acids nor produce D- amino acids like acid and alkaline hydrolysis (Pollock and Frommhagen, 1968; De Groot and Slump, 1969). L-amino acids are preferred by the human metabolism. Finally, the enzymatic hydrolysates are free of toxic substances such as lysino-alanine and ornithino-alanine, which are commonly found in products hydrolysed by acid or alkali (Bohak, 1964; Ziegler *et al.*, 1967; Feron *et al.*, 1977).

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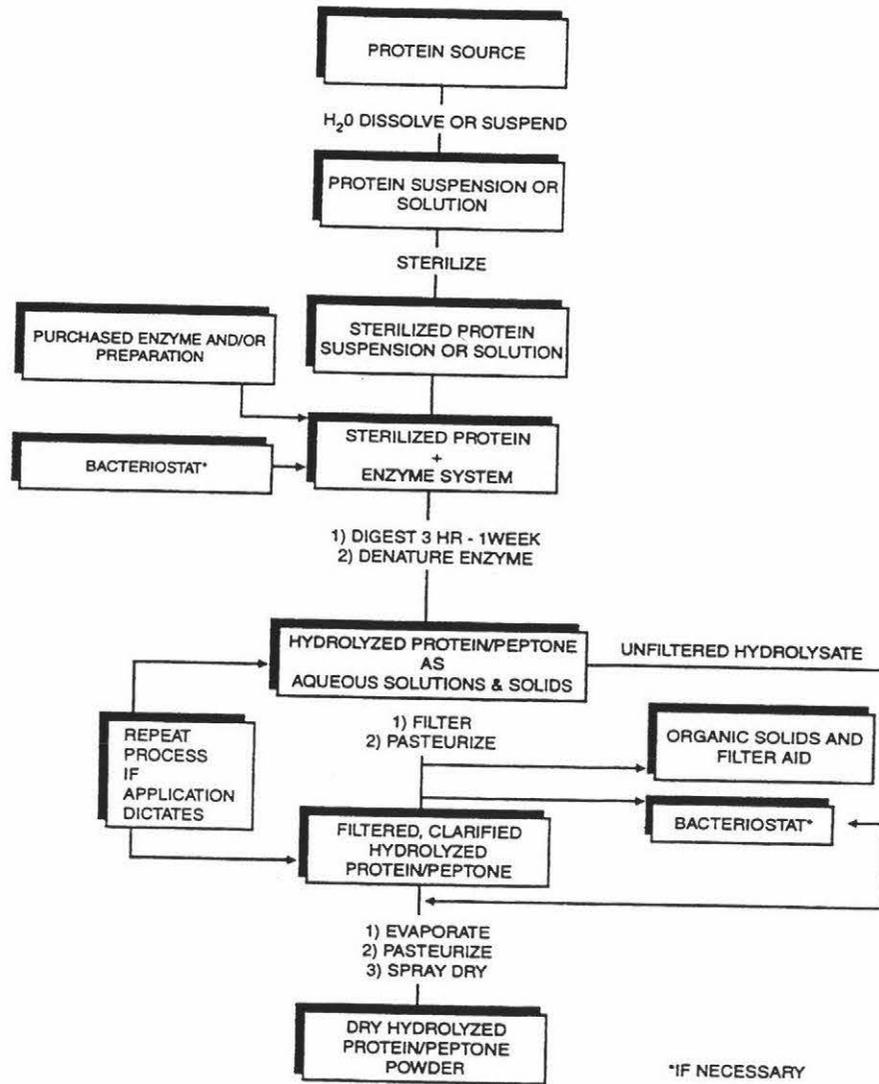


Figure 1. The enzymatic process in the production of hydrolysed milk proteins (From Lahl and Braun, 1994).

## 1.2 Uses of Protein Hydrolysates

The chemical, physical, biological and immunological modifications occurring during hydrolysis make the hydrolysed protein very attractive for the nutrition of consumers with special but not medical needs and also for people undergoing certain medical treatments.

### 1.2.1 Non-Medical but Special Nutritional Needs

Modification of the intrinsic properties of the protein by hydrolysis make hydrolysates desirable ingredients for several products. Extensively hydrolysed proteins are more soluble under acidic conditions than intact proteins (Mahmoud, 1994). Furthermore, the peptide remains in solution even at pasteurisation temperatures (Frøkjær, 1994; Mahmoud, 1994). The viscosity of solutions of hydrolysed proteins is low even at high concentrations (Frøkjær, 1994). Because of their low viscosity and high solubility, hydrolysates have been used increasingly in acid beverages over the last six years. For example, hydrolysed proteins can be used to nutritionally enrich soft drinks and juices (Frøkjær, 1994; Mahmoud, 1994). Commercial examples are Miprodan and Lactoprodan (MD Foods, Viby, Denmark).

#### 1.2.1.1 Hydrolysates for the elderly

Elderly people constitute an important sector of the population in industrialised countries. Censuses performed during 1994 - 1995, indicated that the elderly population in New Zealand, United States of America and Japan was 11.7%, 12.8% and 14.5%, respectively. The population of elderly people in these countries is estimated to increase to 22.5%, 20.0% and 28.0%, respectively, by the year 2030 (Statistics New Zealand, 1998; Smith, 1998; Statistics Bureau and Statistics Centre; Management and Coordination Agency of Government of Japan, 2000).

This increase in the number of elderly people will have an impact on the type of food products demanded in the market. In a period of 30 years, it will become necessary to develop more specialised nutritional products specifically for this elderly population.

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The elderly may require specially designed foods, because physiological changes that occur with age may prevent them from obtaining adequate nourishment from the food suitable for younger persons (Whitaker, 1996). Often, a lack of self-sufficiency and the social environment may cause a reduction of food intake in the elderly. Lack of help, infrequent shopping trips, unaccompanied meals and depression caused by illness, also decrease the appetite of the elderly and consequentially food intake (Gibbs and Turner, 1986). Decreased energy expenditure could be another factor affecting food intake. The loss of weight in people aged 65 to 70 or older has been associated with a decreased intake of food (Frøkjær, 1994). Furthermore, physiological changes occurring with age, such as a loss of sensitivity of the taste-buds, loss of teeth and use of dentures, and a decrease in the HCl of gastric juices, can cause inefficient digestion and absorption of nutrients (Chen, 1986). It has been observed that protein-calorie malnutrition is correlated with a higher rate of mortality and morbidity in the elderly (Pinchofsky-Devin and Kaminski, 1986). Thus for some elderly, hydrolysates might be used to support their nutrition because it is assumed that pre-hydrolysed proteins readily undergo total hydrolysis within the upper gastrointestinal tract (Rosenberg *et al.*, 1989 cited by Linder, 1991; Whitaker 1996).

Contrary to energy requirements, amino acid requirements per kg of body weight for maintenance do not decrease with the age (Young, 1992). Bos *et al.* (1999) reported that short-term protein and energy supplementation of moderately malnourished elderly subjects significantly improved their gain of fat-free mass. Since daily intake of fluid is more easily achieved than solid food (Frøkjær, 1994), protein enriched drinks, often containing hydrolysates, are seen as an attractive development for increasing the daily protein intake in elderly.

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### 1.2.1.2 Hydrolysates for sports people

Even though there is no consensus among scientists about the role of protein as a source of energy (Layman, 1987), proteins do have an important role during the training and performance of athletes. Daily protein requirements increase for people training intensively (Meredith *et al.*, 1989; Phillips *et al.*, 1993). Lemon *et al.* (1999) concluded from a review of studies that the optimum protein intake in a strength athlete is probably between 1.7 and 1.8 g protein/kg/day (approximately 225% of the current recommendations for normal healthy sedentary adults). Diets of athletes in heavy resistance (strength/power) training such as bodybuilders, wrestlers and weight lifters usually consume 3- to 7-fold more than the recommended daily intake for non-athletes (Frøkjær, 1994; Lemon, 1999). It appears, however, that the need for a high protein diet is justified only at the beginning of such training when muscle protein synthesis is increased (Fern *et al.*, 1991; Tarnopolsky *et al.*, 1992). Moreover, anabolism of the muscle decreases at a certain point in power training and subsequently a normal, varied diet will supply enough protein to meet the requirements of these athletes (Frøkjær, 1994).

Marathon runners, cyclists and other competitors performing endurance events, have special needs for protein. The optimal protein intake for endurance athletes is 1.2 - 1.4 g protein/kg/day (Lemon, 1999). During the first three hours in a race, the body of a well-trained cyclist uses its glycogen deposits and plasma glucose to obtain energy. A progressive shift from glycogen to plasma glucose occurs with the duration of the exercise. After three hours the body falls into a hypoglycaemic state (blood glucose concentrations below 2.5 mmol/L) and muscular fatigue develops as a consequence of the low contribution of blood glucose to oxidative metabolism. Drinking carbohydrate solutions delays the fatigue for 30 - 60 min but does not avoid it (Coyle, 1992).

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Frøkjær (1994) reported that after some time during prolonged exercise, the body starts to use muscle amino acids as a source of energy, a state comparable to fasting. The use of body protein means the athlete falls into negative nitrogen balance; the nitrogen excreted is higher than the nitrogen entering the body. Negative nitrogen balance decreases the athletes' performance and the ingestion of dietary protein is desirable to restore the balance.

Ingestion of intact protein in a solid meal is not a measure that can make the body recover immediately. Once eaten, the protein needs to be digested, and the amino acids and peptides absorbed and transported, before any improvement can be noted. Amino acids from pre-digested proteins appear in the blood faster than those from intact proteins (Darragh *et al.*, 1997). Thus, ingesting a pre-digested protein in a meal decreases the time for the body to recover from negative nitrogen status (Frøkjær, 1994). Correcting the negative nitrogen status of the body by eating conventional protein rich foods requires the athlete to stop his or her physical activity while the meal is being ingested (Frøkjær, 1994). Drinks containing hydrolysed protein are a better alternative during endurance performance. They supply small peptides and amino acids, allowing the athlete's nitrogen balance to recover while he is still exercising (Figure 2).

Peptides have received attention for their possible roles as performance enhancers, fatigue reducers and recovery facilitators. Zawadzki *et al.* (1992) observed that after exercise the recovery of the glycogen deposits was more efficient when a protein supplement was added to a carbohydrate supplement than when the latter was taken alone. Hydrolysates are nowadays added to refreshing drinks that people can ingest both during and after exercise. DMV International Nutritionals (The Netherlands) introduced a glutamine peptide to sports foods under the belief that it counteracts the changes caused by the "overtraining syndrome", such as decreased performance,

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depressed mood and increased susceptibility to infection (Sheehy and Morrissey, 1998).

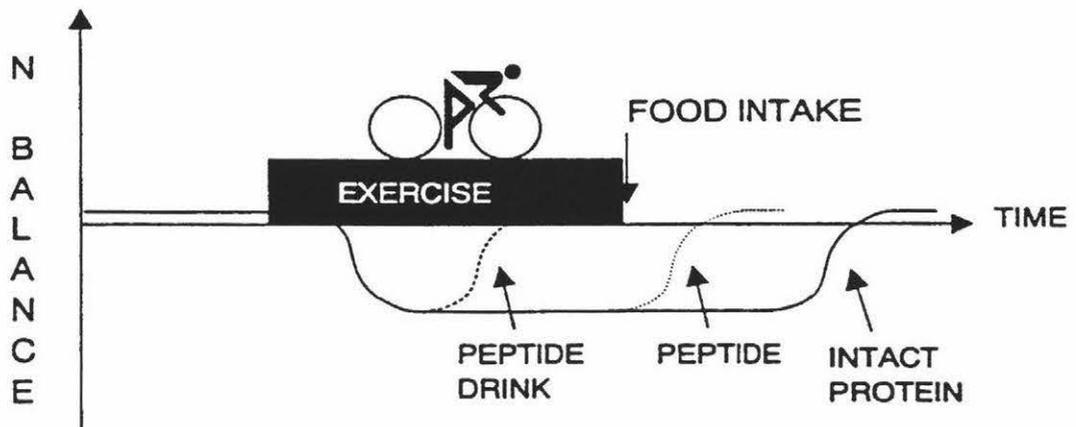


Figure 2. Nitrogen balance during exercise. The possibility of getting a positive nitrogen balance during endurance performances is illustrated by an intake of peptide-based drinks during the exercise, as compared to food intake after the exercise containing either peptides or intact proteins (From Frøkjær, 1994).

### 1.2.1.3 Hydrolysates in weight-control diets

Diets for slimming the body work by reducing the intake of energy and protein, a state comparable to starvation, so that fat deposits must fuel the body. As soon as the intake of protein is reduced the body starts to break down constituent proteins to obtain the amino acids that it needs. After a prolonged period on a diet this phenomenon is observed as weight loss and eventually an unnecessary loss of muscular strength (Frøkjær, 1994).

The body will utilise fat deposits as its main source of energy on low calorie diets if the diets provide adequate protein intake to maintain nitrogen balance. For this reason hydrolysed proteins formulated into soft drinks could be attractive supplements for low caloric diets (Frøkjær, 1994). Furthermore, Hill and Blundell (1989) (cited by

Frøkjær, 1994) observed that meals with a high protein content decreased the appetite of people in comparison to those meals with a high carbohydrate content. Soft drinks containing hydrolysed protein may also help to decrease the appetite of those people that are on low calorie diets based on a high protein intake.

### 1.2.2 Hydrolysates for Individuals with Specific Medical Needs

Hydrolysates have been utilised for the last 45 years to feed individuals with some functional incapacity of the gut. Patients with a reduced intestinal absorption area and/or impaired digestive function can be considered “occasional” consumers of hydrolysates (Frøkjær, 1994).

Ill patients are in constant risk of malnutrition due to blunted palatability, drug medication and depression, which can all, reduce food intake and subsequent nutrient metabolism. Food nutrients are often poorly utilised in ill patients. Drugs can damage the intestinal mucosa, which does not allow it to efficiently transport nutrients into the bloodstream. In addition, key organs in nutrient metabolism, such as the liver, pancreas or kidney, may not be operating effectively in the ill patient. Furthermore, metabolic rate increases with disease. Ill individuals require, therefore, a high calorie and, of course, a high protein intake (Schmidl *et al.*, 1994).

Medical foods have been designed to fulfil or support the nutritional needs of patients whose medical conditions do not allow them to get nutrients from conventional foods. These food products are primarily enteral formulae, whose protein content ranges from 19 to 84 g/L. Together with crystalline amino acids and intact proteins, protein hydrolysates are commonly found as a source of nitrogen in enteral diets (Schmidl *et al.*, 1994). The use of protein hydrolysates in enteral nutrition is based on the assumption that the gastrointestinal absorption of simple molecules such as di- and

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tri-peptides is easier and more effective than that of the intact protein (Ziegler *et al.*, 1990) or free amino acids (Keohane *et al.*, 1985).

### 1.2.2.1 Pancreatic patients

Protein hydrolysates are being used as the source of amino acids for patients with reduced digestive capacity such as pancreatic patients (Frøkjær, 1994). Pancreatic patients suffer from an inflamed pancreas, an organ that produces secretions that are essential for digestion (Tver and Russell, 1989). Pancreatitis is associated with alcoholism and biliary obstruction (Berdanier, 1998). The symptoms of pancreatic patients are tenderness above the stomach, distension, constipation, nausea, vomiting and severe abdominal pain, the latter being stimulated by eating (Ensminger *et al.*, 1995). The treatment for pancreatic patients is based on reducing pancreatic secretions to a minimum. For this reason, these patients are fed intravenously during the first days, and then gradually introduced to soft, bland, and low residue diets (Ensminger *et al.*, 1995). The feeding regimen for pancreatic patients tries to meet nutrient requirements with minimal stimulation of the pancreas. That goal has been partially achieved by either infusing nutrients through a venous catheter for total parenteral nutrition, or through a catheter into the jejunum to provide enteral nutrition (Blackburn *et al.*, 1976; Vidon *et al.*, 1978).

Several studies have demonstrated that feeding amino acids or peptide solutions to pancreatic patients is better for them nutritionally than the conventional intravenous infusion of nutrients (Grant *et al.*, 1984). However, data on the pancreatic exocrine secretion are inconclusive (Schmidl *et al.*, 1994). Some studies have reported that the pancreatic secretions of patients fed with enteral solutions are either inhibited (Keith, 1980), little stimulated (Levy, 1985), or significantly stimulated (Cassim and Allardyce, 1974; Wolf *et al.*, 1975). However, both the quantity of nutrients that can be

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given and the site of administration in the gastrointestinal tract have made enteral feeding gain acceptance as a medical treatment for pancreatic patients (Schmidl *et al.*, 1994). Since di or try- peptides may not need to be digested by enzymes before being absorbed, highly protein hydrolysates appears to be a suitable alternative to support the nutritional status of the pancreatic patients.

### 1.2.2.2 Patients with short bowel syndrome

Patients with short bowel syndrome have experienced massive resection of the small intestine, which consequently reduces their digestive capacity (O'Toole, 1997). Enteral nutrition is usually practised on these patients. The minimum length of small bowel for maintaining satisfactory nutrient intake via the enteral route is 60 centimetres in addition to the duodenum (Pietz, 1956 cited by Anonymous, 1982). In order to make best use of the available area in the bowel, nutrients in the diet should be pre-digested. Since highly hydrolysed proteins require a minimum of digestion and are easily absorbed, they are used to supply amino acids in diets formulated for patients with short bowel syndrome (Schmidl *et al.*, 1994). In addition, Weser (1979) and Kokal (1986 cited by Schmidl *et al.*, 1994) agreed that oral ingestion of pre-digested nutrients such as highly hydrolysed protein is useful during the first stage of re-adapting the bowel to luminal nutrition.

### 1.2.2.3 Crohn's disease

Crohn's disease is a chronic disease characterised by inflammation of the bowel, although its aetiology is unknown. Patients are treated with antibiotics to prevent infection and with anti-inflammatory agents. Patients with Crohn's disease are usually malnourished as a result of both loss of appetite due to illness and also malabsorption (Schneeweiss *et al.*, 1999). This illness is also known as regional

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enteritis, since only some regions of the gut are affected (Bender and Bender, 1995). Highly hydrolysed proteins have been recommended as useful protein sources in the nutrition of sufferers of Crohn's disease (Frøkjær, 1994).

Frøkjær (1994) reviewed the therapeutic effect of elemental diets in Crohn's disease. He concluded that the treatment of Crohn's disease patients with elemental diets represents a more effective and less hazardous therapy than the conventional treatments based on the administration of steroids or surgery. Furthermore he concluded, based mainly on O'Morain *et al.* (1984), that elemental diets might be beneficial to Crohn's patients in one of two ways. (1) Since the components of the diet were mainly absorbed in the duodenal region, the amount of chyme reaching the inflamed and ulcerated gut regions might be reduced. (2) Since the gut mucosa in Crohn's patients is in an ulcerated condition, the reduced amount of large peptides in the elemental diet reduces the risk of secondary immune reactions to food protein that may gain access to immune tissues in the gut wall through the damaged mucosa.

#### 1.2.2.4 Cow's milk allergy

The primary market for milk protein hydrolysates is in hypoallergenic infant formulae. Hypoallergenic hydrolysed infant formulae have been marketed for more than 45 years. They have been classified in "generations", depending on the source of protein and the technology used for their production (Lahl and Braun, 1994). The first generation of hypoallergenic infant formulae were the casein-based hydrolysates. They contained more than 70 mol % free amino acids and the remaining amino acids were present as peptides up to eight amino acids long. The "second generation" was developed 15 years ago based on whey protein hydrolysates. These hydrolysates contained 40 - 60 mol % free amino acids and the peptides were up to 12 amino acids long. The "third generation" has become commercially available only in the past seven

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years. They are hydrolysates based on whey proteins, with less than 20% free amino acids and peptides up to 15 amino acid long (Siemensma *et al.*, 1993).

In the 1980s, formulae containing hydrolysed protein were introduced to support the nutrition of infants with enterocolitis provoked by food proteins other than milk proteins. Nowadays, they are given to infants with allergic reactions to bovine milk or isolated soy proteins (Fomon, 1993). They are also used as a prophylactic measure in infants believed to have a high risk of developing allergic reactions to food proteins (Chandra, 1997). It has been proposed to use the hydrolysates in pre-term infants, in order to reduce the risk of sensitisation to cow's milk proteins and to improve gastric emptying (Lucas *et al.*, 1984).

Hydrolysed formulae are fortified with specific amino acids in order to bring the amino acid composition closer to that of human milk (Lee, 1992). Hydrolysates from cow's casein are fortified with L-cysteine, L-tyrosine, L-tryptophan, taurine and L-carnitine. Their odour and taste are unpleasant because the size of the peptides produced is in the range of 1,500 to 500 Daltons (Fomon, 1993).

The sources of fat are palm oil, soy oil, corn oil, coconut oil, and safflower oil (Fomon, 1993). Cornstarch hydrolysates, sucrose or modified corn starches are usually the source of carbohydrates (Fomon, 1993). Appendix 1 lists some formulae on the market that are based on protein hydrolysates.

As outlined above, a major use of whey and casein hydrolysates is as a therapeutic measure for allergic patients based on the concept that small peptides are less allergic than the intact protein. In order to understand this principle, it is necessary to explain how and why infants develop allergy to bovine proteins.

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#### *1.2.2.4.1 Development of the cow's milk allergy*

In order to understand the development of cow's milk allergy, it is necessary to define some concepts. Antigenicity is the capacity of molecules (antigens) to induce the immune system to produce antibodies that then react with the antigen. Allergenicity is a particular type of immunological reaction in which the allergen induces the production of antibodies that belong to the IgE class of immunoglobulins. Micromolecular sites on the allergen, called epitopes carry antigenic and/or allergenic sequences, which may or may not be destroyed by chemical or enzymatic processing (Cordle, 1994).

After the digestion of cow's milk protein in the gut, the free amino acids and an unknown quantity of di- and tri-peptides are absorbed by the epithelial cells. In addition, small amounts of longer peptides that still remain intact pass through the epithelial layer of the gastrointestinal tract (Savilahti and Kuitunen, 1992). In the interstitial zone, these long peptides (potential allergens) may provoke an immune response, which can be either humoral, cell mediated or both (Savilahti and Kuitunen, 1992). The immunological response starts with the sensitisation of lymphocytes by exposure to allergens. The lymphocytes divide and travel through the mucosal lymphoid tissues eventually maturing into plasma cells that will secrete antibodies that can react specifically with the antigen (Riordan, 1993).

After a period of sensitisation, a further introduction of the protein allergen stimulates further immunological events leading to the production of IgE or IgG and mediators (Bellanti *et al.*, 1988 cited by Guesry *et al.*, 1989). IgE is fixed on mast cells by a specific receptor and by another type of IgE receptor on eosinophils, macrophages and platelets (Taylor and Lehrer, 1996). When an allergen is bound by two molecules of specific IgE, one attached to a mast cell and the other to an

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eosinophil or macrophage, a hypersensitivity reaction is started, which is characterised by the secretion of the active mediators of inflammation (Savilahti and Kuitunen, 1992). Eventually the progression of immunological events is manifested by any number of clinical symptoms of varying degrees of severity such as vomiting, failure to thrive, diarrhoea, atopic dermatitis, bronchial wheezing, colic, and intestinal bleeding with or without diarrhoea, runny nose, cough, asthma, and urticaria (Motil, 1988; Riordan, 1993). In addition to IgE-mediated atopic reactions, allergic reactions may be provoked by cell-mediated immunity (Savilahti and Kuitunen, 1992).

A factor that possibly determines the allergenicity and antigenicity of the cow's milk protein fractions is the permeability of the intestine to proteins during infancy. At birth, non-specific and specific factors that inhibit the passage of cow's milk proteins through the epithelial layer are not present and although these factors develop during the early infancy, cow's milk allergy may be acquired during the first year. IgG antibodies to cow's milk begin to decrease in susceptible infants at one year of age, while IgE-mediated cow's milk allergy disappears slowly after two years, and IgA concentrations fall even more slowly until late childhood. The gradual loss of allergy to cow's milk has been related to the maturation of the gut and to its reduced permeability to antigens (Savilahti and Kuitunen, 1992).

In addition to the changes in intestinal permeability, the degree of development of the local mucosal immune system is another factor that influences the development of cow's milk allergy. This part of the immune system, through its specific mucosal antibody response, inhibits the absorption of allergenic protein molecules. The development of the local mucosal immune system of the gut takes time, the IgA producing component continues to develop for more than two years after birth (Savilahti and Kuitunen, 1992).

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Other factors that influence the development of cow's milk allergy are an inherited susceptibility to atopic disease, presence of other allergenic food proteins, previous episode of gastroenteritis, and others as yet-unknown factors (Chandra *et al.*, 1993).

Both the primary structure and the conformation of the molecule determine the allergenicity or antigenicity of a protein molecule. The amino acid sequences, the structures of side chains and conformational structure of the protein are all components of the antigenic sites recognised and bound by the specific antibodies. This means that if the primary structure or the conformational structure is modified, the antigenicity or the allergenicity of the molecule will be diminished (Lee, 1992).

#### *1.2.2.4.2 Enzymatic hydrolysis and protein allergenicity*

Hydrolysed formulae were designed based on the approach of altering the allergenicity or antigenicity of milk-based formula through the appropriate processing of the raw dietary protein.

Heat denatures proteins such that their conformation is changed and consequently their antigenicity is diminished (Lee, 1992). In addition, under heat treatment, acid is produced from the decomposition of lactose, which can also act on the protein. Despite these changes, heat treatment alone may not yield an infant formula of low allergenicity (Lee, 1992).

The enzymatic hydrolysis of a protein with a mixture of endo-peptidases and exo-peptidases decreases its antigenicity and allergenicity more effectively than heating. Figure 3 shows diagrammatically the enzymatic hydrolysis of a protein. Large peptides result from the partial hydrolysis of a protein, whereas extensive hydrolysis

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yield a mixture of large and small peptides and free amino acids. As explained in an earlier section, large peptides, 5,000 to 10,000 Daltons that resist hydrolysis, are removed by ultrafiltration (Lee, 1992; Lahl and Braun, 1994).

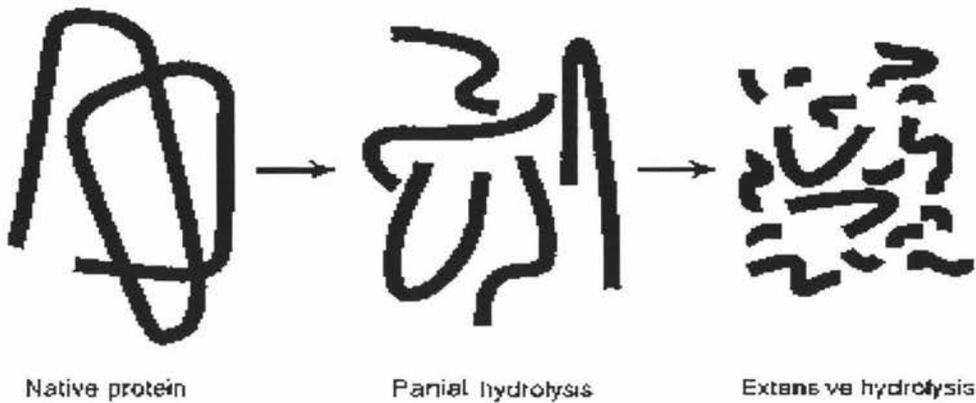


Figure 3. Enzyme hydrolysis of protein (From Lee, 1992).

Because of the destruction of many of the allergens, when a partially hydrolysed formula is ingested by a sensitive infant, the immunological response to foreign proteins is reduced and consequently many of the clinical manifestations are eliminated. However, several studies have reported the continued presence of potential allergens in several hydrolysate-based formulae on the market (Chiancone *et al.*, 1995 and van Beresteijn *et al.*, 1995). The allergenicity and the nutritional value of the hydrolysates will be discussed later. In addition, extensively hydrolysed products have a bitter taste, and many functional properties that are important in the manufacture of formulae (emulsifying, whipping or foaming, or gelling capacities) may be lost. Also, hydrolysis can diminish the availability of amino acids through the induction of lysinoalanine formation or also the Maillard reaction (Lee, 1992). Despite this, hypoallergenic infant formulae are increasingly used because cow's milk allergy plays an important role in the development of atopic illness, one of the highest causes of morbidity in infants and young children. For this reason, interest continues into the

prevention of an allergic response instead of trying to cure the illness after it has developed. Preventive measures include the introduction of hypoallergenic formulae to the babies' diets instead of whole cow's-protein based formulae (Blecker, 1997).

