Endogenous Ileal Amino Acid Excretion in Monogastric Animals

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ABSTRACT

A new method for the determination of endogenous ileal amino acid excretion under conditions of peptide alimentation was refined and evaluated with studies involving the laboratory rat. The refined method was used to investigate aspects of endogenous ileal amino acid flow in the pig. Five studies were conducted, three with rats and two involving the growing pig.

1. Preliminary investigations evaluated the filtration efficiency of ultrafiltration devices, and examined three pre-filtration treatments for rat ileal digesta: trichloroacetic acid (TCA) and perchloric acid (PCA) precipitation, and centrifugation (SPIN). The recovery of nitrogen following ultrafiltration (molecular weight exclusion limit 10,000 Daltons) of fifteen purified protein, peptide and amino acid solutions indicated an effective filtration (>90%) on nominal molecular weight by the ultrafiltration devices. Determination of nitrogen and amino acids in the resulting fractions following TCA and PCA precipitation and centrifugation of rat ileal digesta indicated that PCA was the most effective precipitant. Endogenous ileal amino acid excretions in the growing rat fed an enzymically hydrolysed casein (EHC) based diet with subsequent treatment of the digesta using the ultrafiltration technology were then determined. Twelve 100 g male rats were fed either an EHC-based diet or a protein-free diet and samples of digesta were collected after slaughter. The digesta from the 6 EHC-fed rats were ultrafiltered after centrifugation and the high molecular weight fraction added to the precipitate. The protein-free fed rats had significantly ($P<0.05$) lower amino acid flows than those rats fed the EHC-based diet with subsequent treatment of the digesta.

2. The proportions of endogenous protein-, peptide- and free amino acid nitrogen (N) in digesta N from the distal ileum of the rat immediately after collection or following storage frozen (-20°C and -196°C) were compared. Eighteen growing rats were given a protein-free diet for 6 days, euthanased and samples of digesta were collected from the terminal 20 cm of ileum. The storage of digesta did not significantly affect the proportions of N-containing substances in the precipitate plus retentate or ultrafiltrate fractions. On average, 67% of the total digesta N was in the precipitate plus retentate fraction and 33% of total digesta N was in the ultrafiltrate fraction. Free amino acid N and peptide N were 10.4 and 10.6% of total digesta N, respectively.

3. The effect of using different flushing media for the collection of ileal digesta on the composition of endogenous N was examined. Twelve growing rats were given a protein-free diet and samples of ileal digesta were collected from the

euthanased animal using either distilled water or physiological saline as the flushing medium. There was no significant effect of collection method on the levels of N-containing substances in rat endogenous ileal digesta.

4. The effects of state of body nitrogen balance and the presence of dietary peptides and protein in the digestive tract on the excretion of endogenous amino acids from the ileum of the pig were investigated. Endogenous lysine excretion was determined for pigs given a protein-free diet, an EHC-, a zein- or a synthetic amino acid-based diet. Endogenous flows for amino acids other than lysine were determined for pigs on the protein-free and EHC-based diets. Six male pigs (15 kg liveweight) were allocated to each of the four diets and received the diet for 10 days. The mean endogenous ileal lysine flows for the zein and EHC fed pigs were not significantly different but were higher ($P<0.05$) than those for the protein-free and synthetic amino acid fed pigs whose mean flows were not significantly different from each other. The mean endogenous ileal flows for amino acids other than lysine were higher ($P<0.05$) for the EHC fed pigs compared to the animals on the protein-free diet, except for proline, glycine and arginine.

5. The effect of food dry matter intake on endogenous ileal amino acid excretion of the pig under peptide alimentation was determined. Sixteen male pigs (50 kg liveweight) each fitted with a T-cannula in the terminal ileum were fed at 8 levels of food dry matter intake for periods of 8 days. The experiment involved two trials of 8 pigs each, comprising a cross over design. Each trial involved 4 pairs of pigs with each pair receiving one of 4 sequences of treatment. Each sequence comprised 4 levels of food dry matter intake arranged in a Latin square. The food dry matter intakes were 0.06, 0.08, 0.10 and 0.12, and 0.05, 0.07, 0.09 and 0.11 metabolic liveweight ($W^{0.75}$) day^{-1} for the first and second trials, respectively. There was an increase in ileal excretion of amino acids, nitrogen and dry matter with increasing food dry matter. There were significant ($P<0.05$) linear relationships between endogenous ileal amino acid and nitrogen excretion and food dry matter intake except for lysine, glutamic acid and phenylalanine which increased in a curvilinear manner. These relationships, determined under physiologically more normal conditions than under protein-free alimentation, provide preliminary data on the magnitude of small intestinal amino acid losses in the pig.

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INTRODUCTION

During digestion large quantities of endogenous protein are secreted into the lumen of the gut and mixed with the dietary protein. Both the exogenous and endogenous proteins are digested and amino acids absorbed along the gastrointestinal tract. Endogenous ileal amino acid excretions are defined as the non-dietary amino acids that remain undigested and unabsorbed at the end of the ileum. These amino acids derive mainly from the gastrointestinal tract and include enzymes, mucoproteins, desquamated cells, serum albumin, peptides and free amino acids. Microorganisms and ingested body hair are also included in the measurement of endogenous amino acid excretion even though they are not strictly endogenous. The characterisation and measurement of endogenous ileal amino acid excretions from monogastric animals including humans is important to the understanding of gastrointestinal physiology, as well as having practical application in the determination of amino acid requirements by the factorial method and the determination of true amino acid digestibility.

Net endogenous ileal protein excretion has traditionally been determined following feeding of a protein-free diet or by the regression method. The estimates of endogenous loss determined by the regression method have been found to be similar to those determined under protein-free alimentation. The absence from the diet of such an important nutrient as protein, however, may cause a physiologically abnormal metabolism in the animal. There is evidence that the amount of protein secreted into the gastrointestinal tract is reduced when an animal is fed a protein-free diet.

Recent studies using a variety of techniques (^{15}N tracer, guanidinated protein, peptide alimentation) have provided evidence that endogenous amino acid loss is higher under protein or peptide alimentation in comparison with protein-free feeding. The ^{15}N tracer technique can be criticised, however, for the significant effect the choice of precursor pool may have on the dilution factor. The tracer method can be used to determine the endogenous nitrogen in digesta and faeces, but not the proportions of endogenous amino acids. Work to date using the homoarginine and peptide alimentation methods has been with the rat and using relatively unrefined methodologies. Also, the homoarginine approach provides data only for lysine. In the present work, the peptide alimentation method was refined and evaluated using the rat as the experimental animal. The refined methodology was applied to the pig to further elucidate the effect of dietary peptide, protein and

level of dry matter intake on endogenous ileal amino acid excretion. The overall aim of this thesis was to further understanding of the process of secretion and excretion of proteins from the upper digestive tract of simple-stomached animals generally. The laboratory rat and growing pig were accepted as experimental models for the study of mammalian digestive physiology. The laboratory rat is a widely accepted mammalian model which has been routinely used in investigations of digestive physiology and nutrition. The pig is an important agricultural monogastric species whose digestive physiology and nutrition have been well characterised, and is widely recognised as a suitable model animal for digestion studies in humans.

Chapter 1

REVIEW OF LITERATURE

1.1 INTRODUCTION

Large amounts of endogenous amino acids are secreted into the gut lumen during the course of digestion and are mixed with the dietary proteins. This review first considers the physiology of the digestion and absorption of protein. Second, the nature of the protein secretions into the mammalian gastrointestinal tract, their functions during digestion, the physiological control of and the influence of diet on these secretions are reviewed. Third, the quantities of nitrogen and amino acids secreted by the digestive tract of the pig and the degree of digestion these secretions undergo before they reach the end of the ileum are discussed. Finally, detailed consideration is given to the various approaches that have been used to determine net endogenous ileal amino acid excretions in the pig and the rat.