In conclusion, dietary protein hydrolysates produced enzymatically are less allergenic and antigenic than the whole protein and those hydrolysates produced by heat treatment. Protein hydrolysates may produce less severe immunologic responses in the infant and consequently the clinical manifestations are not present.

Despite their wide use, concern has arisen about the overall allergenicity of protein hydrolysates, which actually remains to be investigated. In the next section, the basis of these concerns is reviewed briefly.

#### *1.2.2.4.3 The hydrolysate formulae and their role in allergic diseases*

Although hydrolysed formulae have been presented as a suitable option for allergic patients, almost since their introduction to the market, there is evidence of anaphylactic-type reactions to them.

The studies of van Beresteijn *et al.* (1995) and Chiancone *et al.* (1995) have contributed greatly to our knowledge of the epitopes that remain after hydrolysis of proteins used in infant formulae and which might be responsible for the allergic reactions presented in babies fed with hypoallergenic formulae. Chiancone and colleagues concluded that these epitopes can be not only allergens but also immunogens in the predisposed baby. Thus that these epitopes are capable of stimulating an immune response, which may involved the production of mediators and other antibodies than IgE (Fudenberg *et al.*, 1978; Webb and Winkelstein, 1978; Roitt, 1997).

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Cézard *et al.* (1996) measured the antigenicity of partially and highly hydrolysed-protein formulae available commercially by the immunodot assay. The allergenicity of partially hydrolysed milk proteins was reduced by only 69.7%, whereas in the highly hydrolysed proteins the reduction was at least 97.5%. These results show that the partially hydrolysed milk proteins contained high concentrations of immunologically active residual peptides.

Whether or not the hydrolysates are hypoallergenic is being debated, because there are several aspects that have to be considered. For instance, the degree of hydrolysis, heat treatment, application of ultrafiltration, as well as the physiological maturity of infant's gut, and any family history of allergies, etc. all need to be taken into account. In order to understand fully about the allergenicity of the protein hydrolysates, more research is needed.

#### 1.2.2.5 Other uses of hydrolysates

Several studies agree that hydrolysates may possess other therapeutic properties, which may be commercially attractive in the near future. For instance Kitts and Yuan (1992) observed that phosphopeptides isolated from casein hydrolysates increase the absorption of calcium in the gut. Schlimme *et al.* (1989) and Daniel *et al.* (1990) found that  $\beta$ -casomorphins act as opoid agonists in the control of gastrointestinal processes such as motility and the stimulation of secretion. This suggests that if they are able to pass through the intestinal epithelium without being digested they may act as anti-diarrheal agents. Also Nielsen *et al.* (1994) observed that peptides in casein hydrolysates enhance whole body protein turnover in rats. The nutritional and clinical consequences of this effect are still being studied.

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Based on the properties outlined above, therapeutic usages are a potential market for the hydrolysates. Further scientific studies are needed, however, to establish their superiority over other protein products on the market.

Irrespective of whether the hydrolysates are used as partial or total substitutes for intact protein in the diet, their nutritional similarity to the latter must be assured. The nutritional response from the ingestion of a hydrolysed protein can be evaluated by comparing the metabolic paths followed in the body by the amino acids and peptides from the hydrolysates compared to those followed after consumption of the corresponding intact protein.

### **1.3 Human Metabolism of Protein Hydrolysates**

To be of any nutritional benefit, the protein hydrolysates must support a similar or better nitrogen balance to that found with the intact protein. As the processes of digestion and absorption are the first stages in protein utilisation, it is important to understand the effect that replacing an intact protein in the diet with a hydrolysed product has on the digestive and absorptive process.

#### **1.3.1 Digestion**

Before the dietary amino acids can be utilised, intact protein must be digested and its components absorbed and transported to the sites of metabolism. The process of digestion of the intact protein starts in the stomach, with the action of hydrochloric acid (HCl) and pepsin. The HCl denatures the protein (unfolding) making it sensitive to pepsin hydrolysis. Pepsin is not, however, a very specific enzyme and it preferentially splits the unfolded protein at the bonds adjacent to aromatic amino acids and leucine. Digestion continues as the chyme passes into the duodenum where it is mixed with secretions from the pancreas and the intestinal mucosa. In the duodenum the partially

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hydrolysed protein is further split by pancreatic enzymes and mucosal peptidases releasing amino acids and small peptides (Bray *et al.*, 1999).

From a hypothetical point of view, the ingestion of either an intact or an industrially hydrolysed protein is not greatly different until their arrival in the duodenum. However, the extent of secretion of gastric, pancreatic and intestinal juices may differ if they are regulated by the presence of peptide bonds. Products of protein breakdown during digestion may directly affect endogenous protein secretion. For example, intestinal protease activities (Maze *et al.*, 1979; Poullain *et al.*, 1989a) and pancreatic protease synthesis and secretion are stimulated by dietary peptides and proteins, which are more effective stimulants than free amino acids (Schneeman 1982; Temler *et al.*, 1983; Puigserver *et al.*, 1986). Butts *et al.* (1993a) studied the effect of dietary peptides (enzymatically hydrolysed casein), a lysine-free protein (zein), a protein-free diet or a diets based on synthetic amino acids, on the loss of endogenous lysine and other amino acids from the ileum of the growing pig. Lysine is commonly used in the nutritional studies because it is the first limiting essential amino acid in diets high in cereals (Friedman, 1977). It is also the limiting amino acid of pig diets and poultry diets. In addition it is suitable to chemical modification (Moughan and Rutherford, 1996). Butts *et al.* (1993a), measure the endogenous flow of lysine at the terminal ileum after feeding pigs their respective diets. Endogenous flow derives mainly from the gastrointestinal tract and contains enzymes, mucoproteins, desquamated cells, plasma proteins, peptides, free amino acids, as well as microorganism (Butts *et al.*, 1993b). In this study Butts *et al.* (1993a), found that that the flow of endogenous lysine did not differed significantly between the hydrolysate and the intact protein. However, the zein and casein hydrolysate induced endogenous flow was significantly higher than that observed with the amino acid or protein-free diets. Therefore, the intact or the peptide form (with molecular weight up to 5,000 Daltons) of the amino acids did not affect the endogenous flow of lysine. However, their presence in the digestive tract

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results in an increased loss of endogenous lysine from the small intestine of the growing pig in comparison to a protein-free diet and/or a diet containing free amino acids. These studies confirmed previous observations in rats (Darragh *et al.*, 1990; Moughan and Rutherfurd 1990; Butts *et al.*, 1991) and pigs (De Lange *et al.*, 1990).

In conclusion, it appears that, the endogenous lysine flow determined at the terminal ileum may not depend on the primary and tertiary structure of protein. In addition, the amount of hydrolysed protein eaten does not affect the loss of endogenous protein at the ileum (Boisen and Moughan, 1996). However, the effect of hydrolysed proteins other than casein and the DH of the protein has not been systematically investigated yet (Boisen and Moughan, 1996).

The pH of the chyme when it reaches the duodenum is important for the action of the digestive enzymes secreted by the pancreas and mucosa, which require a neutral pH. Because the hydrolysis of peptide bonds modifies the pH of the medium (Adler-Nissen, 1986), it is expected that the pH of the chyme reaching the duodenum will differ between the intact and hydrolysed protein. The size of this effect and hence its importance, however, is unknown.

### **1.3.2 Absorption**

Whole proteins can pass through the intestinal cells only under certain physiological conditions such as in the presence of gut disease or in infancy especially if an infant is premature. In these cases, the permeability of the intestine is enhanced, and transfer of whole proteins occurs by passage between the mucosal cells of the villus (Linder, 1991).

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In a normal, healthy individual, however, free amino acids and small peptides are transported from the intestinal lumen into the cell by specific mechanisms (Mathews *et al.*, 1969; Rerat *et al.*, 1988) which require the expenditure of energy and several carrier systems for neutral, basic and acid amino acids. The order in which the individual amino acids are absorbed is: branch chain amino acids and methionine > other essential amino acids > nonessential amino acids > glutamate and aspartate (Linder, 1991).

Intact peptides are absorbed from the intestine at a rate that is related to their size. Matthews and colleagues (Mathews *et al.*, 1969) performed one of the first studies suggesting the transport of intact peptides into the epithelial cells of the small intestine. Since then several animal and human studies have indicated that di- and tri-peptides are more rapidly absorbed than free amino acids (Craft *et al.*, 1968; Adibi and Morse, 1977; Keohane *et al.*, 1985).

From these studies, the dual model of dietary nitrogen assimilation has been established. In this model, only amino acids and di- and tri-peptides produced by luminal and brush border digestion are absorbed via the various transport systems (Figure 4). Tetra and higher peptides need to be further hydrolysed on the brush border before their hydrolysis products can be absorbed (Adibi and Morse 1977). Di- and tri-peptides that are absorbed are then usually hydrolysed by the cytosolic peptidases (Nicholson and Peters, 1979). It has been reported that 30% - 70% of nitrogen absorbed and detected in the portal circulation may be absorbed as a peptide and hydrolysed in the epithelial cells (Schmidl *et al.*, 1994).

Only traces of peptides can be detected in the bloodstream. These peptides that are resistant to brush border and cytosol peptidases are carried to the liver or other

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peripheral tissues where they may be hydrolysed. Some peptides remain unhydrolysed and are excreted in the urine (Hueckel and Rogers, 1970).

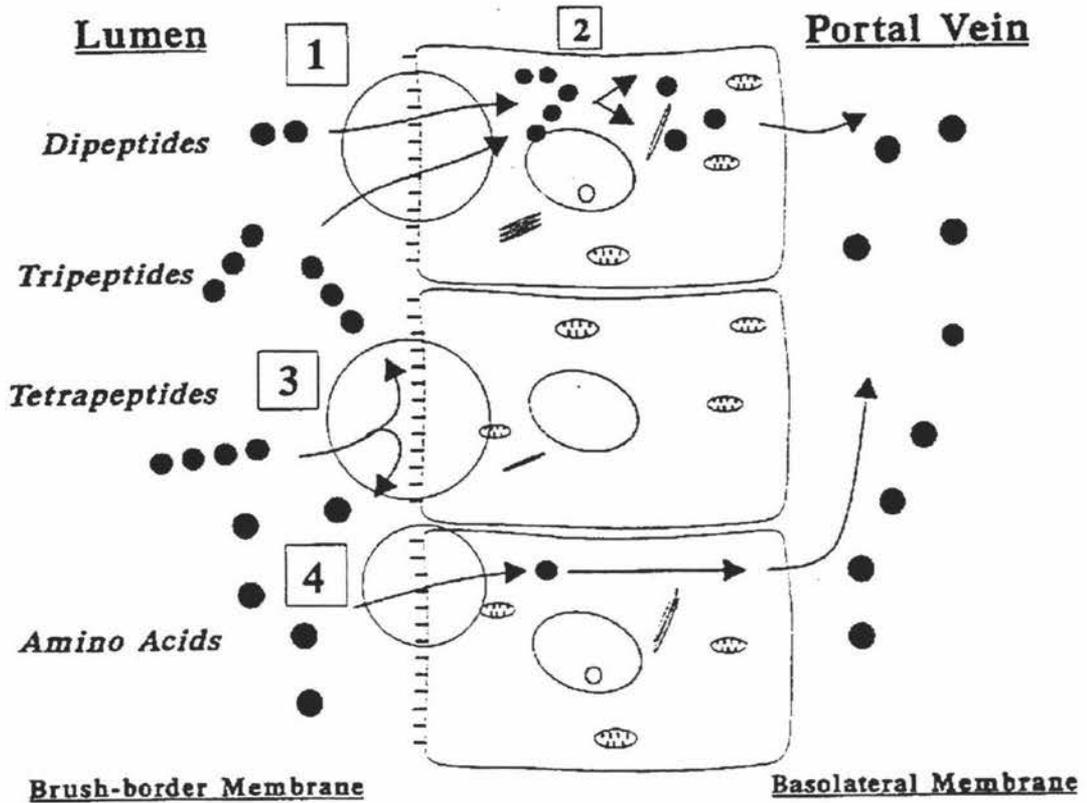


Figure 4. Dual mechanism for the assimilation of dietary nitrogen in the small intestine. Luminal di- and tri-peptides are absorbed by a specific transporter (step 1) and hydrolysed in the cell (step 2). Tetra and higher peptides are hydrolysed by brush-border peptidases (step 3). Free amino acids are absorbed by one of the active L-amino acid transporters (step 4) (From Grimble, 1994).

The speed of protein digestion and amino acid absorption from the gut has been observed to have a major effect on whole body protein anabolism after a single meal (Boirie *et al.*, 1997). Boirie *et al.* (1997) reported that a slowly digested and assimilated protein, such as casein, increases protein deposition by inhibiting protein catabolism without an excessive increase in amino acid concentration in the blood. In contrast, rapidly assimilated dietary proteins, such as whey proteins, increase protein anabolism and also protein oxidation. This has direct implications with regard to the

utilisation of hydrolysed versus intact protein with increasing interest in the effects of feeding a hydrolysed protein on the speed of amino acid absorption, and the consequences of dietary hydrolysates on post-prandial protein synthesis, breakdown and deposition.

Studies comparing absorption kinetics of pre-digested protein and intact protein are scarce. The increased use of hydrolysates as alternatives to the elemental diets for enteral nutrition has meant that most of the studies testing the absorption of amino acids from hydrolysed proteins have compared them to free amino acid diets rather than to diets based on intact protein. The studies that have compared the kinetics of amino acid absorption between intact and hydrolysed proteins are reviewed below.

Moughan *et al.* (1990) determined the absorption coefficients for the dietary amino acids at the end of the ileum and over the entire digestive tract of pigs fed a mixture of intact milk proteins (casein + whey proteins) and its hydrolysate. They found that the absorption, as measured at the end of the ileum, of histidine ( $P < 0.05$ ), arginine ( $P < 0.001$ ), aspartic acid ( $P < 0.01$ ) and glutamic acid ( $P < 0.05$ ) were lower for the hydrolysate than for the intact protein. In contrast, the total nitrogen absorption at the end of the ileum was not different statistically. With the exception of aspartic acid ( $P < 0.05$ ), leucine ( $P < 0.001$ ) and cysteine ( $P < 0.01$ ), the amino acid and nitrogen faecal absorption was similar between the intact protein-based mix and its hydrolysate. Moughan *et al.* (1990) concluded that the hydrolysed milk protein is an acceptable alternative protein source to the intact bovine milk in infant formulae because protein source had little effect on the apparent absorption of the essential amino acids.

Baró *et al.* (1995) performed a study in which the total serum protein concentration and the serum amino acid profile of growing rats at weaning was

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measured using diets with different base proteins, whey protein and casein, that were fed either intact or as their enzymatic hydrolysates. Under similar dietary and protein intakes, the body weight gain and total serum protein did not differ between either type of protein or its molecular form. Irrespective of its form (intact or hydrolysed), rats fed the whey-based diets had higher ( $P < 0.01$ ) concentrations of total amino acids in the plasma than the groups fed with the casein-based diets (intact or hydrolysed). In contrast, the total concentration of branch-chain amino acids (BCAA) was higher in the rats fed the casein-based diets than the whey protein-based diets, while the nonessential/essential ratio was similar in all groups. Based on these results, Baró *et al.* (1995) suggested that the differences in the serum amino acid profiles were due to the amino acid composition of the protein, casein or whey, but not their molecular form. They concluded that the hydrolytic process does not affect the nutritional quality of either whey or casein.

Deutz *et al.* (1996) used the flux of BCAA across the portal drained viscera (PDV) as a parameter for testing the absorption of two structurally different proteins in pigs. In this study, the patterns of appearance of amino acids in the PDV and the total production of BCAA were similar on diets containing either intact or hydrolysed proteins. The PDV BCAA concentration rose rapidly for 15 minutes after feeding pigs either intact whey protein or hydrolysed whey protein and then it started to decrease. Since all the essential amino acids followed the same pattern, Deutz *et al.* (1996) concluded that the amino acid absorption after a meal is not affected by the modification to protein structure.

Deutz *et al.* (1996) also monitored the flux of BCAA across the liver, and the hindquarters by measuring their concentration in the arterial circulation and in the veins draining the tissues. The flux of BCAA across the liver and hindquarters differed only marginally between diets. However, arterial concentrations of BCAA were higher for

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the pigs on the intact diet than for the pigs on the hydrolysed diet. From this study it was concluded that the change in the protein structure by hydrolysis does not affect the nutritional value as assessed by peripheral amino acid uptake (Deutz *et al.*, 1996).

In contrast to the results reported by Deutz *et al.* (1996), the concentration of all amino acids including BCAA in the abdominal aorta were higher for pigs on a diet containing pre-hydrolysed protein than for pigs on a diet containing the intact protein (Darragh *et al.*, 1997). The BCAA increased in concentration in the abdominal aorta for 0.5 hr after feeding and then decreased. Since amino acids other than BCAA are metabolised to some extent in the liver, their appearance in the abdominal aorta did not follow the same pattern as the BCAA. For amino acids such as cysteine and lysine, there was a prolonged rise in concentration in the blood for 2 hr after the meal and then a fall.

Darragh *et al.* (1997) (unpublished data) observed, however, that for pigs fed the intact protein, the rise at 0.5 hr was lower than in those pigs fed the hydrolysed diet and the difference was maintained for over 6 hr. For this reason, they suspected that the pattern of absorption differed between the intact and the hydrolysed protein.

The concentration of urea in the plasma leaving the liver has been used as an indication of amino acid catabolism in this organ (Whitney and Rolfes, 1996). Urea is produced in the liver from the nitrogenous residues released from the metabolism of amino acids. Therefore, the concentration of urea in the hepatic vein is an indicator of the amount of amino acid catabolism in the liver.

Deutz *et al.* (1996) found that between the first to fourth hour after a meal the total liver urea production was higher ( $P < 0.001$ ) in pigs fed hydrolysed protein in comparison to those fed intact protein. However, the blood urea concentrations during

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the fourth hour was similar between pigs fed the two diets. The patterns were characterised by a smooth increase during the first hour and a more pronounced increase between first to second hour. From three hours onwards, the concentration of urea decreased in both groups and came together at four hours. Because the amount of urea produced was of a similar magnitude to the ammonia uptake in the liver, Deutz *et al.* (1996) concluded that the urea produced by the liver after a meal is associated with the ammonia produced from the deamination of glutamine in the gut. Furthermore, they suggested that urea production from the metabolism of amino acids taken up by the liver after the meal might contribute to the urea synthesis, but not significantly.

Similarly, Darragh *et al.* (unpublished, 1997) found that the concentration of urea in the abdominal aorta was higher ( $P < 0.01$ ) in pigs fed hydrolysed protein than in pigs fed intact protein. Urea concentrations differed ( $P < 0.1$ ) at all time intervals after the meal, being more pronounced from 1.5 to 3.5 hr after feeding.

In the study by Darragh *et al.* (1997), however, the shapes of the curves showing the urea concentration after feeding differed slightly ( $P < 0.1$ ) between pigs fed intact whey protein and those fed its hydrolysate. For pigs fed a diet containing hydrolysed whey proteins, there was a peak in the concentration of urea in the plasma in the abdominal aorta between two and three hour after consuming a meal whereas the urea concentration in pigs fed the intact protein remained constant. Based on these results, Darragh and colleagues suggested the possibility of a lower utilisation of protein hydrolysates compared with intact proteins.

It has been suggested that the higher blood urea concentrations on the diets containing hydrolysed proteins are a consequence of differences in the metabolism of amino acids in the gut. Thus, higher ( $P < 0.001$ ) ammonia concentrations in the portal

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veins of pigs fed a hydrolysate as against the intact protein may reflect greater deamination of glutamine and asparagine in the wall of the gut of the pigs fed the hydrolysate (Deutz *et al.*, 1996). Deutz *et al.* (1996) suggest that on diets containing intact protein much of the glutamine is absorbed as peptides, which is protected from deamination. In contrast, more of the glutamine in diets containing hydrolysed protein is absorbed as free amino acid and the  $\alpha$ -amino group is prone to degradation in the mucosal cells. Consequently, the concentration of ammonia in the portal vein is higher in pigs fed the hydrolysed protein than in those fed intact protein. This is reflected in a higher blood urea concentration in pigs given the diets containing hydrolysed protein as a result of conversion of the ammonia to urea in the liver.

#### 1.4.3 Nutritional Evaluation of Protein Hydrolysates

The nutritional value of hydrolysates has been investigated using techniques as nitrogen balance. The nitrogen balance is a measure of the nutritional status of an individual, which in turn reflects amino acid absorption and catabolism. Higher nitrogen balances are observed when the protein is absorbed slowly (Boirie *et al.*, 1997). Thus, diets containing protein hydrolysates, which are characterised by faster rates of amino acids absorption, may be expected to have lower nitrogen balances in comparison to diets containing intact proteins. There are, however, conflicting results concerning the effect of the physical form of the protein in the diet on the nitrogen balance from several studies, which are reviewed in the next section.

Whenever hydrolysates are used to support partially or totally the protein nutrition of an individual, their nutritional value must be evaluated. Usually these studies involve estimating the amount of protein deposited in the body by measuring the amount of nitrogen retained, principle of the nitrogen balance. Hydrolysates used in infant formulae have been evaluated in humans and in model animals such as the

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rat and the pig. Most of the research done in humans have involved infants because of the relevance of hydrolysates in the nutrition of infants with allergies. It is expected that in the near future, research will be extended to other consumers with specific needs for protein hydrolysates such as the elderly and athletes.

In an experiment performed by Yamamoto *et al.* (1985), intact casein was compared to its hydrolysate and to a diet of free amino acids, in rats. They measured body weight gain, protein efficiency ratio (PER), nitrogen balance, total urinary nitrogen (N) and amino acid N. In blood, they measured haematocrit, albumin and urea concentrations. From their study, they concluded, that in normal conditions, intact protein and its tryptic hydrolysate were of similar nutritive value.

Puollain *et al.* (1989) measured nitrogen balance, growth and steatorrhoea, in rats fed iso-nitrogenous protein diets differing only in the physical structure of the protein (intact whey proteins v. hydrolysed whey proteins). Weight gain and concentrations of blood serum proteins and urea did not differ between the two groups. In addition, nitrogen absorption was similar between groups but nitrogen retention was higher ( $P < 0.01$ ) in the group fed the hydrolysate than in the group fed the intact protein. The lower nitrogen retention in the group fed the intact protein was a consequence of greater urinary nitrogen loss.

Moughan *et al.* (1990) carried out a study in which they evaluated infant formulae based on either hydrolysed or intact milk proteins. They measured the development of organs, the activity of digestive enzymes and the absorption of amino acids at the terminal ileum and over the entire digestive tract using the piglet as an animal model for the human baby. They found that the weight/unit body weight of the small intestine, large intestine, liver or pancreas was similar for piglets fed either formulae. The piglets that were fed with the hydrolysed protein formula, however, had

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heavier kidneys ( $P < 0.05$ ) compared to those given the intact milk protein-based formula. The enzymatic activity of pepsin, intestinal trypsin and chymotrypsin and pancreatic chymotrypsin did not differ between the piglets fed the different formulae but pancreatic trypsin activity was lower in the piglets fed the intact milk protein. In addition, the piglets fed with hydrolysates had lower total urinary nitrogen excretion than those fed the formula based on intact protein. This was unexpected given that the large concentration of free nitrogenous compounds in the hydrolysed formula may have been expected to result in a higher excretion of urinary urea. For example, other studies have shown high rates of urinary nitrogen excretion in rats fed hydrolysates (Cézard *et al.*, 1996).

Moughan *et al.* (1990) did not find any nutritionally important differences in ileal and faecal amino acid absorption between the formulae. Therefore they concluded that the hydrolysed bovine milk protein is a satisfactory source of amino acids to replace intact protein in infant formulae.

Vandenplas *et al.* (1993) compared the growth and weight gain in babies fed a formula based on whey hydrolysates with one based predominantly on unhydrolysed whey proteins. Although the ingested volume of whey hydrolysate was less, there was little difference in the nutritional value between the two formulae. Blood analysis for haemoglobin, haematocrit, red blood cell count, white blood cell count, lymphocytes, glycemia, proteins, albumin, prealbumin, calcium, phosphorus, creatine, iron, and vitamins A and E also showed no significant differences except that iron-binding capacity and the concentrations of zinc and urea were higher in babies fed the whey hydrolysate.

In comparison with babies fed a whole protein (casein: whey, 40:60) formula, the concentration of threonine and glutamic acid in plasma were increased and that of

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phenylalanine, leucine, valine and histidine were decreased in pre-term babies fed whey hydrolysates or a whey predominant hydrolysate (casein: whey, 22:78) (Rigo *et al.*, 1995). In pre-term and term infants, nitrogen absorption and nitrogen retention were lower in those fed with hydrolysed protein-base formulae compared with those infants given the whole protein formula (Rigo *et al.*, 1995).

The size of the coefficients of nitrogen utilisation (nitrogen retention/nitrogen absorption) for term infants were highest for breast-fed infants followed by infants fed intact protein, then infants fed hydrolysed whey formula and the lowest coefficient was observed for infants fed hydrolysed casein + whey. Conversely, the coefficient of nitrogen utilisation in pre-term infants was similar for infants fed either hydrolysates or intact protein (Rigo *et al.*, 1995). In addition, the absorption of calcium, fat and phosphorus were lower when the casein + whey hydrolysate formula was fed to the term infant than when the whey hydrolysate-formula was fed. The absorption of fat and phosphorus did not show a significant difference between the hydrolysed whey formula and the whole protein-formula (Rigo *et al.*, 1995).

In the pre-term infant, feeding a hydrolysed formula caused a reduction in the absorption of phosphorus and calcium in comparison with whole protein formula (Rigo *et al.*, 1995). In summary, this investigation (Rigo *et al.*, 1995) clearly showed that the protein hydrolysate formulae did not supply babies with the same nutrients as that provided by the whole protein.

Cézard *et al.* (1996) compared the nutritional value of a mixture of intact proteins (casein + whey) and its hydrolysate using three week-old rats as the animal model for the human baby. They found that absolute nitrogen balance was similar for both diets, which suggested an identical utilisation of dietary protein. However, the rats fed with the intact protein mixture gained more weight than those fed with the

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hydrolysate. Based on several previous studies cited in their paper, Cézard and colleagues suggested that the difference in weight gain could be due to increased fat or water deposition in the rats fed the whole protein formula.

In addition, faecal nitrogen excretion was significantly greater in the rats fed the intact protein diet, which suggested that net nitrogen absorption was greater from the hydrolysed formula. However, the amount of nitrogen excreted in the faeces of rats fed with the intact proteins could be due to an increase in amount of microorganism (Cézard *et al.*, 1996).

Cézard, and colleagues also observed a higher DNA content/10 cm of the gut mucosa which they related to hyperplasia of the mucosa of the small intestine when hydrolysed protein was fed. The reasons for this could reflect differences in the availability of amino acids during their absorption, or in the rate of secretion of enzymes, or secretion of jejunal growth peptide (Cézard *et al.*, 1996). The total activity of intestinal enzymes (sucrase, glucoamylase and N-aminopeptidase), as well as the weight and protein content per 10 cm of intestine was similar for the two diets (Cézard *et al.*, 1996). Based on the absorbed nitrogen balance, however, Cézard *et al.* (1996) concluded that the nutritional value of the protein is preserved after hydrolysis.