1.2 PROTEIN DIGESTION AND ABSORPTION

Comprehensive reviews of the physiology of digestion in the mammalian digestive tract with emphasis on humans have been published by Johnson (1981a), Davenport (1982), and Sanford (1982), for the dog and cat by Strombeck and Guilford (1990), and for the pig by Kidder and Manners (1978) and Low and Zebrowska (1989). Detailed reviews of the nervous and hormonal control of secretion and motility in the gastrointestinal tract have been presented by Kidder and Manners (1978), Walsh (1981), Holst (1986), Greenwood and Davison (1987), Fioramonti and Bueno (1988), and Strombeck and Guilford (1990). The digestion and absorption of protein have also been extensively reviewed (Gitler 1964, Cuthbertson and Tilstone 1972, Erbersdobler 1973, Snook 1973, Rérat *et al.* 1976, Kidder and Manners 1978, Rérat 1981, Hunt and Groff 1990, Rérat and Corring 1991). Protein digestion occurs predominantly in the stomach and proximal small intestine and the main site of absorption is the distal small intestine. Undigested proteins leaving the small intestine are metabolised by the microbial population in the large intestine.

1.2.1 Gastric Digestion

The digestion of protein starts in the stomach with the disorganisation of the protein molecule through denaturation by hydrochloric acid. The stomach also mixes, moistens and stores the food, initiates enzymic hydrolysis, and regulates the rate of digestion by feed-back mechanisms which are controlled by nervous and hormonal processes (Erbersdobler 1973). Enzymic protein digestion in the stomach occurs by the action of the gastric proteinases. The predominant gastric proteinases of adult mammals are pepsin A also commonly known as pepsin, pepsin C, chymosin also known as rennin, and pepsin B (Foltmann 1981). These enzymes have optimal activity under acid conditions, and like other proteolytic enzymes are formed as inactive precursors called zymogens which are activated by hydrolytic removal of a peptide from the amino end of the molecule. The gastric proteinases are non-specific, in that no general or clear-cut rules for specificities have been found, with the exception that peptide bonds between L-amino acids are preferred, and the most rapid rate of hydrolysis occurs for bonds near the aromatic amino acids (Taylor 1968).

Gastric digestion of protein is dispensable but is important to the animal's nutritional state and growth. Its absence causes a reduction in overall amino acid absorption, and total gastrectomy produced a lowering of the apparent digestibility of the proteins by 17-18% in the pig (Cunningham 1967). The influence of dietary protein on the rate of stomach emptying has been reviewed by Rogers and Harper (1964), Porter and Rolls (1971), Snook (1973) and Low (1990). The rate of passage of food proteins from the stomach and thus the degree of initial hydrolysis depends on the amount and type of protein in the diet and generally does not seem to be related to protein quality.

1.2.2 Digestion in the Small Intestine

The contents of the stomach pass into the duodenum where they are mixed with pancreatic, intestinal and bile secretions. All of these secretions are alkaline and thus terminate the activity of the gastric proteinases but they provide the optimal pH range required by the pancreatic proteolytic enzymes. The presence of acid and food in the duodenum stimulates the secretion of a range of proteolytic enzymes from the pancreas, which in conjunction with proteolytic enzymes present in the brush border of the intestinal mucosa and in the mucosal cells, digest the dietary and non-dietary peptides and proteins. Microbial proteases and sloughed intracellular enzymes may have an important digestive function in the lower small intestine and particularly in the digestion of endogenous proteins (Snook 1973).

The intestinal proteolytic enzymes can be classified into three groups (Kidder

and Manners 1978). These are: (1) endopeptidases, which act on a susceptible peptide link wherever it occurs in the protein chain, providing it is accessible to the enzyme; (2) carboxypeptidases, which remove an amino acid residue from the carboxyl end of the chain; (3) aminopeptidases, which remove an amino acid residue from the amino end of the chain. The endopeptidases and carboxypeptidases are secreted by the pancreas as their inactive zymogens, while the aminopeptidases are associated with the intestinal mucosa either on the brush border or within the mucosal cell. The zymogens of all the pancreatic proteases are activated by trypsin. Initially trypsin is activated by enterokinase, an enzyme located on the brush border of the small intestine mucosa, with greatest activity occurring in the duodenum (Antonowicz 1979). The trypsin so produced can activate more trypsinogen as well as activating the other proenzymes.

1.2.3 Mucosal Digestion

There are estimated to be at least eight different peptidases in the small intestinal mucosa (Lindberg *et al.* 1975, Friedrich 1989). Peters (1975) reviewed the subcellular localisation of intestinal peptide hydrolases in the enterocyte, and found that the brush border contains peptidase activity against peptides 3-6 residues in length and possibly large polypeptides, while the dipeptidases appear to be present predominantly in the cytosol but with some present in the brush border. Some of the peptidases from the brush border and cytoplasm of the cells are solubilised in the lumen of the small intestine with the sloughing of mucosal cells, and continue their hydrolytic activity there. Intestinal enzyme activities have been observed to be regionally distinct. For example, alkaline phosphatase is predominant in the duodenum, disaccharidases are predominant in the jejunum, and aminopeptidase is predominant in the ileum (Lindberg *et al.* 1975) suggesting that the completion of carbohydrate digestion occurs in the middle of the small intestine, and the digestion of proteins is nearly complete at the end of the small intestine.

1.2.4 Absorption of the Products of Protein Digestion

The products of protein digestion do not appear to be absorbed from the stomach but are absorbed from the small intestine predominantly as amino acids and peptides. The jejunum appears to have a larger capacity than the duodenum or the ileum for the transport of both free amino acids and peptides. It is now generally accepted that peptides and amino acids are transported across the mammalian small intestine by different systems, however the number of systems involved and the role sodium might play in facilitating absorption of these different substrates requires further clarification.

The mechanisms of amino acid absorption from the gastrointestinal tract have been reviewed by Wiseman (1964, 1968), Booth (1968), Holdsworth (1972), Matthews (1972), Munck (1981), Rérat *et al.* (1980), Adibi and Kim (1981), Smith (1983), Adibi (1985), Silk *et al.* (1985), Steinhardt (1987), Friedrich (1989), Webb (1990) and Rérat and Corring (1991). The transport of amino acids by intestinal enterocytes occurs by simple diffusion, facilitated diffusion (Na^+-independent) and active transport (Na^+-dependent). The precise number of amino acid transport systems in the small intestine is not known, but several different carrier systems have been confirmed with some of them located specifically on the brush border, some on the basolateral membrane, and some on both. Amino acid transport systems are currently classified on the basis of substrate preference. Transport of a particular amino acid is determined by the size, charge and the configuration of the amino acid's side chains, yet amino acids with quite diverse structures often share a transport system. The generally accepted amino acid transport mechanisms include two for neutral amino acids (α-amino-monocarboxylic acids, and imino acids), one for the acidic amino acids, and one for amino acids with their amino group in the beta- and gamma-positions.

The absorption of peptides from the lumen of the gastrointestinal tract has been reviewed by Holdsworth (1972), Silk (1974), Adibi (1975, 1985), Matthews (1975a, b), Adibi and Kim (1981), Smith (1983), Silk *et al.* (1985), Steinhardt (1987), Friedrich (1989), Webb (1990) and Rérat and Corring (1991). Intestinal mucosal uptake of peptides is limited to dipeptides and tripeptides and is mediated by a specific carrier system which is probably driven by an electrochemical proton gradient. This peptide carrier system, which operates against the concentration gradient, shows preference for peptides with bulky side chains and L-stereoisomer amino acid residues in both the amino and carboxyl terminals. The uptake of peptides from the gut lumen is generally faster than the absorption of free amino acids (Matthews 1975a, b, Adibi and Kim 1981, Rérat *et al.* 1985, 1988, Webb 1990). The intestinal peptide transport system is physiologically important because it allows intracellular hydrolysis of dipeptides and tripeptides by the peptide hydrolases in the cytoplasm, thus greatly increasing the efficiency of absorption of the protein digestion products.