There are conflicting opinions about the nutritional value of protein hydrolysates in comparison with that of intact protein. In order to resolve the controversy, future research should focus on the standardisation of an animal model and the search for techniques that can give more detailed data.

In summary, the nutritional evaluation of protein hydrolysates has usually been determined by quantifying the amount of nitrogen retained in the body (e.g. Moughan *et al.*, 1990 and Rigo *et al.*, 1995). Indirect estimates have been made by measuring the

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concentration of nitrogenous compounds in blood (Baró *et al.*, 1995; Deutz *et al.*, 1996; Darragh *et al.*, 1997) or in urine as have already described in previously. Although these studies have provided valuable information, they have not clarified completely if protein metabolism is affected at any metabolic stage when a hydrolysed rather than an intact protein is consumed and if this can cause a different nutritional status in the individual after a long period. The data from a nitrogen balance estimates the nutritional status following the consumption of a particular protein over a period of time during which the protein was consumed. In the same way, measurements of the concentrations of blood proteins and urinary nitrogen following consumption of hydrolysates for a period of time, do not provide information about the metabolism of the protein immediately after ingestion. Deutz *et al.* (1996) and Darragh *et al.* (1997) have followed the acute responses to the ingestion of hydrolysed proteins by determining the blood urea and amino acid concentration for periods of up to 6 hr after feeding. Since the excretion of urinary nitrogen was not measured in these experiments, it was not possible to relate the observations on the concentration of nitrogenous metabolites in the blood to urinary nitrogen excretion. Thus, further studies are needed using techniques that will demonstrate the effect of the form of the amino acids (as intact protein or as a peptide) on their metabolism in the period immediately after ingestion. Moreover, these techniques must involve the use of adequate animal models at an appropriate stage of development. To choose an animal as an experimental model is not an easy task. Several characteristics have to be taken account, including anatomical, immunological and physiological factors.

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## 1.5 The Pig as an Animal Model for Evaluation of Human Nutrition

To undertake nutritional research using human subjects is difficult for ethical reasons. For this reason, it would be more appropriate to use a model animal to allow routine and controlled studies.

Several animal models for studying aspects of human nutrition have been proposed, for example rats, guinea pigs, dogs, swine and non-human primates. Each species has been seriously advanced as the best model for the human being at some stage in scientific history. However, some have been found to be unsuitable as knowledge accumulates about their different susceptibility to human diseases, different requirements for essential nutrients, different growth patterns and their cost of purchase, breeding and maintenance (Darragh, 1995; Zhao *et al.*, 1996; Lindberg *et al.*, 1997).

The cost, availability, short time of reproduction, large size of litters and the possibility of obtaining genetically similar animals gives the pig an advantage with respect to others experimental animals (Rowan *et al.*, 1994). In addition, they are willing to cross-foster between litters, they are omnivorous, they can be weaned soon after birth and be fed from a bottle, their growth is rapid and they quickly become accustomed to being kept in metabolism cages. Moreover, samples of blood and lymph can be taken from the live animal and their digestive tract can be cannulated over several regions in order to take specific intestinal samples (Darragh, 1995).

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### 1.5.1 The Piglet as an Animal Model for the Human Baby

Although at birth, the piglet is less developed than the human and subsequently grows and develops faster, it has been seriously considered since the 1970s as a possible experimental model to study aspects of nutrition of the human baby (Moughan *et al.*, 1990, 1992; Darragh and Moughan 1995; Wykes *et al.*, 1996). Moughan *et al.* (1992) made a detailed comparison of the digestive processes between the pig (birth to 6 weeks of age) and the human infant (birth to 6 months of age) in an extensive literature review, which was complemented by experimental work. They concluded that the anatomical, physiological, histological and microbiological characteristics of its digestive system make the three-week-old piglet an excellent animal model for testing aspects of the nutrition of the three-month-old human infant (Moughan *et al.*, 1992). Two years later the piglet was validated as an animal model by Darragh and Moughan (1995), who compared directly the digestibility of amino acids between the piglet and human baby.

In their study, Darragh and Moughan (1995), found that there were small but statistically significant ( $P < 0.001$ ) differences in the apparent faecal digestibility of dry matter, organic matter and total nitrogen between the species. The figures for faecal digestibilities for most of the amino acids, however, were not significantly ( $P > 0.05$ ) different. These results show that the protein digestibility at the end of the gastrointestinal tract in the piglet seems to be similar to that in the human infant. However, this method for determining the amino acid digestibility underestimates the protein digestibility, because it does not consider the degradation of amino acids in the large intestine due to microbial proteolysis (McNeil, 1988).

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### 1.5.2 The Growing Pig as an Animal Model for Studies in Human Nutrition

Several studies have included the growing pig as an animal model to study the quality of dietary components. Many of these studies have determined absorption (Deutz *et al.*, 1996), utilisation (Darragh *et al.*, 1997), and digestibility (Butts *et al.*, 1993a; Rowan *et al.*, 1994) of dietary proteins as individual ingredients or as a mixed diet. The digestibility of a dietary component is an indirect measure of the disappearance of this component from the gastrointestinal tract. To know the extent of the digestibility and the utilisation of a protein source becomes particularly important when the daily intake of protein by the individual is low. These include people in developing countries or hospitalised or the chronically- ill. Early studies have shown the superiority of the pig as an animal model in this matter. Forsum *et al.*, (1981) evaluated the protein quality of two mixed diets and compared true faecal protein digestibility between adult humans, the growing pig and the growing laboratory rat. Although it was reported that the digestibility of protein was similar between species, several facts limited the comparison. Forsum *et al.*, (1981) noted that there are fewer practical problems in feeding diets of poor protein quality to the pig than the rat. They indicated that the pig is a better animal because it is a meal-eating species and does not practice coprophagy as does the rat.

In a more recent study Rowan *et al.* (1994) evaluated the suitability of the growing pig as a model animal for man in determining ileal amino acid digestibility and the apparent faecal digestibility of dietary energy. Rowan *et al.* (1994) found that there were no significant differences between adult human subjects and pigs for true ileal dietary amino acid digestibility except for threonine, phenylalanine, cysteine and methionine. In addition, they did not find significant differences between species for the ileal digestibilities of dry matter and the faecal digestibility of gross energy. Based

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on these results, Rowan *et al.* (1994) concluded that the growing pig would appear to be a useful model for humans in the determination of dietary protein digestibility. However, more studies must be done using a wider range of foods.

Animal models are necessary for investigate the nutritional aspects of humans. Pigs have been used as a suitable the experimental animal to research the nutritive value of protein hydrolysates. The choice to used the piglet or the growing pig will depend on the end-consumer of the hydrolysate.

## 1.6 Conclusions

Protein hydrolysates are used to meet the nutritional needs of people under medical care. Hydrolysates are also frequently the alternative food for infants with allergies, or for infants at risk of developing allergies. The physicochemical properties of hydrolysates also make them attractive nutritive ingredients in other food products, such as sport drinks. Whereas the studies in the medical field focus on proving the superior absorption of amino acids from diets based on hydrolysates over those based on free amino acids, the studies on allergic patients have two main objectives. One is to assess their hypoallergenicity, and the other their nutritional equality to the intact protein. The latter has received increased interest, as the perceived nutritional benefits of hydrolysates attract other consumers.

The present review shows that comparative data reported on the nutritional benefits of either an intact protein or its hydrolysate peptides are not consistent. There are also limited data on the effects of proteins, and protein hydrolysates on metabolism under similar experimental conditions where factors as the animal model, the enzymes used for the hydrolysis, the degree of hydrolysis and the size distribution of the peptides in the hydrolysates are kept constant. Perhaps this is the reason for the

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inconsistency in the results of measurements of nitrogenous metabolites in blood or urine in various studies.

There is a need to develop a technique that can give more detailed data of the protein metabolism of a protein hydrolysate. Traditional techniques such as the nitrogen balance evaluate the nutritional status of an individual as a long-term response. There is lack of information about the effect that the distinct chemical form of amino acids (intact protein and hydrolysed protein) has on the urinary nitrogen excretion as soon as the respective protein is ingested. Therefore, the present study aims using a novel technique to compare the effect of the molecular form of a whey protein concentrate and its hydrolysate on the urinary excretion of total N, urea N, ammonia N as an immediate response to a meal and to compare it with a long-term response. The growing pig was chosen as a model animal for the human adult in the following trial.

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## **CHAPTER 2**

# **MATERIALS AND METHODS**

This research involved the development of a novel experimental technique for monitoring the urinary excretion of nitrogenous (N) metabolites over time following a meal. Because the technique used had not been described previously, it was necessary to evaluate it in a preliminary trial. The methodology used in the subsequent main trial was based on experience gained in the preliminary trial. As a consequence, the materials and methods section is reported in two parts; one for the preliminary trial work, and the second pertaining to the main trial.

The Massey University Animal Ethics Committee approved all the aspects related to the handling of animals for the preliminary and main trials.

### **2.1 Preliminary Trial**

#### **2.1.1 Animals**

Eight female Landrace x Large White pigs (average age, 105 days; mean body weight  $\pm$  SE,  $44.0 \pm 0.76$  kg) were purchased from a commercial pig farm in Foxton, New Zealand. The pigs were confined throughout the experiment in individual metabolism cages that allowed complete urine collection and the separated collection of faeces. The pigs were housed at a temperature of  $22 \pm 1$  °C and under a constant 10 hour light: 14 hour dark cycle in the Animal Physiology Unit (APU), at Massey University, Palmerston North, New Zealand.

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## 2.1.2 Diet and Feeding

### 2.1.2.1 Experimental diets

Two experimental diets, one containing intact protein, and one containing a protein hydrolysate, were formulated to supply all the nutrients required for the normal growth of the pigs. The diets contained similar concentrations of nitrogen (10% w/w of the diet) and amino acids, but differed in that the source of amino acids in one was a hydrolysate of an intact protein that was the source of amino acids in the other. The hydrolysate consisted of a mixture of free amino acids, small and large peptides.

The diet (IP) containing the intact protein was prepared using a whey protein isolate, lactalbumin (ALATAL 825, Anchor Products HPU, Tirau, New Zealand). The diet (HP) containing the hydrolysate was formulated using a hydrolysed product (ALATAL 821, Anchor Products HPU, Hautapu, New Zealand) prepared from the same whey protein isolate as used for the IP diet. Anchor Products provided the following information about the intact and hydrolysed proteins. The enzymes used for the manufacture of the whey protein hydrolysate ALATAL 821 were from natural sources. Because of commercial sensitivity details about the enzymes used remain confidential. ALATAL 821 is a mixture of amino acids, peptides and polypeptides obtained by controlled enzyme treatment of the whey protein isolate. The mean molecular weight distribution of the peptides was: MW > 20,000: 0%; 20,000 > MW > 10,000: 1%; 10,000 > MW > 5,000: 3%; 5,000 > MW > 1,500: 19%; 1,500 > MW > 1,000: 14%; 1,000 > MW > 500: 21%; MW < 500: 42%. A chromatogram of ALATAL 821, showing the molecular distribution of the peptides is presented in Figure 6. The mean molecular weight distribution of the peptides was determined by exclusion-HPLC using an TSK-Gel G2000 SW<sub>XL</sub> x 2 with TSK Guard Column. The eluent for the column was 36% acetonitrile with 0.1% trifluoroacetic acid. The detection was at  $\lambda$  205 nm in the UV spectrum.

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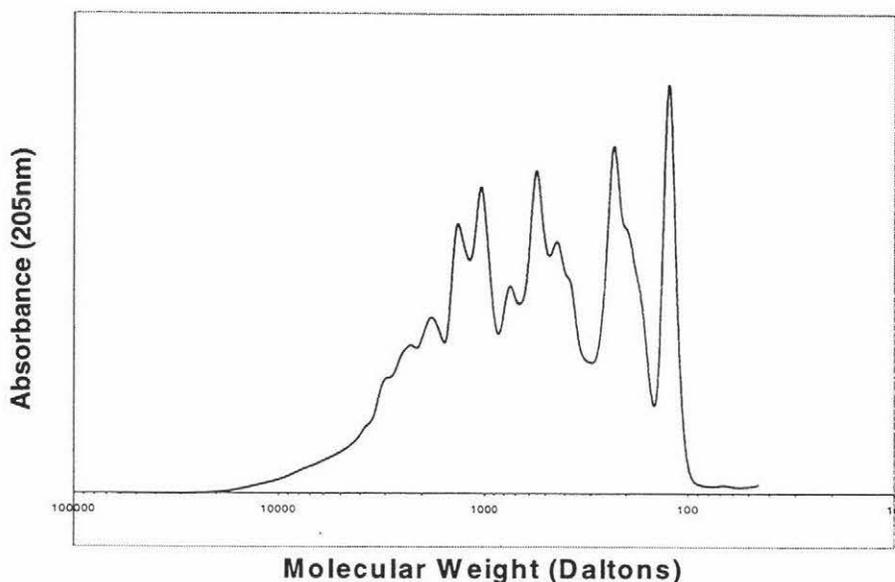


Figure 5. Molecular weight profile of ALATAL 821. Information provided by Anchor Products HPU, Tirau, New Zealand.

ALATAL 821 had a degree of hydrolysis (DH), a measure of the extent of the hydrolytic degradation of the protein (Adler-Nissen, 1986), of 25.2%. The DH of ALATAL 821 was detected by a modified *o*-phthaldialdehyde Method (Frister *et al.*, 1988).

The mean molecular weight for ALATAL 821 was 390 Daltons, as determined by calculation from the results of measuring the degree of hydrolysis. The molecular weight of more than 90% of the peptides was below 5,000 Daltons. The free amino acid content, determined by reverse phase-HPLC, was less than 11%.

The residual  $\alpha$ -lactalbumin was < 0.1% determined by SDS PAGE analysis. The enzymatic hydrolysate of lactalbumin (ALATAL 821) was developed, and is commercially manufactured, especially for use in hypoallergenic infant formulae. Antigenicity of the hydrolysate was 10,000- to 100,000-fold lower than the native whey protein. The antigenicity was determined by competitive ELISA test, in which the

inhibition of the binding between a standard antigen and the antibody by the test protein or its hydrolysate is measured (Figure 7).

A brief description of the basic process for the manufacturing ALATAL 825 and ALATAL 821 is outlined in Appendix 2.

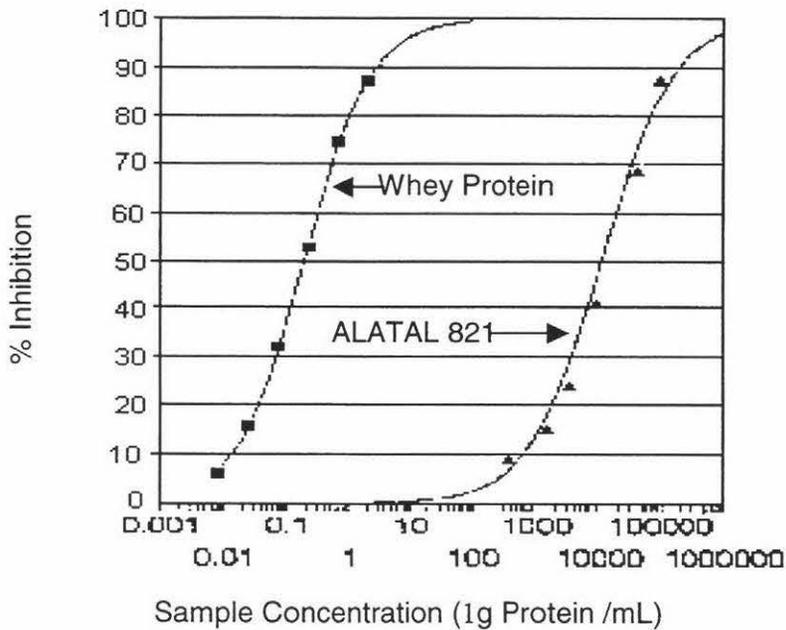


Figure 6. Competitive ELISA of ALATAL 821 and ALATAL 825. Information provided by Anchor Products HPU, Tirau, New Zealand.

A key requirement in this trial was the preparation of an hydrolysate from the same batch of intact lactalbumin used as the source of intact protein. This allowed a comparison between two diets with near identical amino acid composition and differing only in the physical size of constituent peptides. The ingredient and nutrient compositions of the diets are given in Table 3.

Table 3. Ingredient and nutrient composition of the experimental diets <sup>1</sup>

Ingredients	Intact Diet (g/kg as is)	Hydrolysed Diet (g/kg as is)
Intact Lactalbumin <sup>2</sup>	114.0	-
Hydrolysed whey protein isolate <sup>3</sup>	-	115.1
Vitamin & trace element mix <sup>4</sup>	2.5	2.5
Dicalcium phosphate <sup>5</sup>	25.0	25.0
Sodium chloride <sup>6</sup>	3.0	3.0
Potassium carbonate <sup>7</sup>	5.0	5.0
Magnesium sulphate <sup>7</sup>	2.0	2.0
Soybean oil <sup>8</sup>	31.5	34.8
Sucrose <sup>9</sup>	67.5	68.5
Cellulose <sup>10</sup>	50.0	50.0
Starch <sup>11</sup>	699.5	694.1
<b>Nutrients</b>		
Crude protein (g/kg diet, dry matter)	100.00	100.00 <sup>12</sup>
Carbohydrate (g/kg diet, dry matter)		
Sucrose	67.50	68.50
Lactose	2.51	1.50
Fat (g/kg diet, dry matter)		
Soybean oil	31.50	34.80
From protein source	3.53	0.23
Energy (DE kJ /kg as is) <sup>13</sup>	14.33	14.41
Amino Acid Composition (mg/100g) <sup>14</sup>		
Essential Amino acids		
Isoleucine	4.80	4.76
Leucine	11.43	9.88
Lysine	8.95	8.71
Methionine	2.57	2.02
Phenylalanine	3.35	2.95
Threonine	4.64	4.27
Valine	5.09	4.30
Non-Essential Amino Acids		
Histidine	1.77	1.90
Alanine	5.19	4.76
Arginine	2.83	2.55
Aspartic acid	9.78	8.97
Cysteine/cystine	2.29	1.55
Glutamic acid	15.01	12.94
Glycine	1.80	1.64
Proline	4.82	4.49
Serine	3.77	3.02
Tyrosine	3.47	2.69

<sup>1</sup> Experimental diets were calculated to have 100 g crude protein per kg diet supplied by their respective protein source.

<sup>2</sup> ALATAL 825, Anchor Products HPU, Tirau, New Zealand.

<sup>3</sup> ALATAL 821, Anchor Products HPU, Hautapu, New Zealand.

<sup>4</sup> Technik Products, Auckland N.Z. Supplied (per kg diet): vitamin A 7,500 IU, vitamin D<sub>3</sub> 1,500 IU, vitamin E 50 mg, vitamin K 2 mg, vitamin B<sub>1</sub> 1.5 mg, vitamin B<sub>2</sub> 3 mg, vitamin B<sub>6</sub> 3 mg, vitamin B<sub>12</sub> 15 µg, Pantothenic acid 11 mg, Folic acid 0.25 mg Biotin 20 µg, Niacin 15 mg, Choline 135 mg, Manganese 42.5 mg, Iron 100 mg, Copper 180 mg, Zinc 120 mg, Iodine 1.5 mg, Cobalt 2 mg, Selenium 300 µg.

<sup>5</sup> Kemira Kemi AB, BOLIFOR Products, Helsingborg, Sweden.

<sup>6</sup> Iodised Table Salt, Pam's Products Ltd., Auckland, New Zealand.

<sup>7</sup> BDH Chemicals Ltd., Poole, England.

<sup>8</sup> Davis Trading Company Ltd., Palmerston North, New Zealand.

<sup>9</sup> New Zealand Sugar Company Ltd., Auckland New Zealand.

<sup>10</sup> Avicel crystalline cellulose, Asahi Chemical Industry Company Ltd., Osaka, Japan.

<sup>11</sup> Wheaten Cornflour, Manildra Group of Company, Auburn, Australia.

<sup>12</sup> Molecular distribution of the whey protein peptides MW > 20,000: 0%; 20,000 > MW > 10,000: 1%; 10,000 > MW > 5,000: 3%; 5,000 > MW > 1,500: 19%; 1,500 > MW > 1,000: 14%; 1,000 > MW > 500: 21%; MW < 500: 42% (Data on the grading analysis provided by Anchor Products HPU, Hautapu, New Zealand)

<sup>13</sup> Calculated figure from crude protein, fat and carbohydrate content.

<sup>14</sup> Amino acid profile for ALATAL 821 (Whey protein hydrolysate) and ALATAL 825 (Lactalbumin). Results from the analysis performed in the Analytical Laboratory, IFNHH, Massey University, Palmerston North.

### **2.1.2.2 Diet preparation**

The diets were made in batches of 100 kg at the Feed Processing Unit, Massey University. The premix containing the vitamins and minerals, protein, sodium chloride, dicalcium phosphate, potassium carbonate, magnesium sulphate, sucrose, and cellulose, was made in a 50 kg mixer (The Hobart MFG Ltd., England). Then the premix was transferred to a wetmash mixer of 150 kg capacity (The Bonser Engineering Co., England) and the starch powder was added. After being mixed, part of the starch and premix material was transferred back to the 50 kg mixer and soybean oil was slowly added until the mixture was homogeneous and free flowing. Finally, the mix of oil and starch powder was returned to the rest of the material remaining in the wetmash mixer and blended again. To maintain uniformity of the experimental diets throughout the trial, sufficient quantities of all the non-protein ingredients were set aside at the Feed Processing Unit, at ambient temperature, to make all the batches needed. The prepared diets were stored in paper sacks and transported to the APU, where they were held at ambient temperature until required.

### **2.1.2.3 Sampling of diets**

Samples of the diets were collected at the time of preparation. Sub-samples of these diets were stored until sent for analysis of total nitrogen amino acid, and moisture content, to the Analytical Laboratory, Institute of Food, Nutrition and Human Health (IFNHH), Massey University, Palmerston North, New Zealand. See section 2.1.5 for the analytical methods.

### **2.1.2.4 Schedule for the preliminary trial**

The preliminary trial consisted of one 7d adaptation period and two consecutive 7d experimental periods.

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### 2.1.2.5 Feeding.

The pigs were fed their diets at the rate of 10% of each pig's metabolic body weight ( $\text{kg}^{0.75}$ ) per day, with the daily allocation split between two equal meals. This equated to a mean  $\pm$  SE intake by the pigs during the adaptation period, and the first and second 7d experimental periods, of  $1.71 \pm 0.018$  kg/day,  $1.81 \pm 0.026$  kg/day and  $1.98 \pm 0.027$  kg/day, respectively.

The pigs were weighed and metabolic body weights calculated at the beginning of each 7d period. As part of the normal routine, feeding was scheduled to begin at 0730 h and 1530 h. After each meal, the pigs had access to water *ad libitum*.

The pigs were fed according to their ascending number code. The first pig to be fed was pig 1 and the last pig to be fed was number 8. The time between feeding successive pigs was 10 min so that the last pig was fed 70 minutes after the first. This ensured that the researchers were able to apply the same experimental routine to all pigs with exactly the same time interval between meals.

To obtain complete acceptance of the new diets, the pigs were introduced gradually to the flavour and texture of the experimental diets. Thus, during the adaptation period, pigs were fed a combination of a commercial grower diet (Growtec pig grower, Feed Processing Mill, Palmerston North New Zealand), the composition of which is presented in Table 4, and the test diets. The proportion of the experimental diets fed was increased each day so that by day 6 all pigs were receiving an equal mixture of the two experimental diets. The proportion of each test diet and grower diet fed during the adaptation period is presented in Table 5. Feeding a 50:50 combination of the two test diets facilitated the change over and acceptance of the test diet to which each pig had been randomly allocated to after this period. During the experimental

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period, pigs were fed with 100% of their allocated diets. Those pigs fed the hydrolysed diet in the first experimental period received the intact diet in the second experimental period and *vice versa*.

Table 4. Composition of a commercial grower diet fed to 37-53 kg pigs

Ingredients	g/kg of diet
Barley <sup>1</sup>	809.6
Meat and bone meal <sup>2</sup>	100.0
Dried Blood <sup>2</sup>	40.0
Tallow <sup>2</sup>	5.0
Fishmeal <sup>3</sup>	15.0
Soybean meal <sup>3</sup>	25.0
Methionine <sup>4</sup>	0.4
Lysine <sup>4</sup>	1.0
Vitamin & mineral premix <sup>4</sup>	2.5
Salt <sup>5</sup>	1.5

<sup>1</sup> Wrightson Nutrition, Palmerston North, New Zealand.

<sup>2</sup> Lakeview, Levin, New Zealand.

<sup>3</sup> Jossco NZ Limited, Auckland, New Zealand.

<sup>4</sup> Nutritech International, Auckland, New Zealand.

<sup>5</sup> Davis Trading, Palmerston North, New Zealand.

Table 5. Composition of the diet fed to all the pigs for each day of the 7d adaptation period

Day	Grower Diet (%)	Hydrolysed Diet (%)	Intact Diet (%)
1	100	0	0
2	100	0	0
3	70	15	15
4	50	25	25
5	30	35	35
6	0	50	50
7	0	50	50

The powdery nature of the diets made it difficult for the pigs to eat so, from days 1 to 5 of the adaptation period, fresh water (approximately 700 ml) was mixed with the diet in the feeding container. From day 6 of the adaptation period until the end of the experiment a handblender (Handblender MR555MCA, Braun, Spain) was used to mix the powdered diet into a viscous but drinkable "milk shake".

During the adaptation period the pigs were trained to eat their allocated meal in a short time period. This was achieved by offering the pigs food for 15 minutes and then removing anything remaining after this time. By the end of the adaptation period

all pigs were consuming their meal within 6 minutes and during the experimental period the meals were consumed within 3 - 4 minutes.

Spillage of the diet was avoided during feeding by placing an iron sheet in front of the pig that prevented it from having access to the food while the food was being placed in the food container.

### **2.1.3 Experimental Procedure**

The utilisation of dietary protein nitrogen in this study was evaluated following by monitoring the urinary output of nitrogenous metabolites both over an extended period and as an acute response. These two procedures are explained next.

#### **2.1.3.1 Nitrogen balance**

On day 4 of both experimental periods, urine and faeces were collected separately for a period of 24 hr. Urine was collected into a bucket containing 5 ml of 6 M HCl (3.2 ml/L of urine collected) to prevent the growth of microorganisms, which thus avoided the volatilisation of ammonia. The bucket was emptied every 12 hours and the HCl replaced. At the end of the 24 hr collection period, the buckets and funnels used to collect the urine from each pig were sprayed with distilled water, which was also collected. This helped to minimise the loss of urinary nitrogen. The urine volume was measured using a 1 litre cylinder and then the urine was transferred to a plastic container, which was capped and kept in a refrigerator at (4°C) awaiting sub-sampling. Faeces were collected from the wire mesh on the floor of each crate at the end of 24 hr. Faeces were placed in a weighed container, which was closed and frozen until analysed. The amount of food eaten during the nitrogen balance (NB) was recorded and sub-samples of urine and faeces were taken.

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### *2.1.3.1.1 Sub-sampling of urine and faeces*

Representative sub-samples of urine taken from the 24 hr NB were sent to the Physiology Laboratory, IFNHH, Massey University, Palmerston North, New Zealand for analysis of creatinine, ammonia and urea, and to the Analytical Laboratory, IFNHH, Massey University, Palmerston North, New Zealand, for determination of total nitrogen content. See section 2.1.5 for the analytical methods.

The entire frozen faecal output collected from each pig during the 24 hr NB, was freeze dried (Cuddon Freeze Drier 0610, WGG Cuddon Ltd, Blenheim, New Zealand) and ground (Ultra Centrifugal Mill ZM100, Retsch GmbH Co. KG, Haan, Germany). A representative sub-sample was sent to the Analytical Laboratory, IFNHH, Massey University, Palmerston North, New Zealand for analysis of the total nitrogen and dry matter content. See section 2.1.5 for the analytical methods.