Peptides that are resistant to dipeptidase activity, such as hydroxyproline-containing peptides following a gelatin meal, and carnosine and anserine following the consumption of chicken breast, have been detected in elevated levels in plasma (Matthews 1975a, b, Webb 1990). The appearance of peptides in the portal blood may result from absorption from the intestinal lumen or they may be degradation products associated with intestinal protein turnover. Many biologically active

peptides, such as phenoxymethyl penicillin, bile salts and the vitamins, folic and pantothenic acid, are also absorbed intact from the gut (Matthews 1975a, 1975b). A peptide that is absorbed intact from the gastrointestinal tract following ingestion must be resistant to acid and proteolytic enzyme attack. Matthews (1975a,b) lists four possible mechanisms for the absorption of peptides: by the mechanism(s) responsible for absorption of macromolecules and intact proteins; by diffusion through the plasma membranes of the absorptive cell (lipid soluble); by diffusion through aqueous pores in the plasma membranes of the absorptive cells (water soluble); or by a specific carrier mechanism. Peptides that are absorbed intact are probably not hydrolysed in the plasma, but are transported into tissue cells where they are hydrolysed and their constituent amino acids are made available to the cell (Webb 1990). The quantity of peptides that are absorbed intact and the nutritional, hormonal and immunological significance of this absorption are not known.

Proteins and even whole particles can be absorbed intact by the healthy mammalian gastrointestinal tract (Walker 1981, Sanford 1982; Baintner 1986, Magee and Dalley 1986, Seifert and Saβ 1987, Gardner 1988, Friedrich 1989). The absorption of macromolecules in some newborn mammals is a normal physiological process but it is lost or masked as the young animal develops the ability to digest food by acid and enzyme secretion. Four possible mechanisms for macromolecular and particle absorption across the intestinal wall of both mature and immature animals have been postulated: by binding to brush border receptors and then pinocytosis; through the intercellular clefts; via phagocytic cells which migrate into the intestinal epithelium; and via special cells such as the M cells of Peyer's patches of the intestinal epithelium. The amount of absorbed macromolecules or particles has been reported to be small (2% for intact bovine serum albumin) and also quite high (50% for the pineapple protease bromelain). The absorption of large molecules does not occur in sufficient quantities to be of nutritional importance but the small quantities may be of immunological importance, but why this occurs and the precise mechanisms of absorption are not known.

1.2.5 Digestion in the Large Intestine

In the large intestine, there is considerable bacterial deamination, decarboxylation and transformation as well as synthesis of bacterial protein from the amino acids arriving from the small intestine (Fauconneau and Michel 1970, Mason 1980, Wrong *et al.* 1981, Zebrowska 1982, Just 1983). Endogenous proteolytic enzymes play a limited role in the digestion of nitrogenous compounds within the large intestine because there is little protease secretion by the caecum and colon mucosa, and only small amounts of proteolytic enzymes enter the hindgut from the

distal ileum which are rapidly inactivated by the hindgut microorganisms (Zebrowska 1982).

Ammonia is the main nitrogenous compound absorbed from the large intestine. The newborn piglet has been shown to actively transport methionine from the proximal colon (James and Smith 1976) but there is limited evidence of active transport of amino acids in the hind gut of older animals (Binder 1970). There is evidence, however, for limited transfer of a few amino acids into the colon mucosa of pigs which appears to occur by simple diffusion (Zebrowska 1982).

Protein digestion and absorption in the large intestine have been shown to be of little nutritional significance. This was first demonstrated by Zebrowska (1973, 1975) who infused both intact and hydrolysed casein into the terminal ileum of pigs fed a nitrogen-free diet and found that the infused material was digested and absorbed, but the absorbed nitrogen was rapidly and completely excreted in the urine. Just *et al.* (1981) in a similar study infused soya-bean meal, protein concentrate, skim milk powder, meat-and-bone meal and lysine HCl into the caecum of the pig and also found near complete digestion of the nitrogen, but most of this was not utilized and was excreted in the urine. Schmitz *et al.* (1991) reported no trace of homoarginine in the blood following the infusion of homoarginine into the caecum of pigs which also indicates little or no absorption of amino acids from the large intestine of this animal. In contrast, Niiyama *et al.* (1979) found ^{15}N-labelled amino acids in the blood of pigs that were infused with ^{15}N-labelled micro-organisms into the caecum.

In summary, the digestive secretions into the gastrointestinal tract are of vital importance to the assimilation of dietary protein. The quantities and compositions of these gastrointestinal secretions as well as the control of the secretion and the effects of dietary components on their secretion and composition for the rat and pig will now be discussed.

1.3 GASTROINTESTINAL PROTEIN SECRETIONS

Considerable quantities of proteins, peptides, amino acids and other nitrogen-containing compounds diffuse into and are secreted into the lumen of the gastrointestinal tract during the digestion of food (Fauconneau and Michel 1970, Snook 1973, Kidder and Manners 1978, Buraczewska 1979, Low 1982a). The salivary glands, the pancreas and the glandular parts of the stomach and intestine secrete digestive enzymes which break food down to smaller molecules. The liver secretes bile salts with surface active properties which assist the dispersion of fat

and especially the micellar dispersion of the products of fat hydrolysis, which is essential to their absorption. There are mucus secreting cells active along the entire length of the tract, and epithelial cells are continually replaced and the old cells shed from the intestinal mucosa into the gut lumen. Also, plasma proteins, free amino acids, amines and urea diffuse into the gut from the epithelial cells. The secretions from the various glands are regulated by a combination of nervous and hormonal controls which simultaneously regulate motility throughout the gastrointestinal tract. The overall effect of these control mechanisms is to provide secretions in quantities sufficient for the efficient digestion and assimilation of food as it passes through the gastrointestinal tract.

1.3.1 Salivary Protein Secretion

Salivary secretion has been reviewed by Burgen (1967), Ellison (1967), Schneyer and Schneyer (1967), Kidder and Manners (1978), Jacobson (1981), Davenport (1982), Sanford (1982), Van Lennep *et al.* (1986), and Low and Zebrowska (1989). Mammalian saliva is important in cleaning and protecting the teeth, moistening food and oral surfaces, lubricating the oesophagus and food bolus, providing the fluid environment required for taste, and initiating the digestion of starch.

The protein levels in saliva have been recorded to be 0.63-0.95 mg/ml with a volume of 13.1-32.1 ml/100 g of cereal-based diet for 107-110 day-old pigs (Juste 1982). Kidder and Manners (1978) reported the composition of saliva from pigs with fistulas of the parotid salivary gland to be nitrogen 0.40-1.05 mg/ml, dry matter 0.7-1.6% and amylase 12-64 Wöhlgemuth units. Human submandibular and parotid saliva contain only 0.1 and 0.5 g protein per 100 ml, respectively (Davenport 1982).

Many biologically active polypeptides have been found in saliva, including digestive enzymes (α-amylase, ribonuclease, acid phosphatase, kallikrein, rennin), growth factors (nerve growth factor, epidermal growth factor), and gastrointestinal regulatory peptides. Of the various enzymes secreted by the salivary glands, some have a digestive function (α-amylase and lipase) but others are considered mainly protective (peroxidase and lysozyme). Other proteins and nitrogen-containing substances present in saliva include albumin, transferrin, globulin, lactoperoxidase, transaminases, secretory IgA, blood group substances, mucus, antigens, free amino acids, urea and uric acid.

Initiation and maintenance of secretion by the salivary glands are almost exclusively dependent on the parasympathetic and sympathetic nerves, with the parasympathetic system being the stronger stimulus. The protein secretion products, stored in the granules of salivary endpiece cells, are discharged by exocytosis, as in

other exocrine glands. Salivary glands also respond to acetylcholine, noradrenaline (α- and β-receptors present), substance P (SP) and vasoactive intestinal polypeptide (VIP). It is thought that acetylcholine, the α-adrenergic agonist and SP initiate secretion (Van Lennep *et al.* 1986).

The speed of onset, duration of secretion and volume of saliva secreted in the pig have been shown to vary according to the stimulus, and the left and right glands respond independently to stimuli applied to the respective sides of the mouth (Kidder and Manners 1978). The composition of saliva depends on the rate at which different cell types contribute to the secretion and the subsequent modification of the secretion by the duct cells. There are two types of salivary secretion, the thick mucus secretion containing mucopolysaccharides and the watery serous secretion containing amylase. The various salivary glands produce mainly one or other of these secretions.

The quantitative and qualitative collection of saliva poses practical difficulties. The material collected can be contaminated with oral tissue, fluid from the gingival sulcus, bacteria and food remnants. The collection apparatus must maintain its position despite the close proximity of teeth and tongue, and the glands on both sides of the jaw as well as all the contributing glands (submaxillary, parotid, sublingual and submandibular) need to be collected from. There are also secretions from numerous small glands found beneath the oral mucous membrane covering the lips (labial), palate (palatine), tongue (lingual) and cheek (buccal).

The effects of diet composition on salivary secretion have been little studied. This is probably because of the practical difficulties of the qualitative and quantitative collection of salivary secretions outlined above, and the relative insignificance of salivary protein contribution to overall endogenous protein at the end of the ileum. Low (1989a, b) reported an early study of parotid saliva secretion which showed no systematic relationship to changes in diet composition of pigs fed either a protein concentrate, crushed barley, crushed oats or wheat flour.

1.3.2 Gastric Protein Secretion

The anatomy of the stomach and the stimulus of gastric secretion have been extensively reviewed (Ito 1981, Johnson 1981b, Malagelada 1981, Davenport 1982, Sanford 1982, Magee and Dalley 1986, Davison 1989a, Low and Zebrowska 1989, Strombeck and Guilford 1990, Rérat and Corring 1991). In the gastric mucosa, parietal or oxyntic cells secrete hydrochloric acid and intrinsic factor, the chief cells produce proteinases, G cells produce the hormone gastrin, mucous cells produce proteinases and mucus, and surface epithelial cells secrete mucus. Also, there are mucus-containing surface cells in the layer of mucus covering the mucosa, which

have been shed and trapped in the mucus gel. In addition, the gastric mucosa secretes small quantities of other enzymes such as lipase, gelatinase, lysozyme, urease, neuraminidase, and carbonic anhydrase.

Zebrowska *et al.* (1983) calculated the salivary plus gastric secretions in 35 kg liveweight pigs to be 4 and 8 kg per 24 hours for diets based on wheat starch, sucrose plus casein, and barley plus soya-bean meal, respectively. The minimum amount of endogenous nitrogen from this secretion was estimated to be 0.3-0.6 g in 24 hours.

The biochemical characteristics of the gastric proteinases have been reviewed in detail by Foltmann (1981) and Fruton (1987). The gastric proteinases have molecular weights of around 40,000 Daltons (Da) for the zymogens and about 35,000 Da for the active enzymes. They consist of single polypeptide chains with three intramolecular disulphide bridges, and have a high content of dicarboxylic and β-hydroxyl amino acids, but rather low contents of basic amino acids. Intrinsic factor, a mucoprotein with a molecular weight of 55,000 Da, complexes with vitamin B_{12} in the stomach facilitating its absorption. In the pig, intrinsic factor activity is present in both the pyloric and duodenal mucosa (Jeffries 1967, Donaldson 1981). Plasma protein, epithelial cell loss, and mucus secretions throughout the gastrointestinal tract are discussed in Sections 1.3.6, 1.3.7 and 1.3.9, respectively.

1.3.2.1 Physiological Control of Gastric Secretion

The control of the exocrine secretion of the stomach involves the interaction of endocrine, paracrine and neurocrine transmitters. The major endocrine peptides involved in the control of gastric secretion in the pig are gastrin (stimulatory) and somatostatin (inhibitory). The most important neurocrine transmitters are acetylcholine and gastrin-releasing peptide. Chemo- and mechanoreceptors located in the tongue, buccal and nasal cavities are stimulated by tasting, smelling, chewing and swallowing food and stimulate the mucosal cells directly, and indirectly via the vagus nerve, to secrete acid and proteinases. Distension of the stomach and bathing the gastric mucosa with certain chemicals, primarily amino acids and peptides, stimulates pepsinogen secretion directly and acid secretion indirectly via the nervous system. The most potent chemical stimulators of gastrin secretion appear to be ammonia, amines and amino acids while intact proteins, fat and carbohydrate are relatively poor gastrin secretory stimulants. Protein digestion products in the duodenum also stimulate acid secretion via a hormonal mechanism involving gastric inhibitory peptide (GIP). Acid secretion is inhibited by decreasing pH in the stomach causing a decrease in gastrin release. Hormones, including GIP, secretin and somatostatin released from duodenal mucosa by acid, fatty acids, or

hyperosmotic solutions inhibit acid secretion and often gastric emptying. Pepsinogen secretion is also stimulated directly by the presence of acid, and the hormones gastrin and secretin. Mucus production is stimulated directly via chemicals and in response to physical contact and friction from the food. In summary, gastric secretion depends on both extrinsic neural and endocrine regulation, whereas its inhibition is dependent only on endocrine regulation. The understanding of the specific neural and hormonal factors responsible for the components of gastric secretion, the intracellular mechanisms for stimulus secretion coupling and the mechanism for release of the granular contents is far from complete (Low 1990).

Evaluation of the literature on proteinase secretion is complicated because most authors do not discriminate between the individual gastric proteinases, even though there is some evidence that different stimuli may have different actions on the secretion of the individual gastric proteinases (Foltmann 1981). Information on porcine gastric secretion of proteolytic enzymes, as distinct from reports of proteolytic enzyme concentration in the gastric mucosa or gastric contents is relatively limited. Also, it is technically difficult to quantitatively and qualitatively measure the volume of gastric secretion due to the close proximity of the bile duct to the pylorus, and the use of gastric pouches may not be representative of the whole organ and the gastric mucosa in the pouches is not directly influenced by the physical effects of food.

1.3.2.2. Effect of Age and Diet on Gastric Protein Secretion

The gastric secretions are affected by age and dietary composition. Pepsin secretion is low in pigs up to about 3-4 weeks of age after which it undergoes a very rapid increase, and access to solid food before and at weaning also has significant positive effects on the capacity of the stomach to secrete pepsin (Cranwell 1985). Braude *et al.* (1970) showed that enzyme activity of the digesta and stomach wall of piglets was increased by an increased level of feeding. Zebrowska *et al.* (1983) found an increase in pepsin activity in pig duodenal digesta following feeding of a barley and soya-bean meal diet compared to a wheat starch, sucrose and casein based diet. Low (1982b) and Moughan *et al.* (1990a), however, found no effect of protein source on the pepsin activity in the duodenum of 40 kg pigs and gastric contents or tissue of piglets, respectively. Observed increases in pepsin activity may reflect enhanced production or secretion of pepsinogen or it might simply be due to retarded breakdown of pepsin in the presence of dietary protein.

Low (1985,1989a) reviewed the response of the gastrointestinal secretions of the pig to dietary fibre (non-starch polysaccharides, NSP) and concluded that various sources of NSP stimulate gastric secretion. The characteristics of the

hetereogenous sources of NSP made it impossible to determine in detail how these NSP sources exerted their physiological effects. Shah *et al.* (1986) showed that the addition of pectin, guar gum and lignin to a semi-purified diet decreased the total pepsin activity measured in the stomach of rats. Morgan *et al.* (1985) demonstrated that the addition of NSP in the form of guar gum to humans suppressed plasma gastric inhibitory polypeptide (GIP), probably through reduced levels of blood glucose because of the delayed absorption of glucose induced by guar gum, as well as delayed gastric emptying. Lowered levels of GIP may lead to enhanced levels of gastrin secretion and thus greater gastric secretion. Gastrin secretion is also known to be stimulated by the presence of peptides as well as antral distension which would occur following consumption of diets containing a high NSP content.

1.3.3 Pancreatic Protein Secretion

The composition and functions of pancreatic secretions have been described in detail by Cuthbertson and Tilstone (1972), Kidder and Manners (1978), Johnson (1981c), Rérat (1981), Davenport (1982), Sanford (1982), Magee and Dalley (1986), Davison (1989b), Low and Zebrowska (1989), and Strombeck and Guilford (1990). The pancreas secretes a mixture of enzymes in an alkaline solution into the duodenum. The alkaline fluid has a high bicarbonate concentration which neutralises acid entering the duodenum and assists in regulating the pH of intestinal contents. The pancreatic enzymes digest carbohydrate, fat and protein. Pancreatic secretion by its influence on duodenal contents also affects gastric secretion and emptying.