## **2.1.4 Acute Urine Collection**

### **2.1.4.1 Implantation of bladder catheter**

A catheter was implanted under general anaesthesia in the bladder of each pig on day 5 of each experimental period. This allowed for the accurate and regular collection of urine during days 6 and 7. For the remainder of the thesis, this technique will be referred to as the “acute urine collection” (AUC) and will be preceded by a serial number, which indicates the experiment being referred to. For example, “3<sup>rd</sup> AUC” relates to the third experiment of this type. Although 1<sup>st</sup> and 2<sup>nd</sup> AUC were performed during the preliminary trial, only the data for the main trial (3<sup>rd</sup> AUC and 4<sup>th</sup> AUC) are presented in this thesis.

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#### 2.1.4.1.1 Surgery

All pigs underwent surgery, using full aseptic procedures. Food was not available to the pigs for 16 - 17 hr before surgery but water was freely available.

Pigs were sedated using a combination of xylazine hydrochloride (Phoenix Pharm Distributors Ltd., Auckland, New Zealand) with Zoletil 100 (an equal mixture of Zolazepan hydrochloride, 50 mg/ml and tiletamine hydrochloride, 50 mg/ml, Virbac Laboratories, France). The Zoletil 100 and xylazine hydrochloride were mixed in the same syringe and administered at dose rates of 4.0 mg/kg and 2.2 mg/kg, respectively, by deep intramuscular injection into the neck.

The sedated pig was prepared for the surgery using the following procedure. Once anaesthetised, the pig's hindquarters were shaved and the perineal region was cleaned using soap and disinfectant and dried with sterile swaps. The pig was placed in dorsal recumbency, and a sterile Foley Catheter (silicone coated, 2 way, 8 Fr, 3 cc Sherwood Medical, USA) was inserted into the bladder *per vaginum*. This required the operator to palpate the external urethra *os* with the index finger of one hand while the catheter was manipulated into the urethra with the other hand. A water-base lubricant (K-Y lubricant, Johnson & Johnson, Australia) was applied on the balloon ending of the catheter to facilitate palpation of the urethra. As much of the working length of the catheter as possible was advanced into the bladder. Aspirating urine through the catheter indicated that the catheter was correctly placed. Once the catheter was in the bladder, the balloon of the catheter was inflated with 3 ml of isotonic saline and the catheter withdrawn until the resistance of the balloon could be felt in the neck of the bladder. Then a plastic multi-purpose tubing adaptor (Cook 139 308, catalogue; V-PFLLA-VTA-L, Cook Veterinary Products, Brisbane, Australia) with an injection site as a stopper was screwed into one of ducts at the exterior ending of the catheter. The

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surplus catheter was then sutured to the skin dorsal to the base of the tail, and secured with adhesive type. Aseptic techniques were maintained throughout the procedure.

After insertion of the catheter, the pig was transferred to a recovery space until it started to regain consciousness (sounds), and was then transferred to its crate. No water or food was made available to the pigs until they were fully alert, able to stand firmly and to demand their food.

When the pigs had fully recovered from the anaesthesia, they were fed their allocated meal for the evening. The catheters were opened to allow voiding of urine until the time of the sampling, which commenced approximately 16 hours later. Once implanted, the catheters required little maintenance except periodic inspection for blockages.

#### **2.1.4.2 AUC sampling**

After the meal that followed surgery, the pigs were fasted for approximately 16 hr before being fed a half ration of their daily intake at time 0. Pigs were given free access to fresh water at all times. Prior to feeding, and half an hour before the first urine sample was collected, any urine in the bladder was drained through the catheter and the Luer Lock fitting at the end of the catheter was plugged (Luer Lock, Baxter Healthcare Corporation, USA). Thirty minutes later and 30 minutes before feeding, the first urine sample (-0.5 hr) was collected. To do this, the plug in the catheter was removed, and a 100 ml hypodermic syringe was attached to the Luer Lock connection. The urine that had accumulated in the bladder was removed by gentle suction into the syringe. Collection was considered complete when no more urine could be obtained and resistance was felt to increase in the syringe. The volume of urine was measured and the pH of the urine adjusted to between pH 3 - 4 with solutions of either 0.1 M HCl

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or 0.1 M NaOH. Once at the required pH the individual urine samples were stored in plastic containers at 4 °C. The procedure was repeated for each pig at 0 (immediately before feeding), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10, 12, 14, and 16 hours relative to the time of feeding. Pigs received their second half of their daily ration once the collection of the 16 hour sample was completed.

#### **2.1.4.3 Removal of the catheter**

After the last sample of urine was collected the catheter was removed. To do this, the external sutures were removed, the balloon of the catheter emptied and the catheter slowly pulled from the bladder. Pigs were then injected intramuscularly in the neck with Albipen LA (Intervet International, B. V. Boxmeer, Holland), at 0.25 ml/kg body weight, as a prophylactic to minimise the risk of infection of the urinary tract. Once the catheters had been removed, the pigs were weighed and returned to their normal feeding regimen until the next AUC was taken.

#### **2.1.4.4 Preparation of the urine samples for analysis**

In preparation for analysis, the volumes of the urine samples were remeasured and the pH checked with pH indicator paper (Acilit pH 0-6, Merck, Germany). Sub-samples of approximately 5 ml were sent to the Physiology Laboratory, IFNHH, Massey University, Palmerston North, New Zealand and stored at -20 °C until analysed for urea, ammonia and creatinine.

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### 2.1.5 Chemical Analysis of Tested Proteins, Excreta and Diet Samples

#### Total Nitrogen Analysis

The total nitrogen content of the lactalbumin (ALATAL 825), whey hydrolysate protein (ALATAL 821), diet and faecal samples was obtained by the Dumas method (Association of Official Analytical Chemists; AOAC, 1995) using a Leco CNS Analyser (Leco Corporation, MI, USA). Merck analytical reagent grade ammonium ferrous sulphate  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , was used as a standard. Obtaining an estimate of the crude protein content of the protein sources allowed for accurate isonitrogenous formulation of the diets.

The total nitrogen content in the urine samples collected during the 24 hr NB was determined in duplicate by the Kjeldahl method (AOAC, 1995) using the Kjeltac 1030 system (Tecator Ab, Hoganas, Sweden), and a Digester 2020 (Tecator Ab, Hoganas, Sweden).

#### Dry Matter Analysis

The dry matter content of the lactalbumin (ALATAL 825), whey hydrolysate protein (ALATAL 821), diet and faecal samples was determined by the Moisture Air Oven Method (AOAC, 1995). Duplicate samples were dried in a forced air oven at a temperature of 105 °C until a constant weight was achieved, and the dry matter content was determined (AOAC, 1995).

#### Amino Acid Analysis

The analysis of amino acids involves acid hydrolysis of protein, with subsequent identification and quantification of the amino acids. Since fat can interfere with the

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reaction of the acid and the peptide bond, the samples of protein had to be defatted prior acid hydrolysis.

Freeze-dried samples of the lactalbumin (ALATAL 825) and whey protein hydrolysate (ALATAL 821) were placed into a Soxhlet apparatus (AOAC, 1995) and defatted with light petroleum ether (b.p. 60 °C - 80 °C).

Two replicates (5 - 7 mg /replicate) of the defatted lactalbumin or whey protein hydrolysate were accurately weighed into acid-washed pyrolysed (650 °C, overnight) glass test tubes (10 ml) and 1 ml of redistilled 6 M-HCl (containing 1% phenol w/v) was added to each tube. All tubes were closed under vacuum and hydrolysed at the temperature of  $110 \pm 2$  °C, for 24 hr. An internal standard (50  $\mu$ l of 4 mM norleucine : Sigma, St. Louis, MO, USA Code: n-8513) was added to each tube, once the time of reaction was over. The acid-hydrolysed product was further concentrated under vacuum (Automatic Speedvac Concentrator AS290, Savat Instruments Inc., Farmingdale, NY). Prior to analysis, two ml of loading buffer (0.2 M-sodium citrate, pH 2.2) was added to each tube. The samples were then analysed for individual amino acid content using ion-exchange chromatography (Millipore Corporation, Waters, Chromatography Division, Milford, MA, USA). A reaction with ninhydrin was used for detect the amino acids in the UV spectrum (AOAC, 1995).

The amino acids methionine and cysteine were detected separately from the rest of the amino acids by oxidising them prior to the acid-hydrolysis step previously described. Cysteic acid and methionine sulphonate are products of the oxidative reaction of performic acid with cysteine and methionine, respectively (Moore, 1963). The oxidised amino acids were passed through an ion-exchange HPLC system (Millipore Corporation, Waters, Chromatography Division, Milford, MA, USA) with a

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post column *O*-phthalaldehyde derivitisation and fluorescence detection. Tryptophan, which is destroyed during the acid hydrolysis step of amino acid analysis, was not quantified. The concentration in gram weight of each individual amino acid was calculated using the concentration in moles and the free molecular weight (AOAC, 1995).

#### Creatinine, Ammonia and Urea Concentration Analysis

Each sub-sample of urine, from the NB and AUC experiments, was thawed and centrifuged at 10,000 rpm for 30 seconds to remove particulate matter (SORVALL MC12 V, Dupont, USA) before being further analysed. The concentrations of urea, ammonia and creatinine in the urine were measured in duplicate using a centrifugal analyser (CobasFara II Autoanalyser, Roche, USA). These techniques are briefly described next.

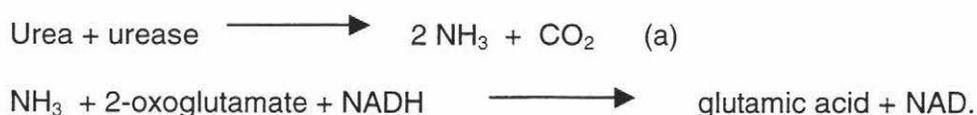
The urinary creatinine, urea and ammonia concentrations were determined using commercial kits. The creatinine concentration was determined by a kinetic modification of the Jaffé reaction according to Larsen (1972) using a creatinine ROCHE Kit (Roche Diagnostic Systems, New Zealand). Urea concentrations were measured with an enzymatic assay (Tiffany *et al.*, 1972) using a commercial available kit (Urea ULTIMATE 5, Roche Diagnostic Systems, New Zealand). The ammonia concentration was determined also enzymatically using a commercially available kit (Ammonia kit no.171-UV, Sigma, USA). The methodology was based on the reaction described by Neeley and Phillipson, (1988) and van Anken and Schiphorst, (1974).

The accuracy and precision in the three assays described above was in part a factor of the standards used and repetitions in each run. Controls of high ammonia (Sigma diagnostics, St. Louis, USA) were used to test the accuracy of the ammonia analysis and Control Serum P (human) (Roche Diagnostics Systems, Basel) was used

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to test the creatinine and urea analyses. The precision (intra-assay variation) of the urea, creatinine and ammonia assays was determined by including the respective standard sample in each run of 22 samples.

Urea was measured by using urease and measuring the amount of ammonia produced, as show below



In the absence of endogenous ammonia, this will give a correct value for urea. However, ammonia is always present in urine samples and the urea result must be corrected for the presence of free ammonia. From equation (a) above, it follows that every two moles of free ammonia present in the urine are incorrectly reported as one mole of urea. By determining the amount of free ammonia present, the correct urea concentration can be calculated using the following formula

$$[\text{Urea}]_{\text{true}} = [\text{Urea}]_{\text{apparent}} - ([\text{Ammonia}]_{\text{meas}} / 2)$$

Where:

$[\text{Urea}]_{\text{true}}$  = true urea concentration.

$[\text{Urea}]_{\text{apparent}}$  = apparent urea concentration

$[\text{Ammonia}]_{\text{meas}}$  = measured ammonia concentration

The total amount of nitrogen excreted was calculated from the volume of urine and the concentration of urea, creatinine and ammonia in the urine on the basis that urea contains 46.7% N, creatinine 37.2% N, and ammonia 82.2% N, on a molecular weight basis.

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All excretions of the urinary nitrogen metabolites were expressed per unit of metabolic body weight (MBW). The MBW was calculated as the body weight (kg) elevated to the power 0.75.

## 2.1.6 Data Analysis

### 2.1.6.1 Body weights, average dietary gains and feed intakes

The mean of body weights, average dietary gains and feed intakes for the intact diet and the hydrolysed diet were compared by a simple one-way analysis of variance in the SAS statistical package, (SAS for Windows version 6.12, SAS Institute Cary, NC, USA). The effects were considered significant at  $P < 0.05$ .

### 2.1.6.2 Nitrogen balance

A Nitrogen Balance ( $\text{g N } 24 \text{ hr}^{-1} \text{ kg}^{-1} \text{ MBW}$ ) was calculated as the difference between daily nitrogen intake and daily nitrogen output (daily urine nitrogen excretion plus daily faecal nitrogen excretion).

The mean nitrogen balances for the intact diet and the hydrolysed diet were compared by a simple one-way analysis of variance using the general linear method procedure (GLM) in the SAS statistical package (described on 2.1.6.1). The effects were considered significant at  $P < 0.05$ .

### 2.1.6.3 Acute urine collection

The data from the 1<sup>st</sup> and 2<sup>nd</sup> AUCs were not reliable because of a problem with the expulsion of catheters and consequently urine collections were incomplete. For this reason these data were not analysed.

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## 2.2 Main Trial

Most of the experimental procedures in the main trial were the same as that established in the preliminary trial with the following exceptions.

### 2.2.1 Animals

Sixteen female Landrace x Large White pigs (average age 90 days; mean body weight  $\pm$  SE,  $37.0 \pm 0.52$  kg) were obtained from a commercial pig farm in Foxton, New Zealand. The pigs were allocated individually into the metabolic cages and kept at the same environmental conditions as in the preliminary trial.

### 2.2.2 Diet and Feeding

The main trial involved a 6d adaptation period followed by three consecutive experimental periods. The first experimental period consisted of 8d whereas the following two consisted of 7d.

The schedule for the adaptation period followed that of the preliminary trial except that it was reduced from 7 to 6 days. The introduction of the diet followed the same schedule as described in the preliminary until day 6 rather than until day 7. On day 7, the first experimental period started.

The composition of the diets, their preparation and daily feeding schedules were the same as those used in the preliminary trial. However, the period of time between feeding one pig and the next was reduced to 5 minutes rather than 10 minutes as in the preliminary trial.

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At the start of the first experimental period, each pig was allocated randomly to either the hydrolysed diet or the intact diet (8 pigs /diet) and they remained on this diet until the end of the trial.

The feeding regimen on the days of acute urine collection (days 6 - 7) followed the same procedure as in the preliminary trial only during the first 7d experimental period but not during the second 7d experimental period. For the second 7d experimental period, pigs were fed half of their daily intake in the morning, time designated as 0, and the other half 8 hours later, as for a normal feeding-day.

### **2.2.3 Experimental Procedure**

After the adaptation period, a NB was performed during the first 8d experimental period and an AUC was conducted at the end of each of the two 7d experimental periods, during days 6 and 7. During the AUC periods, the water used to prepare each meal was fixed at 1.3 ml/g of food. The amount of water added to the dry feed was determined from the experience gained in the preliminary trial as to the proportions needed to give a mixture that was practical to handle and acceptable to the pigs.

In a divergence from the preliminary trial, a sample of each diet was collected during the NB and AUC, rather than only after the preparation of the diets.

#### **2.2.3.1 Nitrogen balance**

The pigs were fed their allocated diets for 4 days and then urine and faeces were collected for a further period of 4 days. This was a deviation on the preliminary trial when the NB was for 24 hr only.

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The daily collection of urine followed the same procedure as that used in the preliminary trial. After 4 days of collection, the total urine collected was sub-sampled for analysis. The faeces were collected twice each day, weighed and kept in a refrigerator at 4 °C for later sub-sampling and analysis.

#### *2.2.3.1.1 Sub-sampling of urine and faeces*

Each daily faecal sample was homogenised and 25% of it taken to make a composite sample for the 4-day collection period. The 4-day composite samples were freeze-dried and ground before being sent to the laboratory for analysis of total nitrogen and dry matter (cf. preliminary trial).

Samples representing 5% of the daily volume of urine for each of the four days were mixed in a container. Once mixed, a 4-day sub-sample was sent to the laboratory for analysis of total nitrogen, urea, creatinine and ammonia content. The nitrogen content of the samples of urine and faeces was reported as mg /24 hr period.

#### **2.2.3.2 AUC sampling**

Catheters were inserted into the bladders on day 5 of each 7d period for the AUC performed during days 6 and 7.

##### *2.2.3.2.1 Implantation of bladder catheter*

In contrast to the preliminary trial, a longer catheter (Foley Catheter two way, 12 cm long; Baxter, Malaysia) with a balloon of greater capacity (30 ml) was used in this main trial. This catheter allowed a more complete urine collection because the larger size of balloon facilitated retention of the catheter in the pig's bladder until the end of the collection period, eliminating the problem of catheter expulsion as happened in the

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preliminary trial. All aspects of implantation of the catheter, however, including preparation of the pig for the surgery, the procedure for implantation of the catheter, care after surgery, the dosage of anaesthetics, feeding after surgery, the fasting periods, and the administration of a prophylactic antibiotic were the same as those described for the preliminary trial.

#### *2.2.3.2.2 Collection of urine samples*

For the 3<sup>rd</sup> AUC, the fasting time before starting sample collection, the time of collection relative to time of feeding, the way of collecting the sample, and the subsequent processing of the samples followed the same procedures as in the preliminary trial. During the 4<sup>th</sup> AUC, however, urine was collected over a 24 hr period. Samples were collected hourly over an 18 hr period, followed by three further collections at two hourly intervals. Furthermore, unlike in the preliminary trial, the sampling started at zero time (first mealtime) rather than 30 minutes prior to the meal. Thirty minutes before the first sample was taken the bladder was emptied and the bladder catheter plugged as in the preliminary trial.

#### *2.2.3.2.3 Removal of the catheter*

Similar procedures were followed to those described in the preliminary trial for the removal of the catheter. At the end of the trial, however, instead of giving the pigs an intramuscular injection of antibiotics, a powder antibiotic containing sulphadimidine and trimetoprim, (Trimsulp powder Phoenix Pharm Distributors Ltd., Auckland, NZ) was mixed with the afternoon meal at a dosage of 25 mg /kg for 3 days. The need for a prophylactic antibiotic was indicated by incidences of urinary tract infections in the preliminary trial in which two of the pigs were infected.

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#### *2.2.3.2.4 Preparation of the samples for analysis and chemical analysis*

Urine volume was measured, the urine sub-sampled, pH adjusted and the sub-sample stored before it was analysed as in the preliminary trial. Urine, faeces and diet samples were analysed chemically as in the preliminary trial.

### **2.2.4 Data Analysis**

#### **2.2.4.1 Body weights, average dietary gains, feed intakes and nitrogen balance**

Data processing and statistical analysis for the body weights, average dietary gains, feed intakes and nitrogen balance in the main trial followed the same procedures as described for the preliminary trial. The ANOVA analysis was done using the general lineal method procedure (GLM) in the SAS package (SAS for Windows version 6.12, SAS Institute Cary, NC, USA).

#### **2.2.4.2 Acute urine collection**

##### *2.2.4.2.1 Transformation of the data from the acute urine collection*

The AUC data were tested for normal distribution, using SAS for Windows version 6.12, SAS Institute, Cary, NC, USA, before being statistically analysed. Whenever the distribution of the data appeared not to be normal, the particular set of data was mathematically transformed to either log, ln, root-square or to the  $x$  power and its distribution from the transformed data evaluated for its closeness to a normal distribution (Gómez and Gómez. 1984; Fernández, 1992). Thus, the data that were finally statistically analysed had a distribution close to normal.

Data from the 3<sup>rd</sup> AUC, and relating to the excretion rate of urea nitrogen and total nitrogen were transformed to the power of 0.5. In the same way, data of excretion rate of ammonia nitrogen were transformed to the power of 0.75. The same procedure

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was performed on the excretion rate of urea and ammonia nitrogen (to the power 0.5) from the 4<sup>th</sup> AUC.

#### 2.2.4.2.2 The statistical design for the rate of excretion

Both the 3<sup>rd</sup> AUC and 4<sup>th</sup> AUC trials were based on a repeated measures design. For data analysis, therefore, a split plot design was used. The effect of diet was included in the main plot, whereas the effects of time and time-by-diet interaction were included in the split plot. If the time-by-diet interaction was found to be significant, a post-hoc analysis (Tukeys's t-test; SAS for Windows version 6.12, SAS Institute, Cary, NC, USA) was conducted to determine at what time point the differences between the two diets occurred. The effects were considered significant at  $P < 0.05$ . The model used to analyse urinary nitrogen excretion for all the metabolites and the total nitrogen can be described as follows:

$$Y_{ijk} = \mu + P_i + D_j + \varepsilon_{ij}^M + T_k + (DT)_{jk} + \varepsilon_{ijk}^S.$$

This can be described as:

$Y_{ijk}$  the ammonia excretion rate associated with the  $i$ th pig, the  $j$ th diet, and the  $k$ th time point

$\mu$  the (unknown) overall mean ammonia excretion rate

$P_i$  the (main) effect due to the  $i$ th pig

$D_j$  the (main) effect due to the  $j$ th diet

$\varepsilon_{ij}^M$  the main-plot error

$T_k$  the (main) effect of the  $k$ th time point

$(DT)_{jk}$  the interaction effect between the  $j$ th diet and the  $k$ th time point

$\varepsilon_{ijk}^S$  the split-plot error (Mead, 1994).

#### *2.2.4.2.3 The accumulative nitrogen excretion*

The accumulative nitrogen excretion for each metabolite is the total amount of the nitrogen excreted in this particular metabolite until the referred sampling time. It was calculated by addition of all single nitrogen excretions at sample times before the referred one. The means of the accumulative urine excretion at each sampling time were compared between pigs fed with the intact protein and those fed the hydrolysed protein using a simple one-way analysis of variance using GLM (SAS for Windows version 6.12. 1989-1996 by SAS Institute Inc., Cary, NC, USA). The effects were considered significant at  $P < 0.05$ .

In order to visualize the pattern of excretion during the sampling period, overall mean rates of excretion for the IP and HP groups, were calculated over the whole sampling period. Solid and dotted horizontal lines in Figures 8 to 15 represent the overall means for the IP and HP groups, respectively.

## CHAPTER 3

### RESULTS

The results presented here are mainly related to the experiments performed in the main trial. From the preliminary trial, only data from the last nitrogen balance performed during the second experimental period are presented because of the multiple modifications and adjustments to experimental methodology between the preliminary and main trial. However issues such as survival and health of the animals, feed intakes, body weights, etc, will be described for both trials.

#### 3.1 Pig Survival

All pigs survived both the preliminary and main trials and were returned to a commercial farm at completion of the trials.

#### 3.2 Animal Health

##### Preliminary trial

The eight pigs used in the preliminary trial remained healthy throughout the trial. During the adaptation period, three pigs developed abrasions around their mouths caused by rubbing against the food container of the crate. The abrasions disappeared in two days without any medical treatment. One pig took an unusually long time to regain consciousness following catheterisation. Microbiological analysis of urine samples taken from the pigs during the second catheterisation revealed urinary tract infection (UTI) in two pigs (pigs 4 and 6). This was indicated by the

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detection of *E. coli* in the urine of these pigs. This meant that the antibiotic dosage given to the pigs after the first bladder catheterisation was not enough to prevent an UTI. Despite the presence of an UTI, pigs' alertness and appetite were not affected.

#### Main trial

One pig (number 5) on the intact diet developed a gastric ulcer at the end of the first experimental period of the main trial, and was excluded from all subsequent trials. The diagnosis of a gastric ulcer was based on this pig's faeces being a darker colour than normal and that fifteen days after arrival the pig vomited blood. The affected pig returned to a farm where it recovered well after it was provided with a grower diet.

A pig (number 14) in the HP group was excluded from the NB study because it was fed the opposite diet to the allocated one on the second day of the NB. This pig, continued however, into the two next experiments, the 3<sup>rd</sup> AUC and 4<sup>th</sup> AUC.

All the catheterised piglets consume a normal feed intake after surgery. They also readily consumed their set daily allowances throughout the experimental period.

One pig (number 10) had the lowest weight gain of all the pigs. Its faeces were a darker colour than normal until the starting of the second experimental period but it exhibited no other abnormalities. During the 3<sup>rd</sup> AUC this pig also hurt its hoof on the crate when demanding food. Subsequently her rate of weight gain was further reduced. The pig finished the experiment with a better growth rate.

During the 3<sup>rd</sup> and 4<sup>th</sup> AUC one pig (number 12 and 7, respectively) had mild diarrhoea on one occasion while urine was being collected. However, both before and after these incidences, their faeces were of a normal consistency.

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Four pigs (numbers 3, 7, 12 and 16) had a small amount of yellow discharge from the vulva 20 hr after the start of sampling in the 4<sup>th</sup> AUC. The remaining pigs appeared healthy throughout the trial.

Once the experimental work was completed, the pigs were housed for one month in the commercial pig unit at Massey University in order to wash out residual antibiotics from their bodies, and to adapt them back onto the grower diet and group living. They were then returned to a commercial farm for finishing.

### 3.3 Average Body Weights

The average body weights (mean  $\pm$  SE) of the pigs at the beginning of each experimental period during the preliminary and main trial did not differ statistically ( $P > 0.05$ ) between the HP and IP group. Table 6 shows the mean body weights of the pigs at the beginning of the experimental period 2 in the preliminary trial. This table also shows the mean body weights of the pigs at the beginning of the experimental periods 1, 2 and 3 and at the end of experimental period 3 in the main trial.

### 3.4 Average Daily Gain

For the main trial, the average daily gain (ADG) was not statistically different ( $P > 0.05$ ) between the IP and the HP group either during the nitrogen balance, the 3<sup>rd</sup> AUC or the 4th AUC. However, the ADG for both pig groups decreased dramatically during the course of the main trial (Figure 8).

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Table 6. Average body weight of female pigs consuming 10% of their metabolic body weight ( $\text{kg}^{0.75}$ ) of a diet containing either intact protein (IP)<sup>1</sup> or a hydrolysed whey (HP)<sup>2</sup> protein. The values are means  $\pm$  SE.

Experimental day	Average Body weight (kg)		
	IP	HP	SS <sup>3</sup>
Preliminary Trial <sup>4</sup>			
7	55.3 $\pm$ 1.25	53.1 $\pm$ 1.16	NS
Main Trial			
1 <sup>5</sup>	37.1 $\pm$ 1.24	37.1 $\pm$ 0.67	NS
7 <sup>5</sup>	43.7 $\pm$ 1.44	43.8 $\pm$ 0.74	NS
14 <sup>6</sup>	48.8 $\pm$ 1.83	48.9 $\pm$ 0.81	NS
21 <sup>6</sup>	50.9 $\pm$ 1.86	51.2 $\pm$ 0.94	NS

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5,000 Da. The antigenicity has been reduced  $10^4$  to  $10^5$  from the intact protein. Degree of Hydrolysis = 25.2%.