The pancreas of the 35-50 kg pig has been reported by Low and Zebrowska (1989) and Corring *et al.* (1990) to secrete 1.2-5.0 and 1.85 litres per 24 hours containing 6-19 and 22 g of protein nitrogen per 24 hours, respectively. Ninety percent of the protein secreted in pancreatic juice is enzymic (Kidder and Manners 1978, Davenport 1982, Magee and Dalley 1986). Mosenthin and Sauer (1991) reported that urea contributed 22-23% of the total nitrogen secreted in pancreatic juice.

The enzymes and bioactive peptides found in pig pancreatic juice have been reviewed by Keller (1968), Kidder and Manners (1978), Davenport (1982), Magee and Dalley (1986) and Puigserver *et al.* (1986a). The enzymes include carbohydrases, endopeptidases and exopeptidases, lipases, and nucleases. The carbohydrases include α-amylase and chitinase. The endopeptidases include trypsin, chymotrypsin A and B, chymotrypsin C, elastase and enterokinase. The exopeptidases include carboxypeptidase A and B. The lipases include triacylglycerol lipase, phospholipase A and cholesterol esterase. The nucleases include

ribonuclease and deoxyribonuclease. Also present in pancreatic secretions are some peptides that act as enzyme cofactors and inhibitors. They are colipase I and II and trypsin inhibitor I and II. Lactoferrin and plasma proteins are also present as well as many other enzymes and bioactive peptides and proteins which have not yet been isolated and characterised.

The endopeptidases and exopeptidases are all secreted as their proenzymes and are activated by proteolysis of trypsinogen by enterokinase. Enterokinase is a very large glycoprotein (M.W. 300,000 Da) found on and produced by the brush border of duodenal surface epithelial cells. It is a very specific enzyme, acting only on the lysine-isoleucine (6-7) linkage of trypsinogen, resulting in a six amino acid peptide called the trypsin activation peptide and active trypsin. Trypsin then activates further trypsinogen and the other proenzymes, as well as acting as a digestive enzyme (Desnuelle 1986).

1.3.3.1 Physiological Control of Pancreatic Secretion

Pancreatic secretion is primarily under hormonal control, with some influence from vagovagal reflexes. Cholecystokinin (CCK) and secretin, secreted from endocrine cells within the intestinal mucosa in response to digesta in the duodenum, stimulate pancreatic secretion into the intestinal lumen (Go *et al.* 1970). Secretin is released in response to acid in the duodenum which stimulates the secretion of fluid and bicarbonate. CCK is released in response to food, particularly protein and fat digestion products in the duodenum, and stimulates the secretion of pancreatic enzymes. Vagal nervous stimulation of the pancreas has both effects (Meyer 1981). Cholecystokinin appears to mediate protease secretion through the feedback regulation of its release by dietary protein. For lipase and amylase, their induction is brought about by the digested metabolites appearing in the blood, which in some way signal the pancreas to increase secretion directly or through their further metabolic products or by the resultant released hormones. Corring and Chayvialle (1987) found the plasma levels of CCK, secretin, pancreatic polypeptide (PP) and somatostatin were unaltered when 41 kg pigs were fed either a starch-rich or a lipid rich diet indicating that these hormones were not involved in the pancreatic adaptation to the large amounts of carbohydrate and fat ingested. Solomon (1981) and Corring *et al.* (1989) concluded from examination of the literature that there is a critical ratio of insulin to glucose, which is required for initiation of increased amylase synthesis following a carbohydrate containing meal. Secretin and ketones are the proposed mediators of pancreatic adaptation to dietary fat (Brannon 1990).

A multitude of effective peptide hormones potentially could be released from gut mucosa by the variety of constituents in chyme. Corring *et al.* (1989) cite evidence for the effect of digestion products acting on the intestinal mucosa which

then secretes a secondary messenger. Similarly, various classes of food components could excite distinctly separate neural reflexes controlling pancreatic output. The potential diversity of mechanisms raises the possibility that individual components of food might evoke individual enzyme responses through separate pathways. Enzyme secretion is also influenced by a positive feedback system whereby the more protein and fat digestion products present, the more secretin and CCK are released. The inhibition of pancreatic secretion has been little studied, although somatostatin has been shown to inhibit all secretory processes of the digestive tract (Low and Zebrowska 1989). The inhibition of pancreatic secretion may involve the sympathetic nervous system, the release of pancreatic polypeptide from the islets of Längerhans, negative feedback mechanisms in the intestine or post-absorptive inhibition (Meyer 1981).

1.3.3.2 Effect of Age and Diet Composition on Pancreatic Protein Secretion

The pancreatic response of the pig to feeding and its variation with dietary constituents has been well studied due to the relative ease with which the secretion can be measured via simple cannulation of the pancreatic duct. The concentrations of individual enzymes in pancreatic juice, however, are highly variable between samples and studies (Kidder and Manners 1978, Rérat 1981, Solomon 1981, Partridge *et al.* 1982, Corring *et al.* 1984). Both the concentration and the amount of a pancreatic enzyme must be determined to fully characterise changes in its rate of synthesis and secretion (Solomon 1981).

Pancreatic secretion (volume, protein and enzyme activities) develops with increasing age of the animal and with alterations in feeding regimen, feed intake and diet composition (Rérat 1981, Low and Zebrowska 1989, Makkink and Verstegen 1990). The adaptations of pancreatic enzymes to dietary changes have been reviewed by Snook (1973), Corring (1980a), Rérat (1981), Schneeman (1982), Corring *et al.* (1989), Low and Zebrowska (1989), Brannon (1990) and Makkink and Verstegen (1990). The increase in protein secretion in response to food was shown clearly in the pig by Corring *et al.* (1972). Braude *et al.* (1970) showed an increase in proteolytic activity in the contents of the small intestine of piglets with increased level of feeding. Hee *et al.* (1988) fed pigs the same quantity of diet but at increased feeding frequencies and found that the volume of pancreatic secretion increased as did the level of amylase. Lipase, however, did not increase. Pancreatic adaptation to diet occurs through changes in the rate of synthesis of enzymes and through changes in the levels of mRNA in the pancreas.

1.3.3.2.a Dietary Protein

A number of studies with rats and pigs have determined the effect of dietary protein on the output of pancreatic nitrogen, enzyme concentrations and activities.

Partridge *et al.* (1982) and Schneeman (1982) found no difference in levels of pancreatic proteins and enzymes secreted for pigs and rats, respectively, fed different proteins at the same dietary level. Increased dietary protein, however, has resulted in increased levels of protein secreted by the pancreas in both the rat and pig (Corring and Saucier 1972, Ozimek *et al.* 1984), and increased levels of some or all of the proteolytic enzymes and sometimes lipase and amylase (Snook and Meyer 1964a, Corring and Saucier 1972, Lavau *et al.* 1974, Temler *et al.* 1983). Corring *et al.* (1984), however, showed no effect of feeding pigs a protein-free diet on pancreatic protein output.

The inclusion of trypsin inhibitor in the diet has been shown to increase pancreatic secretion and the pancreatic content of proteolytic enzymes in rats (Snook 1969, Green *et al.* 1973, Solomon 1981, Schneeman 1982, Green and Nasset 1983). In pigs, the consumption of a raw soybean diet caused an increase in the volume of pancreatic secretion and increased levels of VIP and secretin in blood plasma, but had no effect on pancreatic protein concentration or total protein output (Corring *et al.* 1985, Zebrowska *et al.* 1985). It is thought that CCK release due to the inactivation of proteolytic enzymes is the mechanism by which trypsin inhibitor affects the pancreas.

Snook (1965) found in the rat that the consumption of hydrolysed casein and egg protein evoked a pancreatic secretory response with respect to trypsinogen and chymotrypsinogen which was similar to that of intact casein but was smaller than that for whole-egg protein. Temler *et al.* (1983) also observed similar trypsin and chymotrypsin activities for hydrolysed and intact protein in the rat but the enzyme activities were lower for a free amino acid mixture. In contrast, Schneeman *et al.* (1977) and Green and Miyasaka (1983) showed that free amino acids and protein hydrolysates were weak stimulants of pancreatic secretion in the rat compared with intact proteins, and Leibholz (1981) showed reduced trypsin and chymotrypsin activities in the duodenum and pancreas of piglets given hydrolysed rather than intact protein diets. Yet Konturek *et al.* (1973) found that perfusion of the duodenum with free amino acids in the dog resulted in the release of CCK and increased pancreatic protein and bicarbonate secretion.

1.3.3.2.b Dietary Fat and Starch

Increased dietary fat causes an increase in pancreatic lipase secretion in both rats and pigs (Lavau *et al.* 1974, Ozimek *et al.* 1983, 1985, Corring and Chayvialle 1987, Brannon 1990). The amount of fat in the diet as well as quality (degree of saturation, chain length, degree of oxidation) affects lipase secretion in pigs (Ozimek *et al.* 1983, Makkink and Verstegen 1990). Corring *et al.* (1989) cite evidence for the level of fat being the primary influence on pancreatic lipase and the

type of fat affecting the enzyme secretion below its maximal response. Colipase has been shown both to remain the same and to increase with increased dietary fat (Corring *et al.* 1989). Increased dietary starch predictably causes an increase in pancreatic amylase in rats and pigs (Lavau *et al.* 1974, Corring and Chayvialle 1987, Brannon 1990). The effects of different types of starch (maize, potato, wheat, raw vs cooked), however, are not known.

1.3.3.2.c Dietary Fibre

Increased levels of dietary fibre of various types have been shown for the rat and pig to increase the volume of pancreatic secretion (Zebrowska *et al.* 1983, Zebrowska 1985, Langlois *et al.* 1987, Zebrowska and Low 1987), increase the protein secreted by the pancreas (Isaksson *et al.* 1983a,b, Sommer and Kasper 1984, Zebrowska 1985, Langlois *et al.* 1987, Ikegami *et al.* 1990), and increase the activity and/or the levels of secreted enzymes (Schneeman *et al.* 1982, Isaksson *et al.* 1983a,b, Poksay and Schneeman 1983, Shah *et al.* 1986, Langlois *et al.* 1987, Ikegami *et al.* 1990). In contrast, the studies of Maenhout *et al.* (1987) and Mosenthin and Sauer (1991) using pigs found there was no effect of increased dietary fibre on pancreatic nitrogen, volume or enzyme levels and activities.

Different sources of dietary fibre have different effects on pancreatic secretion. Shah *et al.* (1986) found in rats that dietary pectin decreased trypsin activity, dietary lignin increased trypsin and chymotrypsin activities while dietary guar gum increased chymotrypsin activity only. Dietary pectin (5% of diet) has been found in the rat to decrease trypsin activity in some studies (Sommer and Kasper 1984, Shah *et al.* 1986), and increase pancreatic amylase activity and lipase output in another experiment (Isaksson *et al.* 1983a). The inclusion of pectin at 20% of the diet, however, resulted in increased pancreatic nitrogen and trypsin secretion (Isaksson *et al.* 1983a). Sommer and Kasper (1984) showed that dietary guar gum decreased trypsinogen and protein in pancreatic tissue while Shah *et al.* (1986) found that guar gum increased the chymotrypsin activity in the intestine. Poksay and Schneeman (1983) reported significantly higher lipase, amylase and total proteolytic enzyme activities in the intestine of rats fed guar gum. Sommer and Kasper (1984) showed in rats that dietary wheat bran resulted in decreased protein in the pancreas and increased bicarbonate secretion with no effect on the secreted enzymes, but Sheard and Schneeman (1980) reported that rats fed a diet containing 5% wheat bran had greater lipase, amylase, trypsin and chymotrypsin activities and total protein in pancreatic tissue than rats fed a fibre-free control diet. Langlois *et al.* (1987) found that feeding pigs increased levels of wheat bran increased pancreatic secretion volume and protein as well as increasing the activities of chymotrypsin, trypsin, lipase and amylase, but decreased the protein concentration

of the pancreatic secretion. These effects on pancreatic secretion were accompanied by increased plasma levels of secretin, VIP, somatostatin and PP but not CCK.

1.3.3.2.d Rate of Adaptation of Pancreatic Secretion to Diet

Enzyme adaptations to diet are usually rapid, occurring within 1-2 days following feeding of the new diet, and the changes are complete within one week (Corring and Saucier 1972, Solomon 1981, Rérat 1981, Ozimek *et al.* 1984, Corring *et al.* 1989, Brannon 1990). The short-term adaptation is probably the result of the feedback mechanism which responds directly to the amount of enzymes present in the free form in the gut lumen. In this negative feedback mechanism, the presence of pancreatic juice in the duodenum causes a marked suppression of secretion and its absence enhances secretion (Corring 1974, Kidder and Manners 1978). Corring (1974) showed that proteolytic enzymes only induce this feedback control of exocrine pancreatic secretion and that the duodenum has an important role in this mechanism. Corring *et al.* (1985) found that plasma secretin content rose significantly when pancreatic juice was collected and not returned to the duodenum while plasma levels of gastrin, somatostatin, VIP and PP were unaltered, and that of CCK showed a delayed increase in the portal blood 30 minutes following the removal of the pancreatic secretion. The long-term adaptation of pancreatic secretion in response to feed composition could be explained by other physiological changes occurring after a diet change, such as gastric emptying rate and gut motility (Makkink and Verstegen 1990).

1.3.3.2.e Summary of Pancreatic Adaptation to Diet

In summary, pancreatic adaptation to diet appears to be very complex with each enzyme behaving differently depending on the amount and type of substrate ingested. In general, high carbohydrate diets increase amylase concentration in direct proportion to the fraction of the diet derived from carbohydrate and no other enzymes are increased; high fat diets primarily increase lipase concentration; and high protein diets primarily increase proteolytic enzyme concentration and also tend to increase lipase concentration. Green and Miyasaka (1983) suggested that intact protein and trypsin inhibitor stimulate pancreatic secretion in the rat by reducing the feedback inhibition from luminal pancreatic proteases. It appears that feeding fibres rich in water soluble components such as pectin and guar gum, but not those rich in insoluble components, will elevate the intestinal activity of some pancreatic enzymes (Schneeman 1982). The increased pancreatic secretions following consumption of dietary fibre may be due to a slower rate of enzyme degradation and/or enhancement of enzyme secretion (Poksay and Schneeman 1983) possibly caused by the interference of the dietary fibre on the trypsin-induced feedback regulation (Isaksson *et al.* 1983b).

The synthesis and secretion of the pancreatic proteases appear to greatly exceed the amount which is theoretically required to hydrolyse dietary proteins (Partridge *et al.* 1982). This apparent over-production of digestive enzymes is probably required for the digestion of endogenous proteins which are estimated to equal at least half of the daily intake of dietary protein (Low 1982a). Also, *in vivo* digestion requires high enzyme concentrations because the rate of hydrolysis depends on enzyme concentration in combination with the restricted time the digestive enzymes are able to attack the substrates as they are transported along the gut. Also, the inactivation of digestive enzymes by other enzymes and enzyme inhibitors would decrease the effective concentrations of the enzymes. Snook (1965) estimated that more than 90% of trypsin and chymotrypsin are inactivated by proteolytic enzymes of the gut wall and by microorganisms. The over- or under-production of digestive enzymes could lead to digestive inefficiency resulting in insufficient digestion or unused excess enzymes which could interfere with other digestive processes, with the absorption of digestion products, or cause self digestion of the animal's tissues.

In the many studies that have determined changes in enzyme concentration in response to diet, some of the changes are marked while others have recorded only small increases or no change in enzyme concentration after altering diet composition. Many of these results need to be compared with caution because of differences in experimental technique. The use of different species of animal, inadequate enzyme assay procedures, differences in composition and duration of feeding of the altered diet, differences in tissue patterns of enzyme synthesis which are not reflected by enzyme concentration are some of the potential problems in comparing these studies (Makkink and Verstegen 1990). The determination of enzyme activities in the intestinal contents is influenced by the speed of the specific enzyme reaction, and provides information on the proportion of each enzyme secreted less that which has become inactive due to complexing. Corring (1980a) discussed the physiological significance of digestive enzyme adaptation to dietary change and concluded that it is not important in a healthy well-fed animal but may be of significance during dietary deficiency, particularly that of protein or enzymes. Snook (1973) and Makkink and Verstegen (1990) proposed that adaptation would be useful in decreasing activity when not required, and thereby leading to reduced endogenous protein loss.