<sup>3</sup> SS = statistical significance

NS = non-significant;  $P > 0.05$

<sup>4</sup> IP n = 4, HP n = 4

<sup>5</sup> IP n = 8, HP n = 8,

<sup>6</sup> IP n = 7, HP n = 8,

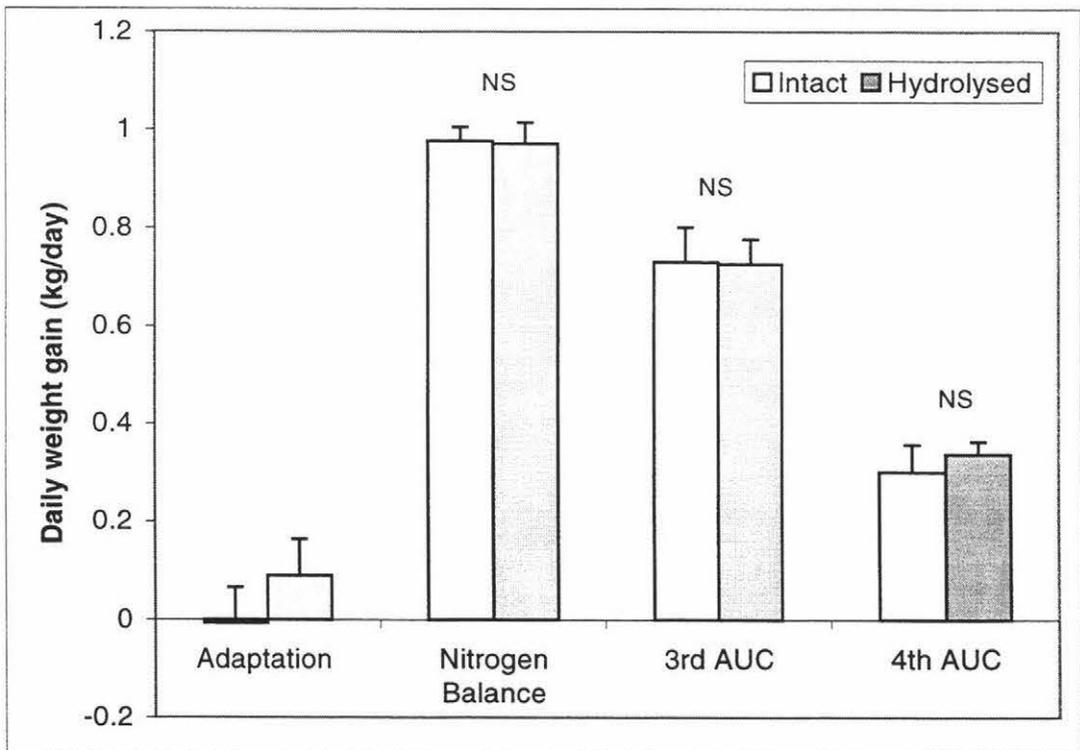


Figure 7. Daily weight gains during the adaptation period, nitrogen balance, the third acute urine collection (3<sup>rd</sup> AUC) and the fourth acute urine collection (4<sup>th</sup> AUC) of the main trial. NS = non-significant at  $P > 0.05$ .

## 3.5 The Mean Daily Intake

### 3.5.1 Preliminary Trial

Pigs were offered an amount of the diet determined as 10% of their MBW ( $\text{kg}^{0.75}$ ). The feed intakes (mean  $\pm$  SE) at the beginning of the nitrogen balance equated to  $2028.50 \pm 34.568$  g ( $31.37 \pm 0.535$  g N) for the IP group and  $1967.50 \pm 32.521$  g ( $32.18 \pm 0.532$  g N) for the HP group.

### 3.5.2 Main Trial

Pigs were also offered 10% of their MBW ( $\text{kg}^{0.75}$ ). Feed intakes (mean  $\pm$  SE) during the nitrogen balance, 3<sup>rd</sup> AUC and 4<sup>th</sup> AUC are depicted in Table 7.

Table 7. Average diet intake (g/day/pig) and the corresponding N intake in female pigs at each experimental stage in the main trial. The values represent the mean  $\pm$  SE.

Experiment	Average Intake (g)			
	Diet Intake (g)		N Intake (g)	
	IP <sup>1</sup>	HP <sup>2</sup>	IP <sup>1</sup>	HP <sup>2</sup>
Nitrogen Balance	$1503.0 \pm 37.95$	$1499 \pm 19.50$	$23.68 \pm 0.598$	$23.46 \pm 0.305$
3 <sup>rd</sup> AUC <sup>3</sup>	$1697.1 \pm 42.75$	$1702 \pm 21.40$	$29.94 \pm 0.754$	$29.20 \pm 0.367$
4 <sup>th</sup> AUC	$1844.0 \pm 53.19$	$1848 \pm 23.08$	$29.00 \pm 0.836$	$29.45 \pm 0.368$

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5,000 Da.

The antigenicity has been reduced  $10^4$  to  $10^5$  from the intact protein.

Degree of hydrolysis = 25.2%.

<sup>3</sup> AUC= Acute urine collection

## 3.6 Variability of the Chemical Analysis

### Total N and Dry Matter Analysis

The difference between duplicates expressed as a proportion of the mean (DDM) was used to evaluate the accuracy of the sampling, mixing and sample handling. The DDM in the determination of analysis of total N and dry matter for the tested proteins were 0.29 % and 0.02%, respectively.

The DDM in the analytical determination of total N in faecal, urine and feed samples were 0.60%, 1.28% and 1.22%, respectively. The DDM for the dry matter analysis were 0.11% and 0.16% for faeces and feed samples, respectively.

#### Amino Acid Analysis

Analysis of all the amino acids except cysteine and methionine had a DDM of 4.80% for the hydrolysed protein whereas the intact protein analysis had a DDM of 0.90%. The analysis for the cysteine and methionine content had DDM of 3.3% and 2.1% for the hydrolysed and the intact protein, respectively.

#### Creatinine, Urea and Ammonia Analysis

The DDM for urea, ammonia and creatinine was 3.59%, 3.29% and 3.07%, respectively, for the samples from the 3<sup>rd</sup> AUC and 2.96%, 2.47% and 2.86%, respectively, for the samples from the 4<sup>th</sup> AUC.

In the analytical determination of the urine samples, the intra-assay coefficients of variability for the analysis of urea, creatinine, ammonia were 1.2%, 2.8% and 5.4%, respectively. In addition, the inter-assay coefficients of variability for the same analysis were 5.31%, 5.15% and 4.83%, respectively.

### 3.7 Nitrogen Balance

The results from two nitrogen balances, one performed during the preliminary trial and other in the main trial are presented in the Table 8. The crude data are presented in Appendix 3.

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Table 8. Comparison of the N intake, N excretion and N balances for female pigs fed with either intact<sup>1</sup> or hydrolysed<sup>2</sup> protein in a preliminary and a main trial. The values represent the means  $\pm$  SE.

Constituents of NB (g N/ kg <sup>0.75</sup> / day)	Preliminary Trial			Main Trial		
	Diet		SS <sup>3</sup>	Diet		SS <sup>3</sup>
	Intact (n=4)	Hydrolysed (n=4)		Intact (n=8)	Hydrolysed (n=7)	
N Intake	1.548 $\pm$ 0.0003	1.635 $\pm$ 0.0003	***	1.570 $\pm$ 0.0037	1.574 $\pm$ 0.0084	NS
N Urine	0.331 $\pm$ 0.0546	0.486 $\pm$ 0.0699	NS	0.410 $\pm$ 0.0294	0.474 $\pm$ 0.0373	NS
N Faeces	0.024 $\pm$ 0.0140	0.036 $\pm$ 0.0125	NS	0.079 $\pm$ 0.0160	0.076 $\pm$ 0.0166	NS
N Balance <sup>4</sup>	1.192 $\pm$ 0.0494	1.113 $\pm$ 0.0794	NS	1.081 $\pm$ 0.0362	1.024 $\pm$ 0.0464	NS

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5,000 Daltons. The antigenicity has been reduced 10<sup>4</sup> to 10<sup>5</sup> from the intact protein. Degree of hydrolysis = 25.2%.

<sup>3</sup> SS = statistical significance

NS = non-significant at  $P > 0.05$

\*\*\* = significant at  $P < 0.0001$

<sup>4</sup> Nitrogen Balance = N Intake - (N urine + N faeces)

### 3.7.1 Preliminary Trial

Dietary N retention by the IP group was not statistically different to that of the HP group. Although total N intake was similar in the two groups ( $P = 0.3253$ ) (data not shown), intake per unit of MBW was significantly greater in the HP group (Table 8). Despite this difference, which was presumed to have occurred during sampling of the feeds, N retention was similar in the two groups.

The total excretion of urinary N during the NB, calculated from urinary metabolites such as urea, creatinine and ammonia, was found to be similar in the two groups (Table 9). The excretions of urea N and creatinine N were not significantly different between the groups, although more urea N was excreted by the HP group. However, those pigs fed the intact protein excreted significantly ( $P < 0.0001$ ) more ammonia than those pigs fed with the hydrolysed protein.

Table 9. Nitrogen (N) excreted (means  $\pm$  SE) as urinary metabolites in pigs receiving either an intact<sup>1</sup> or hydrolysed<sup>2</sup> protein diet during the nitrogen balance study of the preliminary trial.

Urinary N (mg N/kg <sup>0.75</sup> / day)	DIET		SS <sup>3</sup>
	Intact (n=4)	Hydrolysed (n=4)	
Urea N	179.7 $\pm$ 53.84	357.9 $\pm$ 79.92	NS
Creatinine N	41.0 $\pm$ 1.75	41.5 $\pm$ 0.87	NS
Ammonia N	59.9 $\pm$ 5.32	22.7 $\pm$ 2.67	***
Residual N <sup>4</sup>	50.4 $\pm$ 12.74	63.8 $\pm$ 18.82	NS
Total N <sup>5</sup>	331.0 $\pm$ 54.57	485.8 $\pm$ 69.90	NS

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5,000 Daltons,

Degree of Hydrolysis = 25.2%. The antigenicity has been reduced 10<sup>4</sup> to 10<sup>5</sup> from the intact protein.

<sup>3</sup> NS = non-significant at  $P > 0.05$

\*\*\* = significant at  $P < 0.0001$ .

<sup>4</sup> Calculated as: Total N (see Table 8) - (Urea N + Creatinine N + Ammonia N).

<sup>5</sup> Determined analytically by Kjeldahl Method (see Table 8).

### 3.7.2 Main Trial

Body weights at the beginning and at the end of the nitrogen balance trial were not statistically different for the two groups of pigs. Protein deposition ratio (PDR = NB / N intake), the amount of protein retained per g of protein ingested, did not differ statistically between the groups (Table 10).

Table 10. Weight balance and protein deposition ratio (means  $\pm$  SE) in pigs receiving either an intact<sup>1</sup> or hydrolysed<sup>2</sup> protein diet during the nitrogen balance study of the main trial.

	DIET		SS <sup>3</sup>
	Intact (n=8)	Hydrolysed (n=7)	
Live weight beginning, day 1 (kg)	37.07 $\pm$ 1.214	37.06 $\pm$ 0.671	NS
Live weight ending, day 7 (kg)	43.91 $\pm$ 1.271	43.86 $\pm$ 0.853	NS
Daily weight gain for 7 days (kg/ day)	0.98 $\pm$ 0.028	0.97 $\pm$ 0.043	NS
Protein deposition ratio <sup>4</sup>	0.69 $\pm$ 0.023	0.65 $\pm$ 0.028	NS

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5,000 Daltons. The antigenicity has been reduced 10<sup>4</sup> to 10<sup>5</sup> from the intact protein. Degree of hydrolysis = 25.2%.

<sup>3</sup> NS = non significant at  $P > 0.05$

<sup>4</sup> Protein deposition ratio (PDR) = Protein deposition/ crude protein intake.

Protein deposition = 6.25 \* (N Intake - (N urine + N faeces)).

Crude protein intake = 6.25 \* N intake.

With regard to the total urinary metabolite excretion, the HP group excreted slightly more urea ( $P < 0.1$ ) and less ammonia ( $P < 0.0001$ ) than the IP group. The creatinine and total N excretions were not statistically different for the two groups at  $P > 0.05$  (Table 11).

Table 11. N excreted in urinary metabolites (means  $\pm$  SE) in pigs receiving either an intact<sup>1</sup> or hydrolysed<sup>2</sup> protein diet during the nitrogen balance study of the main trial.

Urinary N (mg N/ kg <sup>0.75</sup> / day)	DIET		SS <sup>3</sup>
	Intact (n=8)	Hydrolysed (n=7)	
Urea N	206.57 $\pm$ 25.784	295.05 $\pm$ 34.093	+
Creatinine N	32.24 $\pm$ 1.181	33.73 $\pm$ 1.230	NS
Ammonia N	78.46 $\pm$ 4.945	40.50 $\pm$ 4.522	***
Residual N <sup>4</sup>	92.62 $\pm$ 8.898	104.40 $\pm$ 9.327	NS
Total N <sup>5</sup>	409.90 $\pm$ 29.385	473.77 $\pm$ 37.442	NS

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a size below 5,000 Daltons, Degree of Hydrolysis = 25.2%. The antigenicity has been reduced 10<sup>4</sup> to 10<sup>5</sup> from the intact protein.

<sup>3</sup> NS = non significant at  $P > 0.05$ , + = significant at  $P < 0.1$ , \*\*\* = significant at  $P < 0.0001$ .

<sup>4</sup> Calculated as: Total N (see Table 8) - (Urea N + Creatinine N + Ammonia N).

<sup>5</sup> Determined analytically by Kjeldahl Method (see Table 8).

### 3.8 Rate of Excretion of Urinary Metabolites

The results of the first two urine collections performed during the preliminary experiment are not presented here because technical problems with the catheters meant that the data were considered unreliable.

#### 3.8.1 Third Acute Urine Collection

The rate of excretion of urinary creatinine N, urea N, ammonia N and total N monitored in pigs after a morning meal of either the intact or the hydrolysed protein based diets are shown in Figures 8 to 11. Statistical analysis of the rate of excretion of urinary creatinine N, urea N, ammonia N and total N using a split plot design resulted in ANOVAs such as that showed in Table 12. A summary of the ANOVA of the data showed in Figures 8 to 11 is given in the Table 13 to clarify the explanation of results.

Table 12. An example of the analysis of variance table generated by analysing the results from a split plot design in which effect of diet, time and diet\*time on the rate of urinary ammonia excretion was tested.

Source of variation	df	Sum of Squares	Mean Square	F value	Pr > F
<b>Main plot</b>					
Diet (Main effect A)	1	17.46980598	17.46980598	107.94	0.0001
Pig (Diet) (Main plot error)	12	10.98947336	0.91578945	5.66	0.0001
<b>Split Plot</b>					
Time (Main effect B)	21	17.52132466	0.83434879	5.16	0.0001
Diet*Time (Interaction AB)	21	4.05667774	0.19317513	1.19	0.2569
Error (Split plot error)	248	40.13759229	0.16184513		
Corrected Total (Total)	303	90.17487403			
R-Square	C.V.	Root MSE	POWARE Mean		
0.554892	33.18380	0.402300	1.212338		

Table 13. Summary of the ANOVAs of the effects of diet, time and diet\*time on the rate of excretion of urinary creatinine N, urea N, ammonia N and total N, following a split plot design on the 3<sup>rd</sup> AUC.

	Factors		Interaction Diet * Time
	Diet	Time	
Creatinine N	NS	NS	NS
Urea N	NS	0.0001	NS
Ammonia N	0.001	0.0001	NS
Total N	NS	0.0001	NS

The pattern of creatinine N excretion in the urine over the 16 hour collection for both groups of pigs with respect to time period was statistically not significant at  $P > 0.05$  (Figure 8; Table 13). The effect of diet was not statistically significant ( $P > 0.05$ ), meaning that both groups of pig excreted creatinine at constant and comparable rates over the 16 hr sampling period (Figure 8; Table 13). This was confirmed by the overall mean rates of creatinine N excretion. The IP and the HP had a overall mean rate of excretion over the 16 hr collection period of 0.745 mg N/kg<sup>0.75</sup>/30 min and 0.713 mg N/kg<sup>0.75</sup>/30 min, respectively, which were not statistically different ( $P > 0.05$ ).

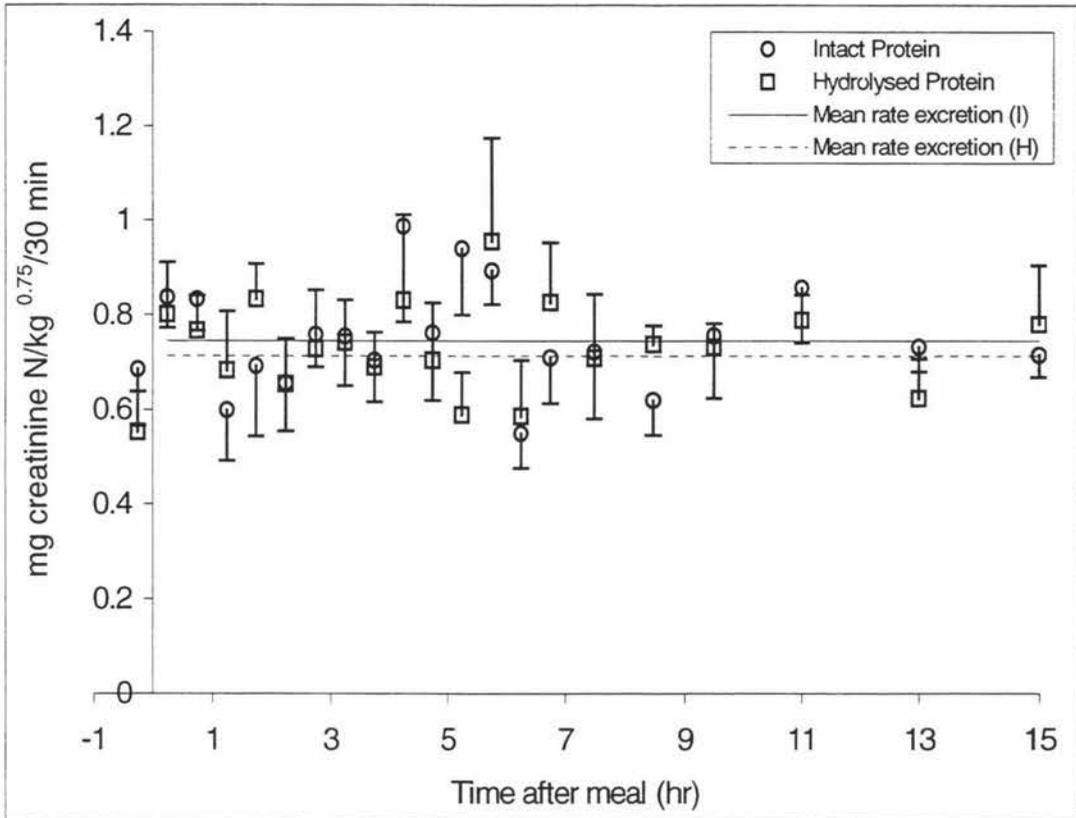


Figure 8. Main Trial; Third Acute Urine Collection (3<sup>rd</sup> AUC); Urinary creatinine nitrogen excreted (mg N/kg<sup>0.75</sup>/30 min) after consumption, by pigs, at time 0 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, ○), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 16 hr sampling period for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>).

Inspection of Figure 9 suggests different patterns of excretion of urinary urea N between the two groups of pigs during the 2 - 4 hr postprandial period. Immediately after being fed their respective diets, there was a rapid and short-lived rise in the excretion of urea N for both groups of pigs. However, 2 hr later, two distinct patterns of excretion are apparent. The rate of urea N excretion by the HP group reached a higher peak than that of the IP group and the difference was maintained, with small periodic falls, for 2 hr. Even though these visual differences, the patterns were not statistically different ( $P > 0.05$ ) with respect to time (Figure 9; Table 13). In addition, the differences in the rate of excretion of urea N in the urine between the two groups were not significant ( $P > 0.05$ ) at any sample time over the 16 hr (Figure 9; Table 13). The overall mean rate of excretion over the 16 hr collection period was 5.559 mg N/kg<sup>0.75</sup>/30 min and 5.946 mg N/kg<sup>0.75</sup>/30 min for the IP and the HP, respectively. These means were not significantly different ( $P < 0.05$ ).

The pattern of excretion of ammonia N in the two groups was statistically similar ( $P > 0.05$ ) with respect to time (Figure 10; Table 13). The rate of excretion of ammonia N over the 16 hr collection period, however, was significantly ( $P < 0.001$ ) greater for the IP group than that of the HP group (Figure 10; Table 13). The differences in rates of excretion were particularly marked between 5 and 16 hours after feeding (Figure 10). The overall mean rates of excretion over the 16 hr collection period, 1.646 mg N/kg<sup>0.75</sup>/30 min and 0.949 mg N/kg<sup>0.75</sup>/30 min for the IP and the HP, respectively, were statistically different ( $P < 0.0001$ ).

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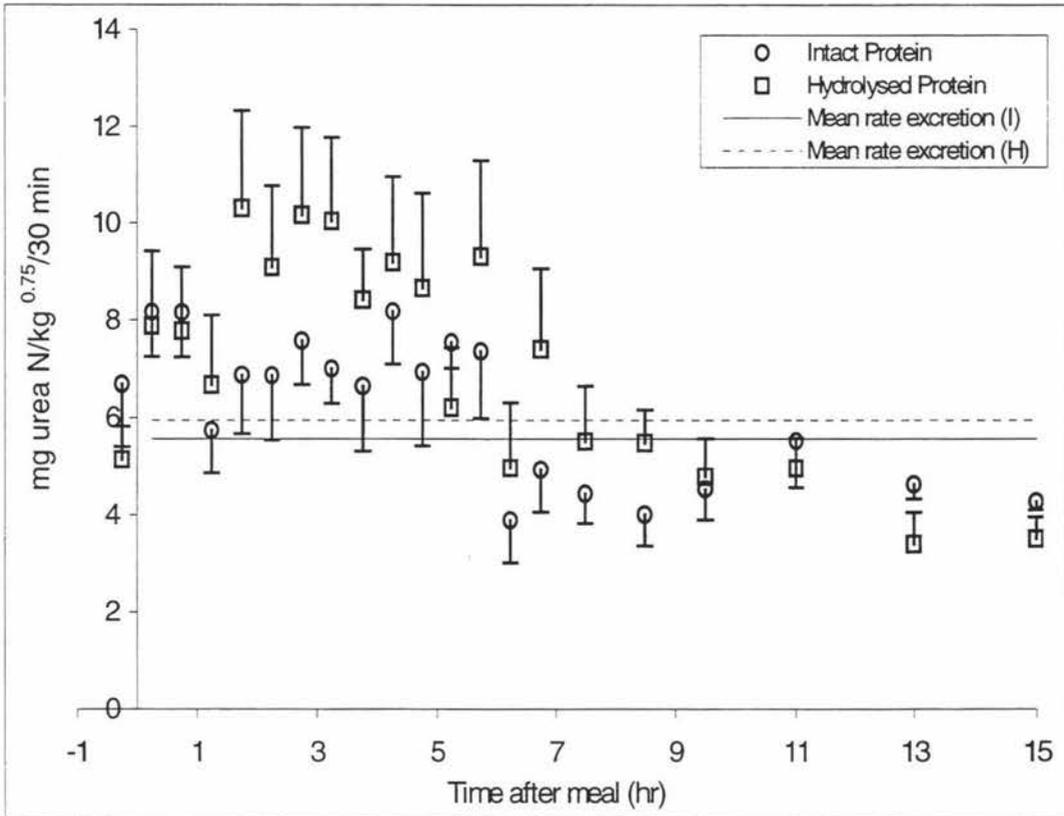


Figure 9. Main Trial; Third Acute Urine Collection (3<sup>rd</sup> AUC); Urinary urea nitrogen excreted (mg N / kg<sup>0.75</sup>/30 min) after consumption, by pigs, at time 0 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, O), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 16 hr sampling period for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>).

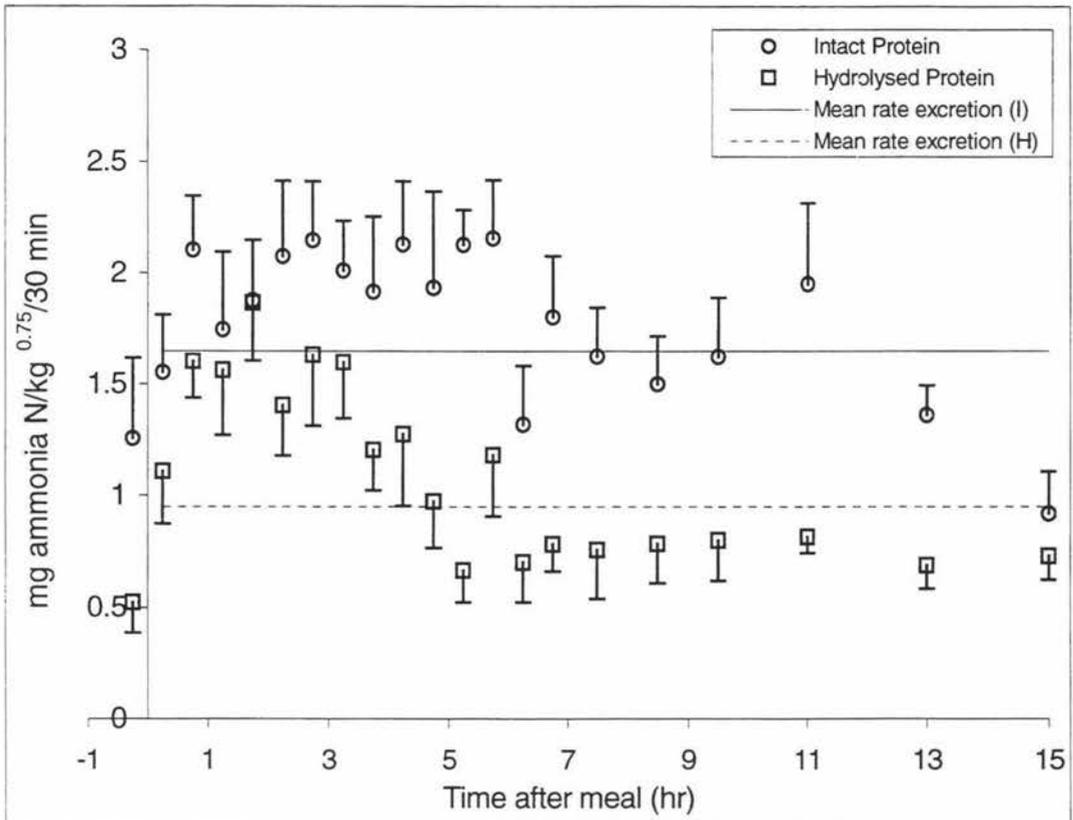


Figure 10. Main Trial; Third Acute Urine Collection (3<sup>rd</sup> AUC); Urinary ammonia nitrogen excreted (mg N/kg<sup>0.75</sup>/30 min) after consumption, by pigs, at time 0 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, ○), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 16 hr sampling period for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>).

The total urinary N excreted during the urine collection was calculated by adding the N component from the urea, creatinine and ammonia excreted. The pattern of excretion of total N is characterised by a small initial and immediate excretion of N for the first 2 hr after the meal, followed by a higher rate of excretion of total N by the HP group for 2 - 5 hr and a similarity in the excretion of N by both groups within 6 hr after feeding (Figure 11). The statistical analysis revealed that the patterns of excretion were not different ( $P > 0.05$ ) with respect to time (Figure 11; Table 13). In addition, the rate of excretion of total N by the two groups was similar over the 16 hr collection period at  $P > 0.05$  (Figure 11; Table 13). The overall mean rate of excretion over the 16 hr collection period, 7.951 mg N/kg<sup>0.75</sup>/30 min and 7.609 mg N/kg<sup>0.75</sup>/30 min for the IH and the HP, respectively, were not statistically different ( $P > 0.05$ ).

The main effect of time was highly significant ( $P < 0.001$ ) for urea N, ammonia N and total N (Table 13).

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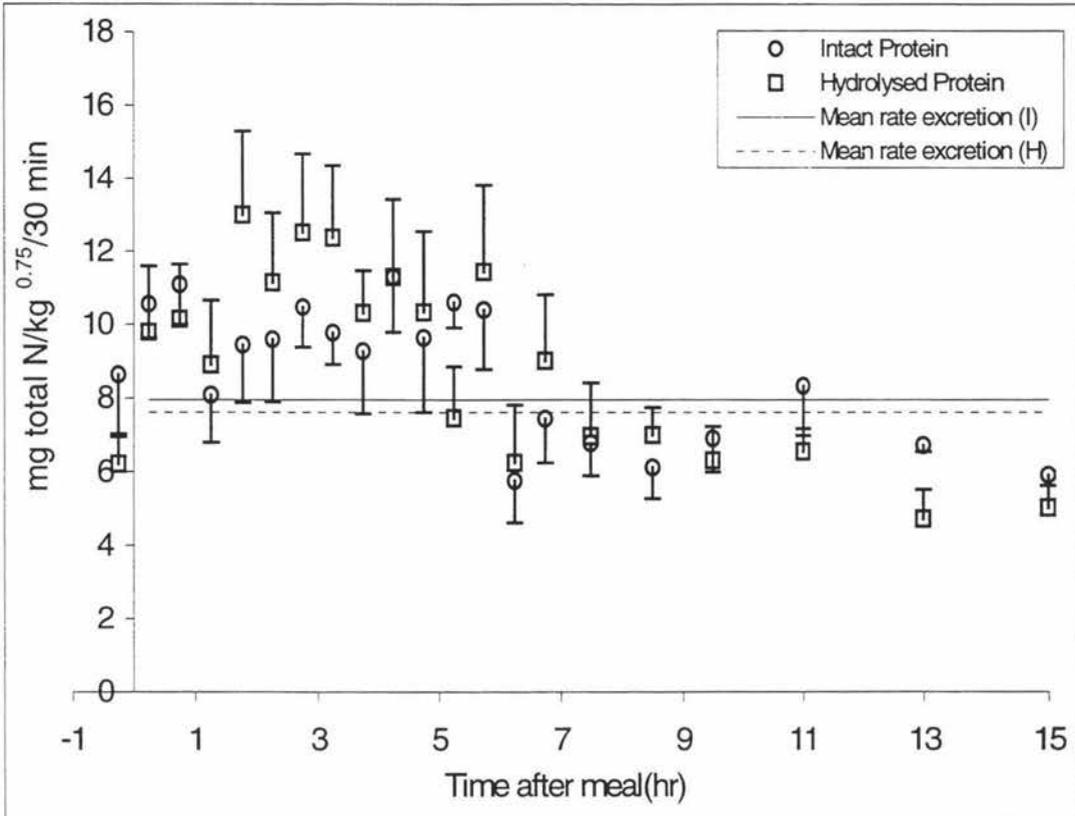


Figure 11. Main Trial; Third Acute Urine Collection (3<sup>rd</sup> AUC); Urinary total nitrogen excreted (mg N/ kg<sup>0.75</sup>/30 min) after consumption, by pigs, at time 0 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, O), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 16 hr sampling period for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>).

### 3.8.2 Fourth Acute Urine Collection

Figures 12 to 15 show the rate of excretion ( $\text{mg N/kg}^{0.75}/\text{hr}$ ) of creatinine N, urea N, ammonia N, and total N in urine of pigs when they were fed according to the normal meal schedule, i.e. a morning and afternoon meal. A summary of the ANOVA of the data showed in Figures 12 to 15 is given in Table 14 to clarify the explanation of results.

Table 14. Summary of the ANOVAs of the effects of diet, time and diet\*time on the rate of excretion of urinary creatinine N, urea N, ammonia N and total N, using a split plot design on the 4<sup>th</sup> AUC.

	Factors		Interaction Diet * Time
	Diet	Time	
Creatinine N	NS	0.0003	NS
Urea N	NS	0.0001	0.001
Ammonia N	0.0001	0.0001	0.0001
Total N	NS	0.0001	0.001

The excretion of creatinine N per hr for the IP and HP groups was similar throughout the day at  $P > 0.05$  (Table 14). The overall mean rate of excretion throughout the day,  $1.592 \text{ mg N/kg}^{0.75}/\text{hr}$  and  $1.616 \text{ mg N/kg}^{0.75}/\text{hr}$  for the IP and the HP groups, respectively, were not different statistically ( $P > 0.05$ ). Although the pattern of excretion for the IP group was characterised by marked oscillations over the first 14 hours (Figure 12), it was not significantly different from that of the HP group at  $P > 0.05$  (Table 14). Even though the effect of the time was significant, a Tukey-Kramer test revealed that the differences between time points were not statistically different ( $P > 0.05$ ).

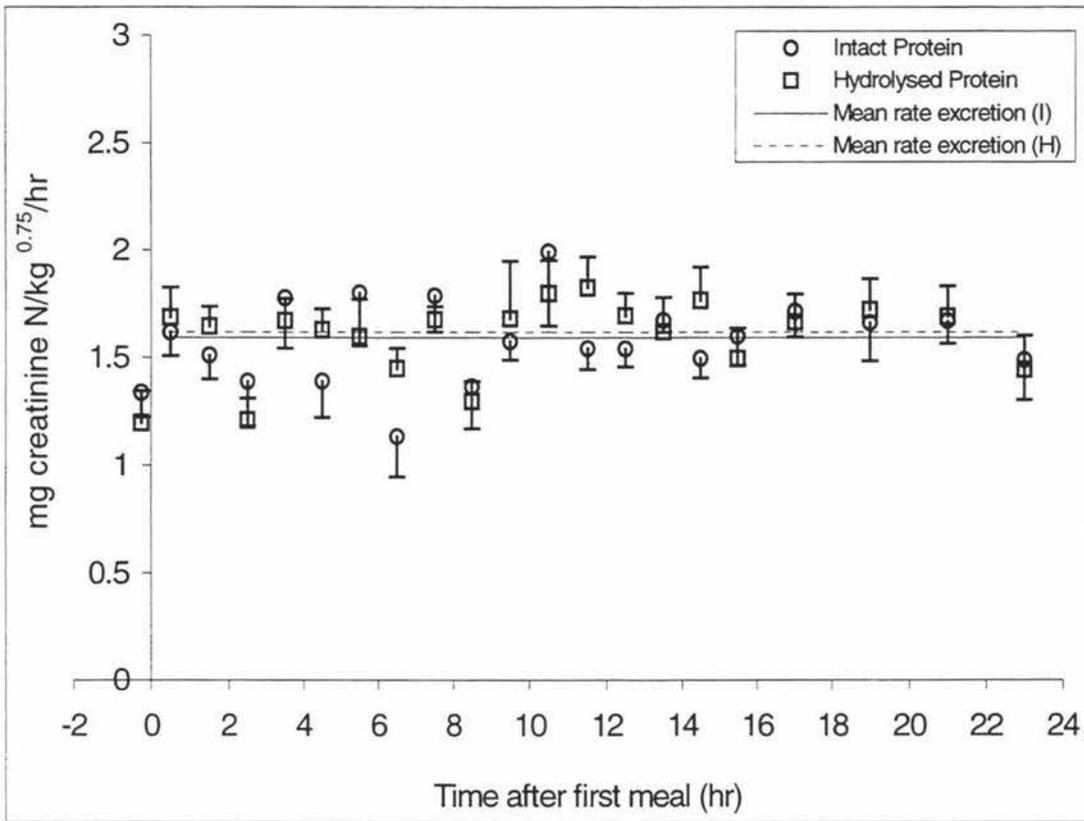


Figure 12. Main Trial; Fourth Acute Urine Collection (4<sup>th</sup> AUC); Urinary creatinine nitrogen excreted (mg N/kg<sup>0.75</sup>/hr) after consumption, by pigs, at 0 and 8 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, ○), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 24 hr sampling period for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>).

The pattern of excretion of urea observed after each meal during the 4<sup>th</sup> AUC (Figure 13) is similar to that following the single meal during the 3<sup>rd</sup> AUC (Figure 9). Over the period from 0 to 16 hr after the first meal (meal 2 at 8 hr), urea excretion was in general greater, but not significantly so, in the HP group compared to that in the IP group. For the pigs fed the intact protein, following the second peak in urea excretion 4 hr after the morning meal, there was a distinctly cyclic pattern of excretion until the next meal. Each peak in excretion rate is alternated with a fall. In contrast, in the group fed with the hydrolysed protein, the urea excretion rate decreased linearly from the peak of excretion reached after each meal. These differences in the pattern of urea N excretion were confirmed statistically ( $P < 0.001$ ). Furthermore, at 15 hr after the first meal, there appeared to be no further difference between the responses of the HP and IP groups. Specifically, however, the rate of urea N excretion for the HP group was significantly higher than that for the IP group at 4.5 hr ( $P < 0.05$ ) and at 11.5 hr ( $P < 0.1$ ) after meal (Figure 13). The overall mean rates of excretion over the 24 hr collection period, 11.12 mg N/kg<sup>0.75</sup>/hr and 13.03 mg N/kg<sup>0.75</sup>/hr for the IP and the HP, respectively, were not statistically different ( $P > 0.05$ ).

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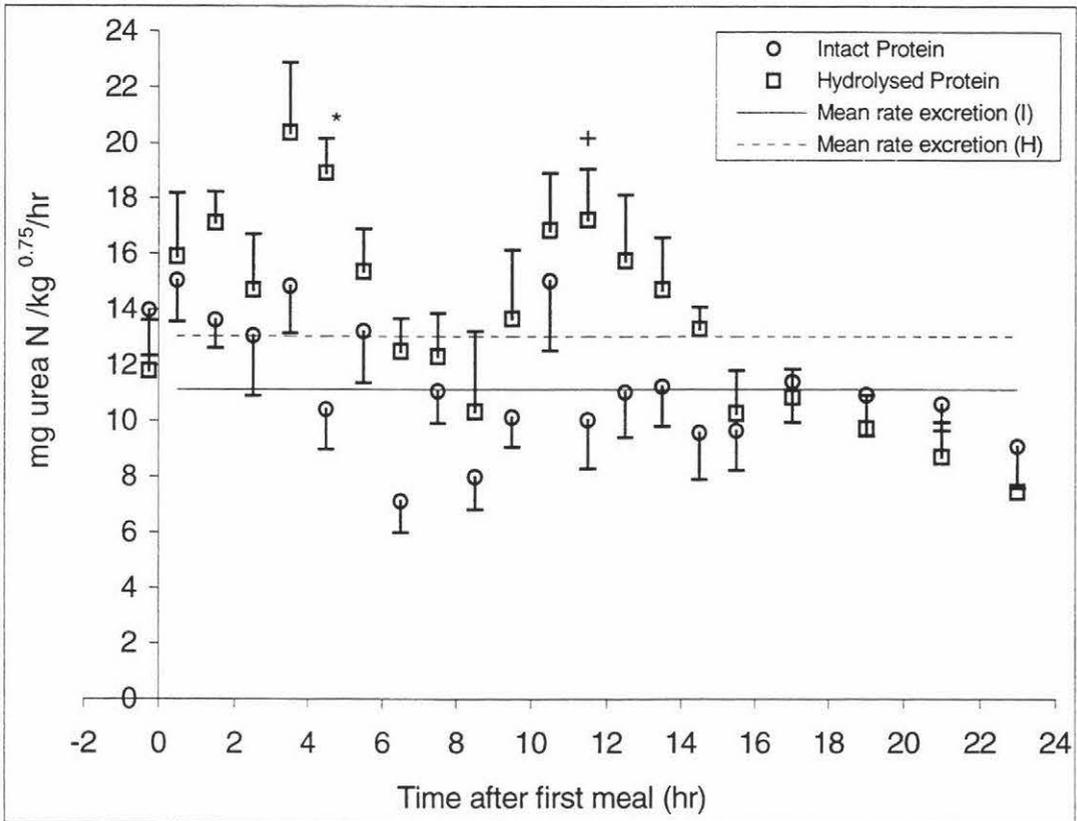


Figure 13. Main Trial; Fourth Acute Urine Collection (4<sup>th</sup> AUC); Urinary urea nitrogen excreted (mg N/kg<sup>0.75</sup>/hr) after consumption, by pigs, at 0 and 8 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, ○), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 24 hr sampling period for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>). + = statistical significance at  $P < 0.1$ . \* = statistical significance at  $P < 0.05$ .

Excretion of ammonia by the HP group was considerably lower and less variable than that of the IP group throughout the day (Figure 14). The pattern of excretion was broadly similar for the first 10 – 12 hr with a rise to an initial peak about 1 - 2 hr after feeding and a gradual fall to 6 hr followed by another peak at 9 – 10 hr, or 1 - 2 hr after the second meal. From 12 hr onwards there was a marked divergence in ammonia excretion between the two groups. The statistical analysis confirmed that the patterns were different as indicated by the time x diet interaction ( $P < 0.0001$ ) (Table 14). In addition, the rate of excretion of ammonia N over the 24 hr collection period by the IP group was significantly ( $P < 0.0001$ ) greater than that excreted by the HP group (Table 14). The pigs fed the intact protein excreted significantly ( $P < 0.01$ ) more ammonia per hr than the pigs on the hydrolysed diet at 5.5, 7.5, 12.5, 13.5, 14.5, 15.5, 17, 19, 21 and 23 hr after the morning meal. This can be summarised by saying that the rate of excretion of ammonia differed significantly between treatments around 5.5 hr and 4.5 hr after the morning and afternoon meals, respectively. The overall mean rates of ammonia N excretion over the 24 hr collection period, 4.401 mg N/kg<sup>0.75</sup>/hr and 2.028 mg N/kg<sup>0.75</sup>/hr for the IP and the HP, respectively, were significantly different ( $P < 0.0001$ ).

Although the treatments did not affect the rate of excretion of total N (Figure 15; Table 14) by the two groups at any time ( $P > 0.05$ ), the interaction between diet and time of sampling revealed that the patterns were different ( $P < 0.001$ ). The overall mean rates of total N excretion throughout the day, 17.115 mg N/kg<sup>0.75</sup>/hr and 16.670 mg N/kg<sup>0.75</sup>/hr for the IP and the HP, respectively, were not statistically different ( $P > 0.05$ ).

The effect of time was significant ( $P < 0.05$ ) for all the parameters evaluated (Figures 13 to 15; Table 14),

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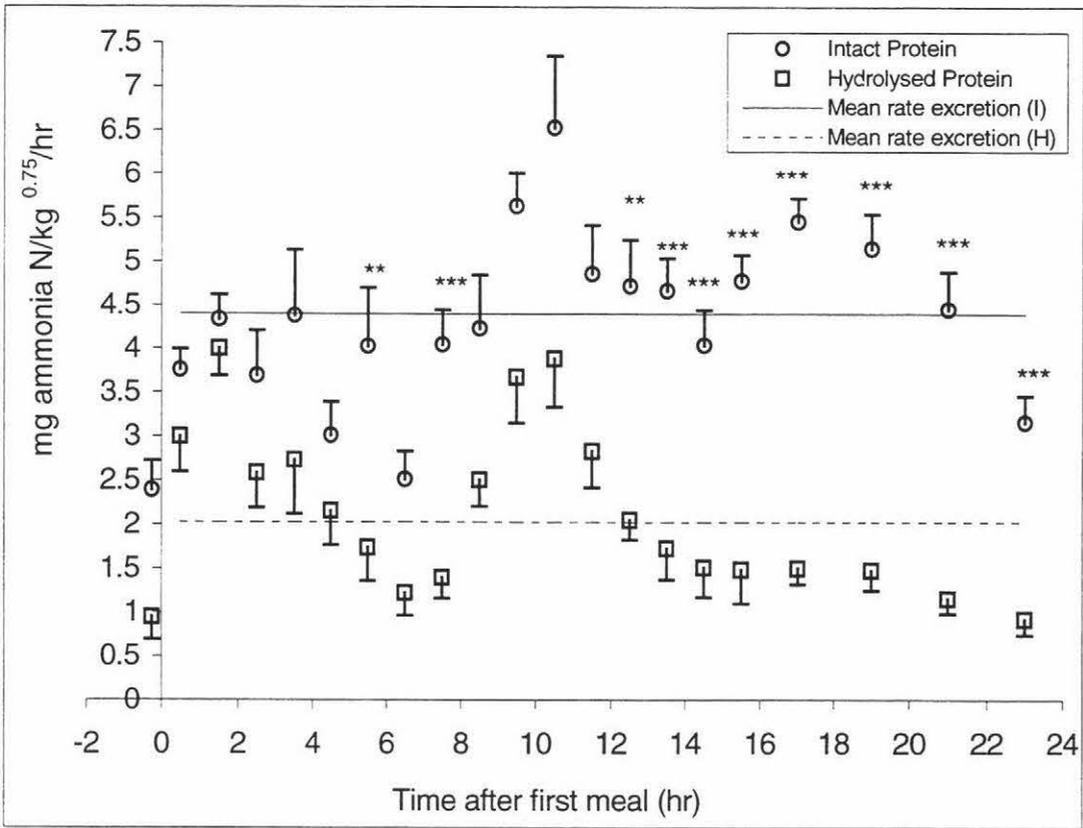


Figure 14. Main Trial; Fourth Acute Urine Collection (4<sup>th</sup> AUC); Urinary ammonia nitrogen excreted (mg N/kg<sup>0.75</sup>/hr) after consumption, by pigs, at time 0 and 8 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, O), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 24 hr sampling for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>). \*\* = statistical significance at P < 0.001. \*\*\* = statistical significance at P < 0.0001.

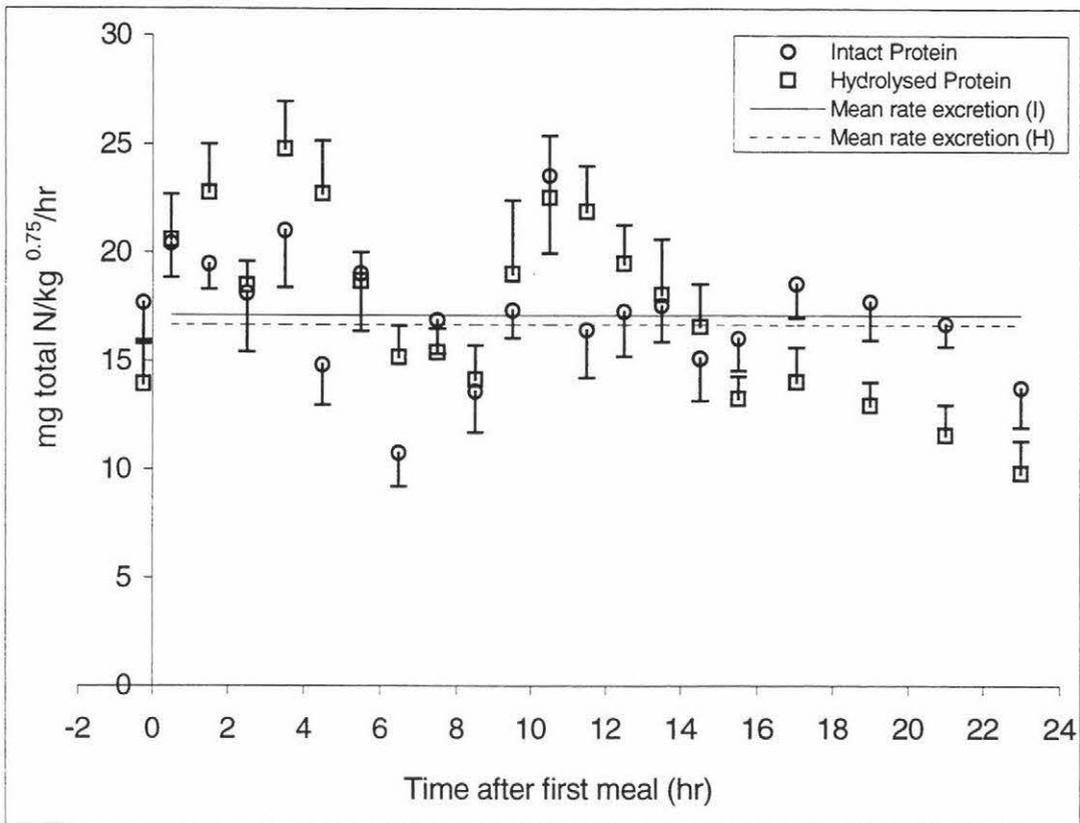


Figure 15. Main Trial; Fourth Acute Urine Collection (4<sup>th</sup> AUC); Urinary total nitrogen excreted (mg N/ kg<sup>0.75</sup>/hr) after consumption, by pigs, at 0 and 8 hr, diets containing either an intact or hydrolysed whey protein. Mean values with associated SEM, diet containing intact protein (n=7, ○), diet containing hydrolysed whey protein (n=8, □). The horizontal lines represent an overall mean excretion rate for the whole 24 hr sampling for each group of pigs eating either the intact protein (I) or the hydrolysed protein (H). Metabolic body weight (MBW) = body weight (kg<sup>0.75</sup>).

## 3.9 Accumulative Excretion of Urinary Metabolites

### 3.9.1 Third Acute Urine Collection

The accumulative excretion of urinary metabolites was calculated by successive addition of the quantities collected at each sampling time. During the 3<sup>rd</sup> AUC the accumulative excretion of creatinine N and urinary urea N was similar for both groups, at each sampling time during the 16 hr sampling period (Table 15).

The accumulative excretion of urinary ammonia N by the IP group, however, was slightly higher ( $P < 0.1$ ) than that of the HP group at 0.5 - 1.0 and 3.0 - 4.0 hr after feeding. After 3.5 hr, the difference in the ammonia N excretion between the two groups increased gradually. By 4.0 - 4.5 hr the difference was significant ( $P < 0.01$ ) and the significance of the difference continued to increase until 16 hr ( $P < 0.0001$ ).

The accumulative excretion of total urinary N, calculated from the addition of the N contributed by urea, ammonia and creatinine, the major sources of N in the urine, was not significantly different ( $P > 0.05$ ) between the groups throughout the 16 hr sampling period.

### 3.9.2 Fourth Acute Urine Collection

The accumulative excretion of creatinine N did not differ significantly between dietary groups at any time over the fourth collection period ( $P > 0.05$ ). The HP group excreted more urea N than the IP group between 4.0 hr and 13.0 hr after the morning meal ( $P < 0.01$ ). Over the next 5 hr, however, this difference became smaller and was no longer significant at 24 hours. The difference in the accumulative excretion of ammonia N between the two groups approached significance 3.0 hr after the morning meal ( $P < 0.1$ ). The difference gradually widened over the sampling period so that by 9.0 hr and until 24 hr the difference was highly significant at  $P < 0.0001$ . The

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accumulative total N excretion calculated from the individual metabolites, urea, ammonia and creatinine was greater in the IP group only at 3.0 and 5.0 hr after the first meal (Table 16).

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Table 15. The accumulated total excretion of nitrogen in urinary metabolites (mean and SE) measured in growing female pigs<sup>1</sup> at certain intervals for 16 hr following a meal<sup>2</sup> of either an intact milk protein (I)<sup>3</sup>, or its hydrolysate (H)<sup>4</sup>.

Urinary Nitrogen Metabolites (mg N/kg metabolic body weight)																			
Urea Nitrogen					Ammonia Nitrogen					Creatinine Nitrogen					Total Nitrogen				
I		H		SS	I		H		SS	I		H		SS	I		H		SS
Mean	SE	Mean	SE		Mean	SE	Mean	SE		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
8.17	0.914	7.88	1.544	NS	1.55	0.257	1.11	0.234	NS	0.84	0.065	0.80	0.110	NS	10.56	0.923	9.79	1.803	NS
16.33	1.574	15.66	2.435	NS	3.65	0.427	2.71	0.295	+	1.67	0.103	1.57	0.133	NS	21.65	1.606	19.94	2.720	NS
22.06	1.558	22.33	3.009	NS	5.40	0.744	4.27	0.360	NS	2.27	0.103	2.25	0.190	NS	29.73	1.769	28.85	3.257	NS
28.93	1.541	32.64	4.178	NS	7.27	0.762	6.13	0.406	NS	2.96	0.174	3.09	0.173	NS	39.16	1.344	41.85	4.383	NS
35.79	2.492	41.72	5.745	NS	9.34	1.027	7.54	0.537	NS	3.62	0.179	3.74	0.239	NS	48.75	2.702	53.00	6.121	NS
43.37	3.221	51.89	6.404	NS	11.49	1.221	9.16	0.701	NS	4.37	0.215	4.47	0.205	NS	59.23	3.477	65.52	6.751	NS
50.37	3.497	61.94	7.533	NS	13.50	1.394	10.76	0.910	NS	5.13	0.306	5.21	0.224	NS	69.00	3.662	77.91	8.007	NS
57.02	4.484	70.36	8.285	NS	15.41	1.662	11.96	1.041	+	5.83	0.277	5.90	0.256	NS	78.26	4.936	88.22	8.794	NS
65.20	3.921	79.55	8.593	NS	17.53	1.681	13.24	1.030	*	6.82	0.366	6.73	0.320	NS	89.55	3.861	99.52	8.843	NS
72.13	4.806	88.21	9.597	NS	19.46	1.861	14.21	1.150	*	7.58	0.424	7.44	0.315	NS	99.17	4.930	109.86	9.863	NS
79.67	4.920	94.42	10.341	NS	21.59	1.922	14.87	1.242	**	8.52	0.522	8.02	0.350	NS	109.78	4.818	117.31	10.647	NS
87.02	5.937	102.56	11.352	NS	23.74	2.031	15.91	1.365	**	9.41	0.522	8.86	0.463	NS	120.17	5.943	127.33	11.854	NS
90.90	6.209	107.53	11.840	NS	25.06	2.255	16.61	1.442	**	9.96	0.524	9.45	0.498	NS	125.93	6.347	133.58	12.352	NS
95.83	6.863	114.93	12.930	NS	26.86	2.369	17.39	1.516	**	10.67	0.540	10.27	0.470	NS	133.36	7.171	142.59	13.433	NS
103.22	7.216	125.95	14.014	NS	29.56	1.834	18.90	1.680	**	11.88	0.749	11.69	0.663	NS	144.66	7.002	156.54	14.615	NS
111.23	7.636	136.90	15.004	NS	32.56	2.189	20.47	1.897	**	13.11	0.714	13.16	0.684	NS	156.90	7.388	170.53	15.539	NS
120.28	8.198	145.27	16.537	NS	35.80	2.186	21.87	2.135	**	14.63	0.918	14.44	0.833	NS	170.70	7.567	181.58	17.403	NS
142.33	9.406	162.60	19.301	NS	43.59	2.477	24.72	2.379	***	18.05	1.111	17.21	1.148	NS	203.97	8.012	204.52	20.495	NS
160.82	10.373	176.22	21.055	NS	49.02	2.918	27.47	2.390	***	20.98	1.201	19.70	1.087	NS	230.83	8.277	223.39	22.424	NS
177.90	10.464	190.28	22.105	NS	52.69	2.802	30.38	2.514	***	23.84	1.333	22.82	1.331	NS	254.44	8.192	243.49	23.578	NS

<sup>1</sup> IP group (n= 7), HP group (n=8)

<sup>2</sup> Meal at time 0 hr

<sup>3</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>4</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5000 Daltons, Degree of Hydrolysis = 25.2%. The antigenicity has been reduced 10<sup>4</sup> to 10<sup>5</sup> from the intact protein.

<sup>5</sup> SS = statistical significance, NS = non-significant at  $P > 0.05$ , + = significant at  $P < 0.1$ , \* = significant at  $P < 0.01$ , \*\* = significant at  $P < 0.001$ , \*\*\* = significant at  $P < 0.0001$

Table 16. The accumulative total excretion of nitrogen in urinary metabolites (mean and SE) measured in growing female pigs<sup>1</sup> at intervals, in a 24 hr period in which the pigs were offered two meals<sup>2</sup> of either an intact milk protein (I)<sup>3</sup>, or its hydrolysate (H)<sup>4</sup>.