1.3.4 Bile Protein Secretion

The secretion and functions of bile have been reviewed by Hofmann (1968), Haslewood (1978), Kidder and Manners (1978), Malagelada (1981), Weisbrodt

(1981), and Shaffer (1989). Bile is secreted by the polygonal cells of the liver into the bile canaliculi which lead into the hepatic bile duct. In most mammalian species bile passes into the gall bladder, an extensible blind side-branch of the bile duct, where it is stored and concentrated. The gall bladder contracts in response to the hormone CCK and the bile is expelled down the common bile duct and through the sphincter of Oddi into the duodenum.

The bile produced by 25-30 kg, 40 kg and 45 kg liveweight pigs has been reported to be 1.2-1.7 litres (Sambrook 1981), 2.0-2.5 litres (Juste 1982) and 1.82 litres per day (Corring *et al.* 1990), respectively. The rates of nitrogen secretion were 1.8-1.9 g, 2.8-3.0 g and 1.7 g per 24 hours, respectively, and comprised mostly (95%) free glycine. Shaffer (1989) reported the protein content of human bile secretion to be 0.10 g/100 ml.

Bile contains water, protein, bile pigments, bile acids, cholesterol, phospholipids, neutral fats, urea, and inorganic ions. The components relevant to bile's digestive function are the bile acids and to a lesser extent, the phospholipids. The bile acids consist of a steroid carboxylic acid conjugated with either glycine or taurine. These bile acids assist in the emulsification of the dietary fat in the lumen of the small intestine, and particularly in the micellar dispersion of the fat digestion products, facilitating their absorption. The bacteria in the lower small intestine deconjugate the bile acids and to some extent alter them chemically, mainly by reduction of some of the hydroxyl groups. The altered and unaltered bile acids are reabsorbed in the lower small intestine, pass into the portal blood and are absorbed by the liver. Newly formed bile acid and the reabsorbed bile acids are then conjugated and again secreted into the bile. Bile also contains lecithin which is hydrolysed in the intestine by pancreatic phospholipase to lysolecithin, a substance which also contributes to the micellar dispersion of the products of fat digestion.

The concentration of protein in bile is quite low. There are, however, a variety of proteins present in bile, many of which are glycoproteins. Albumin is the most abundant protein, with transferrin, gamma-globulin, ceruloplasmin, apolipoproteins, haptoglobulin, secretory IgA, IgM, IgG, insulin, epidermal growth factor, CCK, lysosomal hydrolases, amylase and alkaline phosphatase also being present. Most of these proteins are derived from the plasma pool or the liver cells, and may have immunological and physico-chemical functions. For example, secretory IgA may provide a defence mechanism to the upper gastrointestinal tract, and the apolipoproteins are important in the elimination of cholesterol by stabilising the lipid micelles.

1.3.4.1 Physiological Control of Bile Secretion

Bile secretion is continuous and is greatly increased by the presence in the blood of bile acids, secretin or CCK. Bile acids produce an increase in biliary fluid output while having no effect on electrolyte composition. Secretin stimulates the secretion of water and bicarbonate resulting in a fall in chloride and organic constituents but the sodium concentration remains constant. Cholecystokinin causes increased bile output with a raised bicarbonate concentration and a decreased bile salt concentration. Cholecystokinin is released in response to the presence of fat digestion products in the duodenum and stimulates the contraction of the gall bladder, relaxation of the sphincter of Oddi and thus the release of the bile into the duodenum.

1.3.4.2 Effect of Diet on Bile Secretion

Bile secretion like the other digestive secretions discussed so far is also influenced by diet. Rodriguez *et al.* (1982, 1983) showed that feeding rats a protein-free diet lowered the flow of bile but the concentrations of bile acids, cholesterol, phospholipids and proteins in the bile were unchanged. Villalon *et al.* (1987) studied the effect of feeding a low protein (8%) diet or a normal diet (26% protein) to rats and showed that the low protein diet induced a significantly lower bile flow and lower bile acid and protein secretion rates, while the phospholipid and cholesterol secretion rates were significantly higher. Portman *et al.* (1955) studied bile secretion in rats on a variety of dietary regimens. They reported that cholesterol supplementation significantly increased bile acid and cholesterol excretion, and increasing the levels of the purified cellulose also resulted in an increased bile acid excretion, but the levels of dietary fat did not affect biliary excretion, and feeding a commercial rat diet resulted in a much higher bile acid excretion than feeding a synthetic diet. The substitution of egg albumin for casein in the synthetic diet resulted in an increased bile acid excretion, as did the substitution of starch for sucrose in the basal diet. Sambrook (1981), however, reported no difference in the total bile nitrogen from pigs fed either a barley-based or a semi-synthetic casein-based diet. Similarly, Hagemeister *et al.* (1985) showed no effect of dietary protein (casein or soy isolate) on bile secretion except for an increase in bile phospholipids after soy isolate feeding.

Sheard and Schneeman (1980) reported no difference in bile acid levels in rats fed diets with (5%) or without wheat bran. Valette *et al.* (1989) showed that pigs fed a diet containing 40% wheat bran resulted in significantly higher bile and bile ion secretions than were obtained from pigs fed a bran-free diet. They also reported increased secretin and VIP levels in plasma following bran consumption but concluded that hormonal regulation was not involved in the resultant bile secretion.

Dietary fibres, particularly the lignins and the viscous gelling fibres, tend to absorb bile acids and cholesterol (Vahouny and Cassidy 1985).

Corring *et al.* (1989) who reviewed the response of biliary secretion to dietary fibre and fat with emphasis on humans, concluded that an increase in dietary long-chain triglycerides usually induced an increased secretion rate of bile ions, phospholipids and cholesterol, and dietary fibre depressed the bile acid pool in the hindgut and altered the bile acid composition. The mechanisms underlying biliary responses to dietary lipid, protein, starch and fibre have not been extensively investigated and consequently remain poorly understood. Regulatory peptides are thought to be of some importance and it is likely that modified bile synthesis, intestinal transit, absorption rate and bacterial metabolism may account for the long-term changes in bile secretion in response to dietary lipids and fibres.

1.3.5 Intestinal Secretion

The mucosa of the small intestine produces a secretion into the gastrointestinal tract (Kidder and Manners 1978, Low and Zebrowska 1989). A large part of this secretion is produced by Brunner's glands (duodenum), goblet cells and crypts of Lieberkühn (throughout the small and large intestines), and contains glycoproteins, amylase, enterokinase, lysozyme, sloughed epithelial cells, microorganisms, plasma proteins and brush border enzymes.

Horszczaruk *et al.* (1974) determined the intestinal secretion in 70 kg liveweight pigs to be about 6 litres per 24 hours, containing 8-12 g of nitrogen. Buraczewska (1979) estimated that 50-70 kg liveweight pigs secreted about 15 g nitrogen into the small intestine in 24 hours. It is difficult to measure the amount and composition of intestinal secretion because of the simultaneous processes of secretion and absorption occurring in the intestine.

Intestinal secretion is affected by changes in dietary composition. Horszczaruk *et al.* (1974) using pigs fitted with two pairs of re-entrant cannula in the proximal small intestine measured the secretion from the intestine between the cannulas following feeding of the pigs either a 16% crude protein diet or a protein-free diet, and estimated that 62-73 and 50-62 g of crude protein was secreted into the small intestine during 24 hours, for the two diets, respectively. Low and Rainbird (1983, 1984) using 40 kg liveweight pigs fitted with two re-entrant cannulas in the jejunum determined the amount of nitrogen secreted in the isolated jejunum following perfusion with glucose or maltose and with or without guar gum, and they found that there was no effect of the sugar used but guar gum significantly increased the flow of nitrogen. The intestinal enzymes adapt to changes in the diet by altering their biosynthesis in a similar way to the pancreatic enzymes (Nicholson *et al.* 1974,

Kimura *et al.* 1977, Puigserver *et al.* 1986b).