Urinary Nitrogen Metabolites (mg N/kg metabolic body weight)																			
Urea Nitrogen					Ammonia Nitrogen					Creatinine Nitrogen					Total Nitrogen				
I	H	SS	I	H	SS	I	H	SS	I	H	SS	I	H	SS	I	H	SS		
Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
15.04	1.479	15.90	1.827	NS	3.76	0.230	3.00	0.405	NS	1.62	0.109	1.69	0.140	NS	20.41	1.555	20.59	2.079	NS
28.65	1.792	33.00	3.693	NS	8.09	0.419	7.00	0.656	NS	3.13	0.186	3.33	0.179	NS	39.87	1.690	43.34	3.771	NS
41.70	2.250	47.71	4.508	NS	11.78	0.768	9.59	0.993	NS	4.51	0.269	4.54	0.249	NS	58.00	2.195	61.84	4.411	NS
56.54	2.650	68.08	5.802	NS	16.16	0.801	12.32	1.533	+	6.29	0.285	6.21	0.290	NS	78.99	2.504	86.62	5.590	+
66.95	3.226	87.00	7.901	*	19.18	0.852	14.48	1.833	*	7.68	0.326	7.84	0.262	NS	93.81	2.915	109.32	7.441	NS
80.16	4.592	102.36	8.612	*	23.20	0.984	16.22	2.121	*	9.48	0.386	9.44	0.328	NS	112.84	4.624	128.02	7.865	+
87.26	4.775	114.86	10.004	*	25.71	0.840	17.44	2.289	**	10.61	0.528	10.89	0.344	NS	123.59	4.658	143.19	9.043	NS
98.32	5.494	127.18	10.992	*	29.76	0.957	18.84	2.486	**	12.40	0.595	12.56	0.358	NS	140.47	5.484	158.58	9.872	NS
106.29	5.821	137.51	12.081	*	33.99	0.832	21.34	2.728	**	13.76	0.600	13.86	0.391	NS	154.04	5.499	172.71	10.848	NS
116.42	6.717	151.17	13.654	*	39.63	0.991	25.02	2.889	***	15.33	0.675	15.54	0.589	NS	171.38	6.469	191.73	12.272	NS
131.44	7.560	168.02	15.536	+	46.16	1.266	28.91	3.008	***	17.32	0.821	17.34	0.460	NS	194.92	7.631	214.26	13.942	NS
141.47	8.215	185.24	17.242	*	51.01	1.671	31.74	3.122	***	18.86	0.877	19.16	0.573	NS	211.34	8.555	236.15	15.363	NS
152.48	9.351	201.00	18.778	*	55.73	2.131	33.80	3.208	***	20.40	0.946	20.86	0.607	NS	228.61	10.214	255.65	16.584	NS
163.71	10.583	215.72	20.953	+	60.40	2.485	35.53	3.330	***	22.07	0.998	22.48	0.641	NS	246.18	11.760	273.72	18.721	NS
173.28	12.046	229.02	22.260	+	64.43	2.723	37.04	3.495	***	23.57	1.034	24.24	0.658	NS	261.28	13.496	290.30	19.794	NS
182.93	13.291	239.29	22.621	+	69.21	2.779	38.53	3.725	***	25.16	1.153	25.74	0.744	NS	277.30	14.826	303.56	19.836	NS
205.71	15.482	260.99	25.388	+	80.12	2.824	41.54	3.848	***	28.59	1.338	29.07	0.814	NS	314.42	16.867	331.59	22.518	NS
227.56	16.552	280.45	26.498	NS	90.41	3.014	44.50	4.171	***	31.92	1.614	32.51	0.932	NS	349.88	18.093	357.46	23.164	NS
248.73	17.971	297.89	28.515	NS	99.31	2.943	46.82	4.191	***	35.26	1.711	35.89	0.964	NS	383.30	19.187	380.61	25.280	NS
266.91	19.987	312.79	30.229	NS	105.64	3.249	48.67	4.211	***	38.23	1.975	38.78	1.096	NS	410.77	21.556	400.24	27.312	NS

<sup>1</sup> IP group (n= 7), HP group (n=8)

<sup>2</sup> The times from meal were zero and 8 hr.

<sup>3</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>4</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a molecular weight below 5000 Daltons, Degree of Hydrolysis = 25.2%. The antigenicity has been reduced 10<sup>4</sup> to 10<sup>5</sup> from the intact protein.

<sup>5</sup> SS = statistical significance, NS = non-significant at  $P > 0.05$ , + = significant at  $P < 0.1$ , \* = significant at  $P < 0.01$ , \*\* = significant at  $P < 0.001$ , \*\*\* = significant at  $P < 0.0001$

## CHAPTER 4

### DISCUSSION

The present research introduces a new technique for assessing the nutritional value of a protein. The AUC technique evaluates protein utilisation by individual quantification of the main nitrogenous metabolites excreted in the urine at different postprandial times. The theoretical basis of this technique is explained below.

Protein metabolism is a constant process that requires a continuous supply of amino acids. Since amino acids can only be stored in proteins, the regular ingestion of proteins containing essential amino acids is required. After absorption through the gut, amino acids are available to synthesise new proteins and replace body proteins that have been broken down. If there is lack of a specific amino acid amongst those absorbed, the body uses amino acids that have been released by the breakdown of body protein. The protein, which is catabolised, loses its functionality and releases its amino acids. These in turn, can either be used to synthesise other proteins or be oxidised for energy as will be explained below. Moreover, when an individual is growing, their requirement for essential and non-essential amino acid(s) is increased. Thus, the body requires a constant supply of amino acids for maintenance and growth.

In a normal individual not all circulating amino acids are used for protein synthesis. Both high dietary amino acid supply and an imbalance of essential amino acids in the diet cause a higher amino acid flux than is needed for synthesis or specific metabolic reactions. In such cases, amino acids are converted to energy storage compounds such as carbohydrates or fats. During this transformation, which occurs in

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the liver, amino acids lose their amino groups, which form ammonia, a compound that is very toxic to the body. Almost all the ammonia is converted to urea, a less toxic substance, before it can leave the liver. Finally, urea is excreted in the urine and the amount excreted is proportional to the amount of protein metabolised. Thus, urea has been used as an indicator of protein utilisation in an individual eating adequate protein for her/his maintenance and growth. When nutritional studies are performed to assess the nutritional value of a protein, protein intake is regulated, avoiding excess since in a healthy individual, a high protein intake causes high urea excretion. Urea is not the only nitrogenous urinary metabolite, but it accounts for 80% to 90% of the total nitrogen in the urine (Gibson, 1990). Creatinine, ammonia, uric acid and other minor nitrogenous compounds are also present. Their excretion, however, is fairly stable on a general diet (Gibson, 1990). Creatinine is formed irreversibly from creatine, a nitrogenous metabolite generated in constant amounts during the breakdown of muscle and other body proteins. Most of the ammonia that is excreted in the urine is produced in the kidney primarily from glutamine deamination (Hladky and Rink, 1986). In a normal individual, ammonia excretion is a compensatory mechanism involved in the regulation of acid-base balance in the body.

By following the nitrogenous compounds generated from the catabolism of amino acids and body proteins, it is possible to assess the efficiency of utilisation of dietary amino acids for protein synthesis. Thus, if the production of creatinine and urea are followed under normal conditions, creatinine excretion will reflect the pattern of protein breakdown in the muscle. This is expected to be constant in an individual at rest and receiving the N required for maintenance; whereas, the pattern of urea excretion will reflect metabolism of the dietary amino acids. Large quantities of urea are indicative of low utilisation of dietary protein and *vice versa*. Therefore, estimates of urea excretion allow an evaluation of the nutritive value of a dietary protein.

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In this research the null hypothesis (Ho) being tested was that the effect of a commercial enzymatic hydrolysis process does not affect the nutritional value of an intact protein. This hypothesis comes from the previous study of Darragh *et al.* (1997). They found in pigs fed a diet containing 10% hydrolysed whey proteins, a peak in the concentration of urea in the plasma from the abdominal aorta between 2 - 3 hr after consuming a meal whereas the urea concentration in pigs fed intact whey protein (10%) remained constant. Since a high concentration of urea in the plasma is an indication of amino acid catabolism (Whitney and Rolfes, 1996), Darragh and colleagues (1997) suggested the possibility of a lower utilisation of protein hydrolysates compared with intact proteins. They suggested that this difference could be caused by not only a faster absorption but also a faster catabolism of amino acids from the hydrolysed protein in comparison with the intact protein that did not allow the amino acids to be extensively utilised by the body.

Darragh *et al.* (1997) did not support their findings by monitoring the urinary N metabolite excretion occurring in their experimental pigs. Even though a NB would have indicated whether or not the two proteins were utilised similarly, it would not have shown any differences in the pattern of protein utilisation in the period that follows a meal. This latter question could only be answered by developing a technique in which the N excreted in individual urinary metabolites (urea, ammonia and creatinine) could be quantified at short intervals after the ingestion of the protein. Therefore, in the current study a new technique was developed where metabolic differences caused by the chemical form of the amino acids in the protein sources could be detected as a difference in the pattern of excretion of nitrogenous metabolites, as they are produced. Thus, if the amino acids from the hydrolysed protein were metabolised faster than those from the intact protein, then the increase in N excretion in individuals consuming hydrolysed protein would be earlier than that of those consuming the intact protein. Alternatively, if the chemical form of the protein had not affected the rate of absorption

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and amino acid metabolism, then the pattern of nitrogen excretion would not have differed between the two groups.

In the present study, we compared the utilisation of both proteins by using a novel technique (AUC, described in section 2.1.4) for measuring the pattern of excretion of nitrogenous compounds in the urine and comparing the results with a traditional nitrogen balance and daily weight gain record.

Total nitrogen excretion obtained by the sum of urea N, creatinine N, and ammonia N gives an estimate of the protein utilisation. This is because urea, ammonia and creatinine account for the 80% to 90%, 7.4% and 6.4%, respectively, of the total nitrogen in the urine (Gibson, 1990). And the nitrogen losses in the faeces, skin, hair and nails are relatively constant and account for less than 5% to 10% of the total nitrogen losses in the body (Gibson, 1990). Therefore an AUC not only gives valuable information about the protein utilisation during the immediate postprandial period but it can also give comparable information to the NB if it is carried out over a 24 hr period.

The features of the present research that have not been combined in previous experiments included: (1) The use of a hydrolysed protein obtained from the same industrial source as the intact protein (same batch). (2) The use of growing pigs, which have been used extensively as models in human nutritional studies. (3) Measuring a response in animals that have been subject to minimal surgical interference and are thus behaving normally.

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### Growth rate and daily weight gain

When a trial is performed over short time periods, such as the NB in this study, body weight changes are of limited value to compare two diet groups. Therefore, the results discussed here are only indicative of the relative performance of the pigs.

Growth rates were similar for the two groups of pigs averaging 0.66 kg/day for the IP group and 0.68 kg/day for the HP group over the three periods of the main experiment. However, both groups grew slower than the growth standard (0.78 kg/day) reported by Whittemore (1987) for pigs of similar age. There are several possible reasons for the reduced growth rates and they are discussed with the weight gain results.

The similarity of the daily weight gains (Figure 7) agree with results reported by Baró *et al.* (1995) who found that growing rats consuming hydrolysed whey proteins achieve comparable weight gains to those rats ingesting the native whey protein. Vandenplas *et al.* (1993) and Rigo *et al.* (1995) also noted similar average rates of daily gain between term infants who were fed either a whey protein hydrolysed formula or an intact protein-based formula. Vandenplas *et al.* (1993) also found that the length of the infants was comparable between diet treatments.

The daily gain during the adaptation period for the NB in the main trial was expected to be higher than the 90 g /day recorded (Figure 7) and the gain during the actual NB to be lower than 980 g /day. It is suspected that this is the result of a weighing error in the adaptation period. This conclusion was strengthened by putting the data into a pig growth model (De Lange, 1997), which estimated the expected daily weight gain during the NB for the HP and IP groups based on the diet consumed. The model predicted that the HP and IP group should be gaining 631 and 633 kg daily,

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respectively, over the NB period (De Lange, 1997). The estimated gains are very similar to the actual gains measured over the whole experiment. The growth model also predicted that the protein and energy in the diet during the NB would meet the nitrogen requirements for maintenance and growth of the pigs. Therefore, it is assumed that the weighing error does not invalidate the results of the experiment during the NB.

The pig growth model (De Lange, 1997) also estimated that during the 3<sup>rd</sup> AUC, HP and IP pigs would gain 707 and 708 g per day, respectively, which are close to the daily weight gains that were observed experimentally (Figure 7).

During the last 7d period in which acute urine samples were collected from the pigs, the daily weight gain for both group of pigs decreased significantly (Figure 7). The model estimated that the weight gain should be 760 kg and 761 kg for the IP and HP group, respectively. This reduction could be due to the stress induced from the intensive handling of the pigs housed in metabolism cages during the AUC period or a weighing error again caused by the scales. In any case, the relative growth rates of the pigs are not critical for interpreting the results from the AUC measurements.

The accuracy of the measurement of nitrogen intake is a crucial factor in any study of nitrogen balance and efficiency of nitrogen use. Although nitrogen intake between the two groups was identical in the main trial, it was not the same during the preliminary trial. The reasons for the difference in the nitrogen intake during the preliminary trial and how this error was corrected in the main trial are explained below.

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### Intake sampling

A number of adjustments to procedures were made during the course of the preliminary trial, particularly in the preparation of meals and the sampling of diets.

The diets were formulated to contain 10% protein equivalent (w/w) using data provided on the nitrogen content of the two protein sources. Samples of the diets fed to the pigs were collected and analysed for nitrogen content after the trials finished. This meant that it was not possible to adjust the food intake for any differences between the two diets in their nitrogen content.

The nitrogen contents of the two diets fed in the preliminary trial, when analysed in the laboratory, were statistically different. There was a particularly large discrepancy for batch 2 two used in the preliminary trial. Consequently, the calculated intake of protein was significantly different between the groups for the preliminary trial (Table 8). The difference may have been due to errors made either in making up the diets or in sampling the diets for nitrogen analysis. Possibilities include inaccurate values for the nitrogen content of the protein sources, inaccurate measurement of the ingredients or inadequate mixing of the ingredients while making up the diets. During the preliminary trial, the order of blending the materials was modified several times and it is possible that during the manufacture of batch two, the order in which the materials were added to the mix did not allow a homogenous dispersion of the intact protein among the other ingredients. In the main trial, the nitrogen content of the diets, although variable between batches, were similar between diets (Table 8).

In addition, during the preliminary trial, only one sample per batch was taken to analyse for the nitrogen content of each diet whereas two samples per batch were analysed for the main trial. Furthermore, the average nitrogen content between the

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batches was variable indicating either a problem with obtaining a homogenous mixing of the diets or analytical variation (See Appendix 4).

#### Validation of the technique

In order to get an accurate pattern of the rate of excretion of nitrogenous metabolites, it was necessary to assure a complete collection of urine from the bladder at every sampling time. For this reason, the accuracy and completeness of collection of all the urine from the bladder is a crucial factor in this technique. Incomplete collection of the urine from the bladder during the sampling period will lead to an underestimation of the rate of excretion of the nitrogenous metabolites for the period. The completeness of collection can be assessed by measuring creatinine excretion.

The rate of creatinine excretion is proportional to the amount of creatine in the muscle and the rate at which it is irreversibly converted to creatinine. Therefore, it is similar between animals fed similar diets and at the same stage of growth (Duggal and Eggum, 1978). For this reason, urinary creatinine excretion has been used as an indicator of the completeness of the collection of urine (Moughan *et al.*, 1990). In the present study, the pattern of excretion of creatinine was also used to demonstrate the degree of emptiness of the bladder at each sampling time.

Creatinine collected at each sampling period was not different statistically for the two dietary treatments ( $P > 0.05$ ) and was almost constant between the sampling periods within treatments (Figure 8 and 12). These results support the conclusion that the collection of urine from the bladder was reasonably complete at each sampling time. The fact that the effect of time on the creatinine N of 4<sup>th</sup> AUC was found significant, it may be related to changes in blood flow through the kidney. Therefore it does not represent a bias in the experiment. Furthermore, the total accumulative excretion of

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creatinine by both groups of pigs was not significantly different at any sampling time ( $P > 0.05$ ) which also indicates that urine collections and hence the collection of other urinary metabolites such as urea and ammonia were complete.

The validity of the present research is further supported by the fact that comparable values of rate of excretion of creatinine for pigs of similar weight have been reported in the literature. The mean urinary creatinine excretion for pigs fed the intact whey protein and those fed hydrolysed whey protein in the present study ranged from 32.24 to 41.46 mg N/kg<sup>0.75</sup> per day for pigs between 43 - 55 kg (Tables 9, 11, 15, 16). These data are comparable to those of Duggal & Eggum (1978) for pigs of similar body weights. Figure 16 shows a graph of the results of the present experiments superimposed on the results of a study by Duggal & Eggum (1978) in which creatinine was determined in the urine of pigs of several weights. From the graph, it is concluded that creatinine excretion and hence muscle protein metabolism during the present trials was comparable to that of pigs of the same weight reported by Duggal and Eggum (1978).

It is concluded that the technique used is a reliable method to detect the pattern of nitrogen excretion in the urine and hence the rate of protein metabolism following a meal. In the present research, the results were also compared with those obtained from a conventional nitrogen balance as outlined below.

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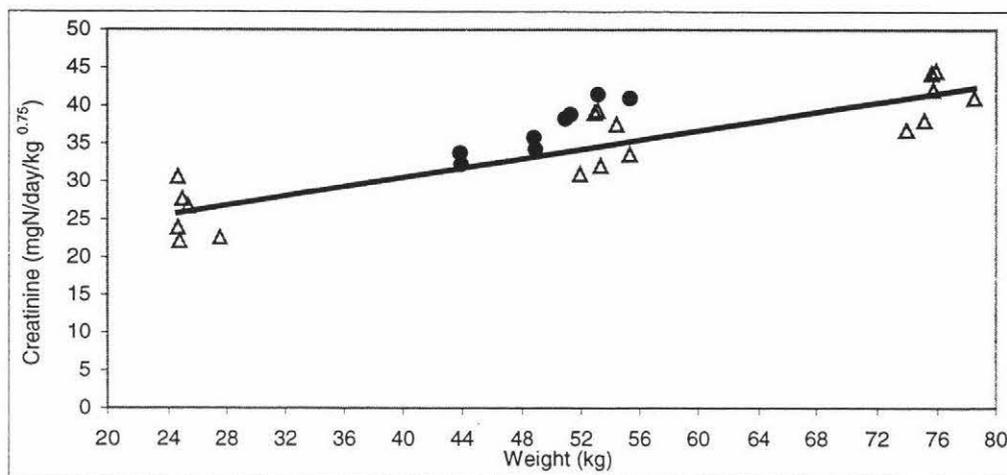


Figure 16. Rate of excretion of urinary creatinine ( $\text{mgN/day/kg}^{0.75}$ ) in Duggal and Eggum study ( $\Delta$ ) (1978) and the present study ( $\bullet$ ). The regression equation between body weight and daily excretion of creatinine is  $y = 0.3081x + 18.184$   $r = 0.8046$  for the data of Duggal and Eggum (1978). The data of the present research corresponds to the NB in the preliminary and the main trial, the 3<sup>rd</sup> AUC and the 4<sup>th</sup> AUC. The rate of excretion for the 3<sup>rd</sup> AUC was calculated using the mean rate of excretion per 30 min.

#### Retention of nitrogen in the body and excretion of total urinary nitrogen

The results from the nitrogen balance study indicate that the commercial enzymatic treatment did not affect the overall nutritional value of the native protein, given that the means for the nitrogen balance and protein retention were not significantly different between treatments ( $P > 0.05$ ). These results agree with those of Cézard and colleagues (1996) who also noted that nitrogen retention in rats fed an infant formula based predominantly on whey proteins was similar to that of rats fed its hydrolysed counterpart. In contrast to the results presented here, Poullain *et al.* (1989) and Moughan *et al.* (1990) reported that the retention of the hydrolysed protein in rats and pigs, respectively, was higher than that of the intact protein. Whereas Rigo *et al.* (1995) reported, that retention was lower in the hydrolysed versus the intact protein in rats. Differences in protein absorption and utilisation may explain the discrepancies between the results presented here and those of Rigo *et al.* (1995) and Poullain *et al.* (1989). The results of Poullain *et al.* (1989), Rigo *et al.* (1995) and Cézard *et al.* (1996)

studies are summarised in Table 17. The study of Moughan *et al.* (1990) is not summarised because it was not possible from their data to calculate the information presented in Table 17.

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Table 17. Nitrogen balances<sup>1,2</sup> comparing alimentary proteins and their hydrolysates.

	Poullain <i>et al.</i> (1989)		Rigo <i>et al.</i> (1995)		Cézard <i>et al.</i> (1996)		Quintino (2000)	
	Rats		Term infants		Rats		Growing pigs	
	I <sup>3</sup>	H <sup>4</sup>	I <sup>3</sup>	H <sup>4</sup>	I <sup>3</sup>	H <sup>4</sup>	I <sup>3</sup>	H <sup>4</sup>
N ingested (mg/kg/d) <sup>5</sup>	900 ± 0	900 ± 0	484 ± 63	438 ± 54	385 ± 20	345 ± 15	1570 ± 3.7	1574 ± 8.4
N absorbed (mg/kg/d) <sup>5</sup>	878 ± 3	881 ± 2	431 ± 60	362 ± 44	326	336	1491 ± 18.3	1498 ± 17.0
N retained (mg/kg/d) <sup>5</sup>	461 ± 22	831 ± 7 *	283 ± 60	223 ± 37*	283 ± 8	287 ± 15	1081 ± 36.2	1024 ± 46.4
N utilised (%)	52.5	94.3	65.7	61.6	86.81	85.42	72.5 ± 2.1	68.3 ± 2.7

<sup>1</sup> From Poullain *et al.* (1989), Rigo *et al.* (1995) and Cézard *et al.* (1996).

<sup>2</sup> Data are expressed as mean ± SEM

<sup>3</sup> I = intact protein

<sup>4</sup> H = hydrolysed protein

<sup>5</sup> Poullain *et al.* (1989) and Cézard *et al.* (1996) results are expressed as (mg/d).

\*  $P < 0.01$  as compared to the intact protein.

N absorbed was calculated from the difference between daily dietary nitrogen intakes and the determined levels of daily nitrogen excretion in the faeces.

Approximately 90% to 95% of the daily nitrogen losses are excreted in the urine (Gibson, 1990). Since urea N accounts for 80% to 90% of the total urinary nitrogen and the excretion of non-urea N components remains fairly stable (Gibson, 1990), Total Urinary Nitrogen (TUN) reflects any changes in the urea excretion and therefore in protein utilisation. A comparison of TUN between the present study and the studies from Cézard *et al.* (1996), Poullain *et al.* (1989) and Moughan *et al.* (1990) show this. The TUN in Cézard *et al.* (1996) and in the present study, were similar on both diets and therefore protein utilisation was assumed to be similar. Whereas, Poullain *et al.* (1989) and Moughan *et al.* (1990) observed lower TUN excretion in rats and pigs, respectively, when they were fed with hydrolysates in comparison with those animals fed the intact protein. Therefore, in the studies by Poullain *et al.* (1989) and Moughan *et al.* (1990) the hydrolysed protein was better utilised than the intact.

There are a number of possible reasons for the discrepancies observed between these various studies. These include the animal model used, the source of the protein and the degree of hydrolysis and molecular weight distribution of the peptides in the hydrolysate. It is not immediately apparent from the information presented in Table 18, however, that these provide an explanation for the differences.

To conclude this section, the results of the NB indicate that the nutritive value of the intact and the hydrolysed proteins are similar, as assessed by the total amounts of nitrogen retained and excreted. Furthermore, the growth rate of the groups was not statistically different.

From the data of the NB and the TUN excretion, the null hypothesis could be accepted. However, the lack of a difference between IP and HP observed in the total nitrogen excreted during the NB does not guarantee that there were no differences in

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the excretion of individual nitrogenous metabolites and therefore in amino acid catabolism. Moreover, the objective of the research was to use the AUC to quantify the pattern of nitrogen metabolism following a meal in terms of total nitrogen, urea, and ammonia excretion in the pig's urine.

Table 18. Comparison between the experimental conditions of several studies that evaluate the nutritional value of intact versus hydrolysed protein.

	Poullain <i>et al.</i> (1989)	Moughan <i>et al.</i> (1990)	Cézard <i>et al.</i> (1995)	Rigo <i>et al.</i> (1996)	Quintino, (2000)
Experimental Unit	Rat (250-300 g)	Piglet (4.5 kg)	Rat (80-90 g)	Term Infants (15 days)	Pig (37 kg)
Source of protein					
I	W	W/C (60:40)	W/C (60:40)	Conventional formulae	W
H	W	W/C (60:40)	W/C (60:40)	W and W/C	W
Residual antigenicity (%)	Not given	Not given	0.2 immunodot	Not given	0.1 SDS PAGE
Peptide distribution (%)					
0 – 200 D	} 65	} 53	5	} 42	} 21
200 – 300 D			26		
300- 400 D			} 25		
400 – 500 D					
500 – 600 D			} 44		
600 – 1,000 D	} 14				
1,000 – 1,500 D					
1,500 - 2,000 D	} 30	} 15	} 19		
2,000 – 5,000 D				13	
5,000 – 10,000 D	} 5	7	} 3		
> 10,000 D		12		1	
Protein in the diet (% w/w)	29	55	15	*	10
Feeding regimen	<i>Ad libitum</i>	5 rations	<i>Ad libitum</i> (5 p.m. to 9 a.m.)	On demand	2 meals/day
Daily intake	<i>Ad libitum</i>	168 ml (16% DM)/kg BW	<i>Ad libitum</i>	On demand	10% MBW
N retention	H>I	H>I	H=I	H<I	H=I

W = whey, C = casein, W/C = whey/casein ratio, BW = body weight, MBW = metabolic body weight, H = Hydrolysed protein. I = Intact protein

\* 1.6 g per decilitre (dry matter content do not given)

Urinary excretion of individual nitrogenous metabolites; their contribution to the total nitrogen.

Regardless of whether a 16 hr fast followed a meal (3<sup>rd</sup> AUC) or whether the normal feeding regimen was followed (4<sup>th</sup> AUC), there was no difference between groups in the total nitrogen excreted in either AUC indicating that the proteins appeared to be similarly utilised (Tables 15 and 16). Thus, the same conclusion is drawn from measuring the total nitrogen in urine collected by catheter as that reached from the NB.

By using AUC, however, it was possible to detect differences and similarities in the pattern of N excretion between pigs fed with the intact and the hydrolysed protein. The lack of a significant diet x time interaction over the 16 hr period after a single meal (Table 13, Figure 11) indicates that the pattern of excretion of total nitrogen did not differ between diets, but it was different (Table 14, Figure 15) when a normal feeding schedule was followed. This indicates that during a normal day, the pattern of excretion of nitrogen is affected by the nature of protein in the diet. The reason for this difference is other than different protein utilisation because the different pattern did not affect the total nitrogen excretion, which was not significantly different between diets following either feeding regimen (Tables 13 and 14). The difference in the pattern of excretion of total nitrogen is indicated in Figures 11 and 15, for 3<sup>rd</sup> and 4<sup>th</sup> AUC, respectively. In Figure 11 patterns for the two groups have a similar shape as indicated by the deviation from the line representing the mean rate of excretion. Whereas in the Figure 15, the pattern followed by the intact protein is different to that of the hydrolysed protein. The patterns of excretion of total nitrogen are the sum of the patterns of excretion of the individual metabolites, in particular urea and ammonia.

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## Urea

In a previous study, Darragh *et al.* (1997), found that plasma urea levels were higher in pigs consuming a diet containing hydrolysed lactalbumin compared to those consuming the intact protein. Darragh *et al.* (1997) suggested that higher concentrations of plasma urea in pigs on diets containing hydrolysed protein might be reflected by a higher excretion in the urine, although they did not confirm this experimentally. The present research supports the suggestion made by Darragh *et al.* (1997); in fact, pigs consuming the hydrolysed diet excreted statistically higher amounts of urinary urea compared to those pigs on the intact counterpart. In addition, at two time points (4 hr and 11 hr) the rate of excretion of the HP was higher than the IP. Vandenas *et al.* (1993) also observed an increase in plasma urea after feeding hydrolysed protein, which led to an increase in the urinary excretion of urea.

It would have been very desirable to have measured the concentration of urea in the blood over the collection period so that the patterns of excretion of urea in the urine could be compared with the concentration of urea in the blood. However, acute attempts to collect peripheral blood from the pigs were unsuccessful. The findings from this research indicate that in future studies blood samples should be collected so that the pattern of urinary excretion of nitrogenous metabolites and their concentration in the blood can be compared.

With regard to the patterns of excretion of the individual components, Darragh *et al.* (1997) found that the blood patterns of urea were different statistically between pigs fed the hydrolysed protein and the intact protein. The shape of the excretion curves for urea (Figure 9) found for the IP and the HP groups resembles those observed by Darragh *et al.*, but the interaction diet x time was not statistically different (Table 13). However, following a normal twice-daily feeding regimen (Figure 13), the interaction

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was different statistically (Table 14). This means that in a normal day, the patterns of excretion of urea differ between the groups fed the two diets. This is in accordance with the study performed by Darragh *et al.* (1997). The results presented here strongly indicate that in a normal day the pattern of urea formation and excretion is affected by the form of dietary protein. Longer-term experiments would be desirable to confirm the result.

Over the 6 hr period following a meal urea excretion by pigs in both the HP and IP groups was maintained above their respective mean rates of excretion (Figure 9) indicating a period of rapid amino acid absorption and catabolism. At 8 hr, close to the normal time for the next meal, excretion fell below the mean line suggesting that little amino acid absorption and subsequent metabolism was occurring. Therefore, AUC is a technique that allows the observation of the pattern of production of urea directly related to the catabolism of dietary amino acids.

The shift in the rate of urea excretion from above to below the mean rate of excretion (Figure 9) may indicate the time at which the animal enters negative nitrogen balance because of a lack of essential amino acids coming from the diet. Thus, it may be possible to use this technique to estimate the optimum time between meals, in particular for those products that are used by individuals such as newborn infants, and enterally and parenterally fed hospitalised patients.

Darragh *et al.* (1997) hypothesised also that the high plasma urea level in pigs consuming hydrolysed diet might be an indicator of inferior nitrogen utilisation in the liver and hence of lower nitrogen retention. The present results do not support this hypothesis because the rate of growth, the daily weight gain and the total nitrogen retention were not different between groups ( $P > 0.05$ ). Thus higher urea

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concentrations in the pig's plasma reported by Darragh *et al.* (1997) and the greater amounts of urea excreted in the urine of pigs fed hydrolysed protein as reported in the present study may not have been associated with protein degradation. Rather they may have been due to a higher rate of glutamine and asparagine breakdown in the gut of pigs fed the hydrolysate (Deutz *et al.*, 1996).

Deutz *et al.* (1996) associated higher liver urea production after meals of pre-digested protein or free amino acids with increased ammonia production in the portal visceral drain (PVD) which in turn was due to a marginally higher intestinal glutamine breakdown (not significant). They suggested that the differences in the breakdown of glutamine in the gut following meals of intact protein or its hydrolysate might be due to the molecular structure of the amino acids in the diet whereby the intact protein might protect glutamine and asparagine from deamination. In the partially digested protein molecule, however, glutamine might be present in a peptide with free  $\alpha$ - amino groups, which are then more labile to degradation. In future studies, it would be interesting to determine the contribution of glutamine and asparagine from hydrolysed protein to end products such as urea.

In conclusion, the results in this section indicate that under a normal schedule of diet, the pattern of urea excretion in urine changes when a protein is hydrolysed. The total urinary excretion of urea per hr is increased more immediately after a meal when protein is hydrolysed compared to when it is left intact. This higher excretion of urea is assumed to be not a consequence of inferior utilisation of the protein as evidenced by comparable body weight and total nitrogen retention data. Rather, the reason for a high urinary urea level, as suggested by Deutz *et al.* (1996) may be due to a higher gut metabolism of the glutamine and asparagine of the hydrolysed protein in comparison to the intact protein. Since the AUC technique indicates the pattern of amino acid

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catabolism, it may be possible to develop the technique to assess the optimum time between meals for a protein or commercial product.

#### Ammonia.

The total excretion of ammonia in the 3<sup>rd</sup> AUC and the 4<sup>th</sup> AUC differed significantly ( $P < 0.001$ ) between the two groups of pigs (Tables 13, 14 and Figures 10, 14) but the time x diet interaction was not significant ( $P > 0.05$ ) for the 3<sup>rd</sup> AUC but was highly significant ( $P < 0.0001$ ) for the 4<sup>th</sup> AUC.

When a normal feeding schedule was followed for the 4<sup>th</sup> AUC, the ammonia excretion of pigs fed the intact protein oscillated, over the postprandial period, closer to the mean rate of excretion than the pigs fed the hydrolysed protein (Figure 14). The ammonia excretion by the pigs fed the hydrolysed protein, however, had a more regular and defined pattern (Figure 10 and 14). In addition, the rates of ammonia excretion were significantly different at several time periods (Figure 14). The HP group excreted less ( $0.145 \text{ mM/hr/kg}^{0.75}$ ) ammonia nitrogen in the urine than that reported by Scott & McIntosh (1975), for pigs of similar size on standard diets ( $0.314 \text{ mM/hr/kg}^{0.75}$ ), whereas the IP group excreted comparable amounts ( $0.359 \pm 0.0723 \text{ mM/hr/Kg}^{0.75}$ ). Differences in both the amount and pattern of ammonia excretion probably indicate an imbalance in dietary electrolytes.

The reduction in excretion of  $\text{NH}_4^+$  in the HP group suggests that the pigs are experiencing a metabolic alkalosis (Scott and McIntosh, 1975), which they are correcting by increasing renal excretion of sodium and potassium ions and decreasing the excretion of ammonia. A temporary mild occurrence of metabolic alkalosis usually follows the ingestion of a meal as a consequence of the secretion of a large quantity of HCl into the stomach that increases the alkalinity of the extracellular fluids (Hladky &

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Rink, 1986). Subsequently, the pancreas secretes substantial amounts of sodium bicarbonate and re-establishes the acid-base homeostasis. Such a mechanism does not explain, however, the large difference in the pattern of excretion of ammonia nitrogen between the two groups after a meal. A metabolic alkalosis induced in this way would be similar for both groups and it would be mild and temporary. It would not be reflected in an observable difference in renal function. For these reasons, another explanation was sought for the differences observed in the excretion of ammonia between the groups.

The reduction in the excretion of ammonia N in the HP group can be explained by an excess of cations in the hydrolysed diet. An analysis of the mineral content of the proteins used in the diets revealed that the hydrolysed protein contained an excess of cations relative to anions whereas the concentration of minerals was much lower in the intact protein with equal concentrations of cations and anions. Consequently, the diets consumed by the HP group during the AUC contained more unbalanced cations than that consumed by IP group. The diets consumed by the HP group during the 3<sup>rd</sup> AUC and the 4<sup>th</sup> AUC, had an excess in cations respect to the intact protein of 74 meq and 91 meq, respectively.

The introduction of a fixed base, such as Na<sup>+</sup> or K<sup>+</sup> ions, into the body can cause metabolic alkalosis characterised by a high urinary excretion of HCO<sub>3</sub><sup>-</sup> and low urinary excretion of NH<sub>4</sub><sup>+</sup>. The excess Na<sup>+</sup> and K<sup>+</sup> have to be excreted through the kidneys but they must be accompanied by an anion. There are two major ways of increasing the availability of anion, one is to increase the excretion of HCO<sub>3</sub><sup>-</sup> and the other is to reduce the output of NH<sub>4</sub><sup>+</sup>.

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The addition of excessive cations into the diet has been shown to cause a reduction in the urinary excretion of ammonia (Scott, 1971). A graph of the percent reduction in urinary ammonia excretion following an increase in dietary cation excess plotted against the increase in cation for the preliminary and main nitrogen balances, and the 4<sup>th</sup> AUC together with data for individual pigs reported by Scott (1971) is presented in Figure 17. Since the data from Scott, (1971) were reported on a 24 hr base, the results for 3<sup>rd</sup> AUC could not be included in this comparison. The strong relationship with a significant linear correlation ( $r = 0.88$ ), supports the conclusion that the cation excess in the HP group most likely caused a reduction in the urinary excretion of ammonia.

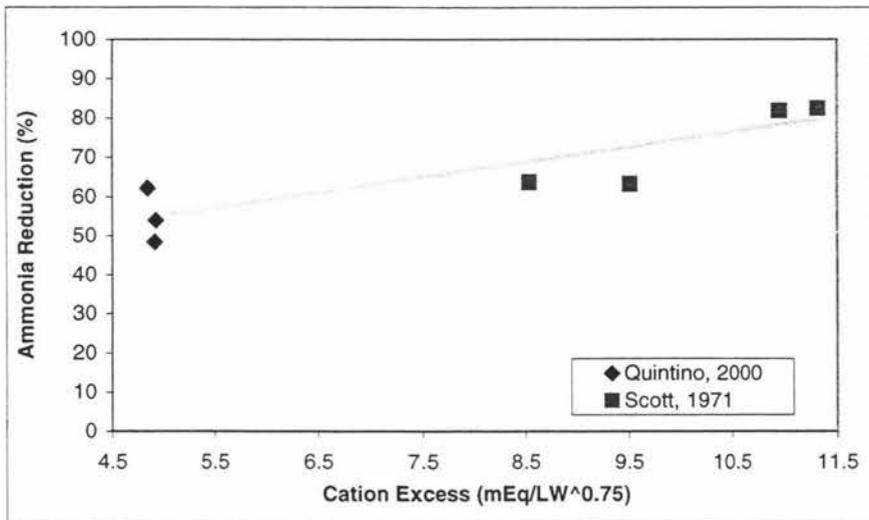


Figure 17. The percentage reduction in ammonia excretion when fixed cations are added in excess to the diet. Data from Scott (1971) when 200 mEq of cation were supplemented to two pigs weighing 67 and two pigs weighing 46 kg together with the mean data for the preliminary and main balances and 4<sup>th</sup> AUC trials, in the present study.  $Y = 34.65 + 3.86x$ ,  $r = 0.88$ .

Similar reasoning was used by Yamamoto *et al.* (1985) to explain the higher excretion of urinary  $\text{NH}_4^+$  by rats on an elemental diet compared with rats fed diets based on either hydrolysed casein or intact casein. The elemental diet was formulated

using hydrochlorides of lysine, histidine and arginine and consequently had an excess of fixed anions (Yamamoto *et al.*, 1985).

It has been observed that the utilisation of basic amino acids is affected by the dietary acid-base content (Austic and Calvert, 1981). The dietary “cation-anion balance” includes all the macrominerals and is defined as:  $\text{Meq} (\text{Na}^+ + \text{K}^+ + \text{Ca}^{++} + \text{Mg}^{++}) - \text{Meq} (\text{Cl}^- + \text{SO}_4^{--} + \text{H}_2\text{PO}_4^- + \text{HPO}_4^-)$ . Dietary electrolyte balance (DEB), however, is described in practical terms as  $(\text{Na}^+ + \text{K}^+ - \text{Cl}^-)$  and expressed as mEq/kg diet (Morgin, 1981). Austic and Calvert (1981) observed that the utilisation of amino acids in pigs improves as the electrolyte balance increases in diets that are limited in amino acids such as tryptophan. This response was observed, however, only when the lysine was largely deficient in diets (Patience *et al.*, 1987). Because the two protein sources tested in the present research contained similar amino acid compositions and contained all the essential amino acids in adequately amounts, the DEB (see Table 19) should not have affected the utilisation of basic amino acids in both diets.

Table 19. Dietary electrolyte balance in protein sources used in the experiment, and in human milk, bovine milk and infant formulae.

	DEB
Preliminary NB (mEq/kg diet)	
I	-3.20
H	73.40
Main NB (mEq/kg diet)	
I	-2.35
H	55.95
4 <sup>th</sup> AUC (mEq/kg diet)	
I	-9.13
H	68.94
Human Milk (mEq/L) <sup>1</sup>	10.08
Bovine milk (mEq/L) <sup>2</sup>	24.60 - 42.42
Formulae (mEq/L) <sup>3</sup>	
Whey predominant	10.30 - 14.50
Whey protein hydrolysate	12.84

<sup>1</sup> Mature milk (30 days) at 12 % solids (From Riordan, 1993).

<sup>2</sup> From Swaisgood, 1996.

<sup>3</sup> at the commercial dilution (370 kcal) (From Fomon, 1993).

However, the imbalance of electrolytes in the hydrolysed protein tested in this research, may have affected the performance of the pigs. Metabolic pathways and enzyme systems are modified wherever the acid-base balance in an individual changes significantly from the normal conditions (Ravindran, 1999). In these cases, the metabolic processes are modified to maintain the pH with the possible consequence of depressed performance. Hence, it is important to achieve optimum acid – base compositions in the diets. There are several studies (Patience *et al.*, 1987; Hayton *et al.*, 1990; Ravindran *et al.*, 1996) indicating that the weight gain and feed intake of pigs increases as DEB increases from 0 to 400 mEq/kg diet. Patience *et al.*, (1987) found that at a negative DEB such as –85 mEq/kg, growth and feed intake decreased ( $P < 0.05$ ). Since the DEB of the diet containing the hydrolysed protein in the present study was higher than that of the diet containing the intact protein (Table 19), a greater rate of gain may have been expected for the pigs fed the hydrolysed protein. That there was no difference in the rate of gain may have been due to a compensatory benefit of intact protein in comparison with the hydrolysed protein. This supposition may change the concept of the hydrolysed protein in this research, and agree with Rigo *et al.* (1995) who observed that the hydrolytic process decreases the utilisation, retention or absorption of amino acids of dietary protein. Because of the insufficient information about DEB in the study of Rigo *et al.* (1995), further studies in which the two protein source have the same DEB are necessary to clarify the situation. To this end, it is important that in future studies, DEB should be considered.

The difference in the cation-anion balance between the two proteins arose from the industrial process itself. Since during processing the pH of the reaction media must be maintained at an optimum for the enzyme, the consumption of acid or base contributes significantly to the dry matter composition of the end product (Adler-Nissen, 1986). Since ALATAL 821 and 825 are used as a base of infant formulae, the

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electrolyte balance ( $\text{Na}^+ + \text{K}^+ - \text{Cl}^-$ ) must be adjusted so that total intake of electrolytes is close to those for the breastfed infant. An infant formulae based on whey hydrolysate (Good Start) has 12.84 mEq per L whereas a whey predominant formulae (Enfamil, SMA) has 10.30 to 14.50 mEq per L at the standard dilution (667 kcal) (Fomon, 1993). Human milk has a DEB of 10.08 mEq per L (12% solids) or 8.4 mEq per kg milk solids (Riodan, 1993).

To conclude this part, the pattern of excretion of ammonia differed between treatments. The patterns reflected differences in the acid-base equilibrium in the body that were generated by different dietary electrolyte balances. It is suggested that an excess of cations in the hydrolysed protein reduced the excretion of ammonia. Further studies are needed to confirm this conclusion, which could include the measurement of ammonia balance across the kidney and the excretion of cations in the urine when either intact or hydrolysed protein is fed. In addition, it is possible that pigs fed intact protein would have had better performance (NB and weight gain) than the pigs fed hydrolysed proteins if the DEB in the diets had been similar. For this reason, it is important that in future studies, DEB from the protein source be taken in account in the formulation of the diets.

The results presented here demonstrate considerable variation in the distribution of nitrogen excretion between urea and ammonia which may explain inconsistencies between urea excretion in urine, its concentration in blood, nitrogen balance and weight gain found by other researchers such as Vandenplas *et al.* (1993). Vandenplas *et al.* (1993) reported similar daily weight gain and length gain in infants fed with either an intact or a hydrolysed protein. However, the plasma urea and the urinary urea were higher in those infants consuming the hydrolysed protein. It is possible that

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the higher excretion of urea in the hydrolysed diet was accompanied by a lower excretion of other nitrogenous metabolites that they did not quantify.

#### Other nitrogenous metabolites

Approximately 22% of the total nitrogen excreted in the pig's urine during the nitrogen balance corresponded to other nitrogenous metabolites (Table 11). Purines, such as allantoin may account for this residual nitrogen between the urinary total nitrogen determined analytically and that computed from the nitrogen in the urea, ammonia and creatinine (Hladky & Rink, 1986). Although these other urinary nitrogenous compounds were excreted in considerable amounts, it is assumed that their constant urinary excretion between diet treatments, would not have affected the results presented here.

The null hypothesis was that the effect of a commercial enzymatic hydrolysis does not affect the nutritional value of an intact protein. This research demonstrate that based on the quantification of the total nitrogen excreted either as an acute response to ingestion or as a long term response, the form (intact or hydrolysate) does not affect the protein utilisation.

It was also hypothesised that the chemical form (intact or hydrolysed) of the nitrogen source would not affect absorption and the pattern of excretion of urea would be the same. The differences in the patterns of excretion when the normal meal schedule was maintained, however, suggest that amino acids from the two sources were being absorbed differently. This difference in absorption was not it reflected in a difference in protein utilisation or difference in weight gain. Further studies must be done to determine the significance of the difference in the pattern of absorption to individuals consuming either hydrolysates or intact proteins.

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The technique proposed here allows the comparison of the utilisation of different sources of protein. It provides an estimate of protein utilisation by measuring the pattern of excretion in the urine of the major nitrogenous compounds during the postprandial period. This technique leads to comparable conclusions, about the utilisation of proteins, to those reached by measuring total nitrogen excreted in the urine in a traditional nitrogen balance. The technique also provides additional data by measuring the pattern of excretion of the urinary nitrogenous metabolites that reflect the time course of amino acid metabolism in the body or other factors such as alkalinity in the diet. Therefore, it represents a promising scientific tool to study aspects of the protein metabolism and acid-base status induced by the diet during the period following a meal. This technique may be useful to determine the optimal interval between meals. That makes it a commercially valuable tool in the evaluation of a protein source as the infant formulae.

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## CHAPTER 5

# RECOMMENDATIONS

The similar utilisation of nitrogen from the intact and hydrolysed protein, found in this research may increase confidence in the use of the latter as an alternative protein source. Hydrolysates would be particularly valuable for hospitalised patients whose medical conditions do not allow them to ingest intact proteins or as a nutritional supplement for the elderly. However, the reason for the more rapid increase after a meal in urea excretion in the urine must be determined before it is recommended.

The possibility of higher ammonia production by the gut when a hydrolysate is ingested is of concern because of the lack of information about the ability of the liver of the infant to handle it at the age that they consume infant formulae. Studies for longer periods of ingestion of the hydrolysed protein should be done using 3-week-old piglets, as a more valid model for the human baby. In the case that a negative effect is confirmed, it may be necessary to develop a hydrolysate in which the production of ammonia by the epithelial cells is reduced.

It has been proposed that athletes may recover faster from a negative nitrogen status during exercise if they consume hydrolysates, than if they consume intact protein. The findings from this research indicate that at rest, the protein utilisation is similar for hydrolysed and intact proteins and further research would be needed to assess whether there was a benefit from consumption of hydrolysates during exercise.

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The cation-anion balance of ALATAL 821 is different to that of ALATAL 825. It is possible that this can affect the acid base homeostasis of individuals consuming products in which the acid base content is not controlled. In the present research the imbalance in ALATAL 821 caused mild alkalosis in pigs at dosages of 1% of MBW. It is important that this is taken into account when formulating products using the hydrolysed protein.

The AUC a useful technique for studying the nutritive value of dietary proteins. AUC determines the protein utilisation, leading to the same conclusion from the nitrogen balance. In addition, AUC can determine differences and/or similarities in the protein absorption whenever they are reflected in the pattern of excretion of urinary metabolites such as urea. AUC is a technique that gives fast and detailed information about the protein metabolism in the post-prandial period. Therefore it is recommended as an experimental tool that could revolutionise the traditional techniques for assessing of the nutritional value of dietary proteins.

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## APPENDIX 1

### INFANT FORMULAE BASED ON HYDROLYSED PROTEIN

COMPANY NAME	PRODUCT NAME	PREDOMINANT PROTEIN	CHARBOHYDRATE	FAT	IRON (mg/L)
MEAD JOHNSON Canada	Enfalac Nutramigen	Hydrolysed casein	Glucose polymers corn starch	Corn, soy	13
MEAD JOHNSON USA	Nutramigen	Hydrolysed casein	Corn syrup solids, modified corn starch	Palm olein, soy, coconut, high oleic, sunflower	12
MEAD JOHNSON Canada	Pregestimil	Hydrolysed casein	Corn syrup solids, dextrose, modified corn starch	MCT, corn, soy, high oleic safflower	13
MEAD JOHNSON USA	Pregestimil	Hydrolysed casein	Corn syrup solids, dextrose, modified corn starch	MCT, corn, soy, high oleic safflower	12
CARNATION USA	Carnation Good Start	Partially hydrolysed whey	Lactose malto-dextrin	Palm olein, soy, coconut, high oleic, safflower	10
MEAD JOHNSON Canada	Carnation Good Start	Partially hydrolysed whey	Lactose malto-dextrin	Palm olein, soy, coconut, high oleic, safflower	10
ROSS LABORATORIES.	Alimentum	Hydrolysed casein	Sucrose tapioca starch	MCT, safflower, soy	12

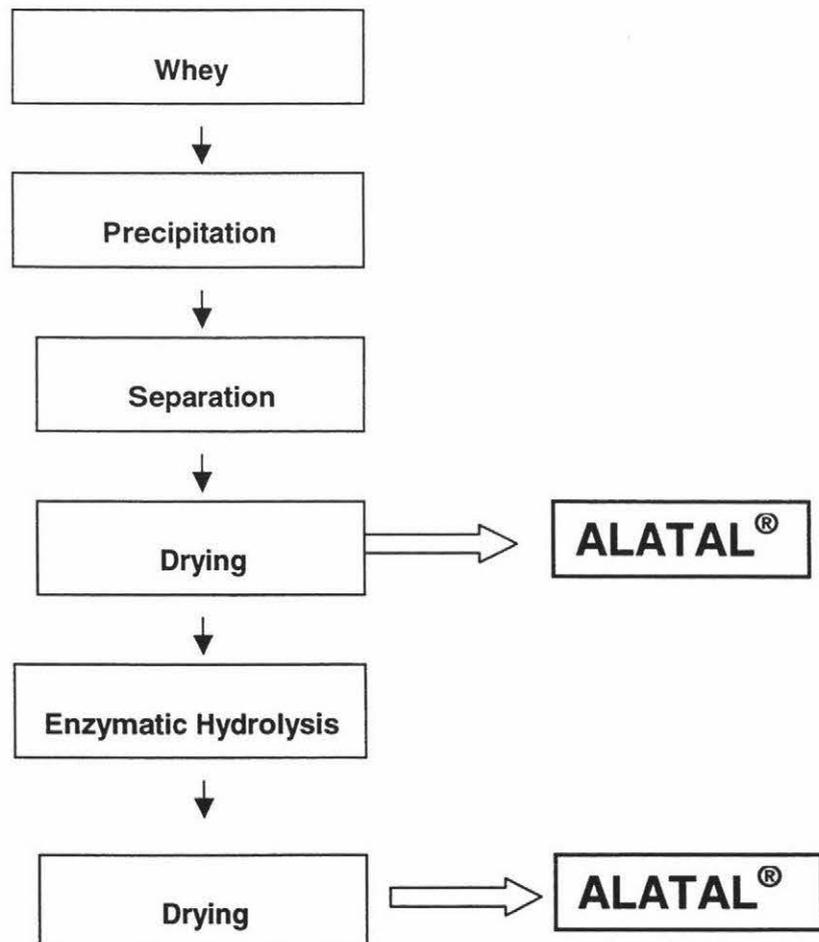
Source: L. Clark-Lowry (1999). Infant Formulas- An Update. HINS Articles. 18/10/1999 <http://www.hins.org/inform.htm>

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## APPENDIX 2

### ALATAL<sup>®</sup> 825 and ALATAL<sup>®</sup> 821 Outline manufacture

ALATAL<sup>®</sup> 825 is the lactalbumin base material used for the production of ALATAL<sup>®</sup> 821. ALATAL<sup>®</sup> 821 is an enzymatic hydrolysate of lactalbumin developed and manufactured especially for use in hypoallergenic infant formula. The basic process is outlined below. The enzymatic hydrolysis is carried out under mild and controlled conditions (temperature and pH) using GRAS enzymes from non-genetically modified organism sources (personal communication, Julie O'Sullivan, Anchor Products, Hautapu 14/12/99).



## APPENDIX 3

### Raw nitrogen balance data for the main trial

Pig	Diet	Body weight (kg)		Intake (g/4 days)	N Eaten <sup>3</sup> (gN/kg <sup>0.75</sup> /day)	Urinary N (gN/kg <sup>0.75</sup> /day)	Faecal N (gN/kg <sup>0.75</sup> /day)	N Retention (gN/kg <sup>0.75</sup> /day)	% N Faecal DM	% N Urine
		Initial	Final							
1	I <sup>1</sup>	37.7	45.2	6088	1.565	0.476	0.019	1.070	1.75	0.13
2	H <sup>2</sup>	37.0	45.0	6000	1.575	0.392	0.039	1.144	1.75	0.15
3	I	36.4	43.0	5936	1.567	0.296	0.067	1.203	1.64	0.18
4	H	35.9	42.1	5864	1.575	0.446	0.088	1.040	2.00	0.29
5	I	39.0	45.7	6288	1.576	0.452	0.071	1.053	2.93	0.29
6	H	37.4	43.6	5904	1.538	0.664	0.039	0.834	1.20	0.20
7	H	39.1	46.6	6240	1.572	0.452	0.041	1.079	1.59	0.19
8	H	36.2	43.4	6048	1.614	0.422	0.072	1.121	1.80	0.22
9	H	39.4	46.1	6256	1.567	0.393	0.093	1.081	1.51	0.19
10	I	29.2	35.5	5024	1.564	0.540	0.135	0.890	2.10	0.20
11	I	37.0	44.5	6000	1.564	0.362	0.149	1.054	2.18	0.20
12	I	39.2	45.2	6264	1.564	0.457	0.085	1.022	2.36	0.26
13	H	34.4	40.2	5680	1.575	0.546	0.160	0.869	1.66	0.17
<sup>4</sup> 15	I	40.4	47.2	6408	1.564	0.333	0.028	1.203	2.39	0.24
16	I	37.7	45.0	6088	1.565	0.364	0.076	1.125	2.15	0.19

<sup>1</sup> Lactalbumin, isolated from pure whey protein by heat precipitation.

<sup>2</sup> Hydrolysed whey protein isolate. More than 90% of peptides had a size below 5,000 Daltons.  
Degree of Hydrolysis = 25.2%.

<sup>3</sup> The nitrogen eaten was computed using the data for the batch 3 in the Appendix 4.

<sup>4</sup> Pig 14 (H) was not considered into this experiment.

## APPENDIX 4

### NITROGEN CONTENT OF THE DIET

Analysis of nitrogen content for all the diet batches used during the experiment. Batches 1 and 2 were used during first and second 7d period of the preliminary trial. Batches 3, 4 and 5 were used during the experimental periods 1, 2 and 3 of the main trial.

	Diet containing the intact protein			Diet containing the hydrolysed protein		
	Dry Matter (%)	Nitrogen (%)	Nitrogen (% DM)	Dry Matter (%)	Nitrogen (%)	Nitrogen (% DM)
Batch 1 (Manufacturing)	89.84	1.62	1.80	89.42	1.59	1.78
Batch 2 (Manufacturing)	90.11	1.55	1.72	89.48	1.64	1.83
Batch 3 (Manufacturing) (NB <sup>1</sup> )	91.02 90.44	1.62 1.58	1.78 1.74	91.02 89.61	1.58 1.56	1.73 1.75
Batch 4 (Manufacturing) (AUC <sup>2</sup> )	91.77 90.37	1.62 1.59	1.76 1.76	91.52 90.74	1.56 1.56	1.70 1.72
Batch 5 (Manufacturing) (AUC <sup>2</sup> )	91.69 89.75	1.59 1.57	1.73 1.75	91.59 91.00	1.63 1.59	1.78 1.75

<sup>1</sup> NB = Nitrogen Balance

<sup>2</sup> AUC = Acute Urine Collection

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