1.3.5.1 Brush Border Enzymes

The intestinal brush border enzymes include sucrase-isomaltase, maltase, lactase, neutral endopeptidase, dipeptidyl peptidase IV, aminopeptidases A, N and P, carboxypeptidase P, nucleotidase, nucleosidase, and alkaline phosphatase (Puigserver *et al.* 1986b). Intestinal aminopeptidase A and N are responsible for the hydrolysis of acidic and neutral amino-terminal residues, respectively, from the peptides resulting from protein degradation by pancreatic enzymes. They are among the more abundant enzymes of the brush border membrane constituting 4% and 10% of the total membrane proteins for aminopeptidase A and N, respectively (Maroux *et al.* 1984).

Brush border peptidase activity is fully developed at birth for the pig and rat (Lindberg *et al.* 1975). The effects of dietary free amino acids, peptides and proteins on various brush border enzymes have been determined for the rat. Solimano *et al.* (1967) showed for rats fed a low protein or a protein-free diet that the jejunal dipeptidase activities decreased and the disaccharidase activities increased compared to those from rats fed a 25% protein diet. Peptide hydrolase, leucineaminopeptidase and disaccharidase activities were increased per unit length of intestine with increased dietary protein or amino acid content (Nicholson *et al.* 1974, Kimura *et al.* 1977). Also, dietary casein caused greater disaccharidase and leucineaminopeptidase activity per unit length of intestine than gluten, gelatin and a protein-free diet, in decreasing order (Kimura *et al.* 1977). Aminopeptidase activity has been shown to be greater following protein and peptide alimentation than after feeding a free amino acid diet (Maze *et al.* 1979, Poullain *et al.* 1989). Cytosol peptidase activity, however, was higher in rats following the consumption of a free amino acid diet (casein hydrolysate) compared to an intact casein-based diet (Maze *et al.* 1979), and sucrase and maltase activities were higher in rats fed a free amino acid- or a peptide-based (hydrolysed whey protein) diet than those for rats fed an intact whey protein-based diet (Poullain *et al.* 1989). It has been shown with rats that the intestinal brush-border hydrolases sucrase-isomaltase and lactase increased markedly following the feeding of a carbohydrate-rich diet after 15 h and 2 days, respectively, while aminopeptidase increased markedly after 6 days on a protein-rich diet (Puigserver *et al.* 1986b).

Vahouny and Cassidy (1986) reviewed earlier studies with rats on the effect of fibre on brush border enzymes and reported that the addition of cellulose to diets had little effect on intestinal enzyme activity, while the addition of pectin induced some changes on intestinal enzyme activities which depended on various experimental conditions. In their own study, Vahouny and Cassidy (1986) reported

there were no consistent effects on enzyme activities of the brush border of the small intestine of the rat following the addition of various sources of dietary fibre. Although the adaptability of the activity of brush border digestive enzymes has been well documented, the mechanisms underlying these changes are still largely unknown.

Investigation of the brush border membrane is made difficult by the fact that the enterocytes are generated in the form of undifferentiated cells in the crypts around the base of each villus, the morphological differentiation and the simultaneous expression of genes coding for the functional proteins of the brush border membrane occur only during the migration of the cells towards the tips of the villi where they are desquamated into the intestinal lumen. This migration is relatively rapid (2-3 days in the rat) due to the high mitotic activity of crypt cells, which ensures a constant renewal of the cells along the villus-crypt axis. Also, the brush border enzymes are large glycoproteins which are difficult to purify, and the levels of these proteins in adult animals are modified by lumenal proteases and perhaps by lysosomal enzymes (Alpers 1984).

1.3.6 Plasma Protein Secretion

The mucosa of the gastrointestinal tract secretes immunoglobulins, predominantly IgA, which adhere to and protect the mucosal surface (Strombeck and Guilford 1990). Jeffries and Sleisenger (1968) reviewed plasma protein loss into the digestive tract and estimated that only 10% of the catabolism of albumin and ceruloplasmin can be accounted for by this route. Cuthbertson and Tilstone (1972), however, estimated that 10-20% of total albumin catabolism occurs in the intestine. Proteins that enter the lumen of the stomach or intestine are usually rapidly hydrolysed to their constituent amino acids which are then reabsorbed into the intestine. With the exception of IgA, which appears to be actively secreted with a carrier molecule, there is no evidence that plasma proteins are actively transported across the mucosal epithelium from the lamina propria. It would seem that lymph escapes from the lamina propria at the apex of the intestinal villi with desquamating cells. The levels of intestinal and colonic IgA have been reported to be modified by the type of dietary fibre consumed (Vahouny and Cassidy 1986).

1.3.7 Epithelial Cell Loss

The replacement of the mucosal cells of the gastrointestinal tract has been reviewed by Wiseman (1964) and Alpers (1983). Mucosal epithelial cells normally desquamate from the tips of the villi and are replaced by cells migrating from the crypt cells. The number of cells in any one villus is determined by the rate of

production of new cells and the rate of loss of mature cells. An enterocyte has an estimated life span of 48 hours, resulting in the gastrointestinal tract mucosa having one of the most rapid turnover rates of any tissue in the mammalian body (Johnson 1988). The replacement time of total cell population is estimated to be 4-6 days in the dog and human, and 3-7 days in the rat. The whole human small intestine has been estimated to lose 20-50 million cells per minute (Croft *et al.* 1968), which contributes 90% of the total cells lost over the entire epithelial surface of the stomach, small and large intestines, and is estimated to be 287 g of cells every 24 hours (Croft and Cotton 1973). Da Costa (1971) estimated 180 g of nitrogen per day is lost from the desquamation of intestinal cells in humans, while Rérat *et al.* (1976) reported a lower value of 8-29 g per day. Souffrant (1991) reported that 1.4-2.0 g of nitrogen per day was secreted from sloughed epithelial cells in the pig.

The gastrointestinal mucosa is exposed to dietary constituents and its own secretions, and these contain a variety of molecules and factors that influence the growth of the mucosa (Johnson 1988). Dietary fibre stimulates mucosal growth (Jacobs 1983, 1986, Vahouny *et al.* 1985, Johnson 1988) and intestinal cell turnover rate (Vahouny and Cassidy 1986, Low 1989a, Johnson *et al.* 1984). Dietary whole protein has been shown to produce more rapid mucosal growth in the rat than hydrolysed dietary protein or free amino acids (Poullain *et al.* 1989). Physical or chemical trauma increases cell loss (Badawy *et al.* 1957) as can changes in the composition of the microflora. Normal bacterial flora reduce the average life of a cell to half of what it would be if no microorganisms were present, while protozoal infection increases epithelial cell turnover (Strombeck and Guilford 1990).

1.3.8 Microbial Protein

The populations of microorganisms in the gastrointestinal tract and their nutritional significance have been reviewed by Cranwell (1968), Donaldson (1968), Ratcliffe (1985), and Savage (1986). The levels of microorganisms in the ileum as estimated by Mason *et al.* (1976) using the levels of diaminopimelic acid (DAP) appear to be negligible. Graham *et al.* (1986), however, reported that more than 10^7 lactobacilli could be found per gram of fresh duodenal and ileal digesta. Also, Wünsche *et al.* (1991) using DAP and bacterial fractionation methods estimated that 25% of total nitrogen in ileal digesta was bacterial nitrogen.

1.3.9 Mucus Secretion

Gastrointestinal mucus secretion, composition and functions have been reviewed by Horowitz (1977), Allen (1981, 1984), Allen *et al.* (1984), Forstner *et al.* (1984), Forstner and Forstner (1986), and Mantle and Allen (1989). Mucus

represents the major organic secretion into the gastrointestinal tract, in terms of its output by weight and the energy requirement for its biosynthesis. Mucin is produced by various cell types in the gastrointestinal tract including the mucus-secreting cells in the cardiac, fundic and pyloric glands in the stomach, and Brunner's glands in the duodenum, the goblet cells and crypts of Lieberkühn in the small and large intestine, and the surface-epithelial mucus-secreting cells found throughout the gut. Under resting conditions, the mucus layer is sustained by a slow, continuous release of mucin by simple exocytosis. In response to stimulus, more rapid secretion of mucin occurs by compound exocytosis, apical expulsion and cell exfoliation. Baseline secretion appears to be independent of neuronal control. Secretory activity, however, can be increased by neuronal stimulation, chemical and/or mechanical stimulation, and mediators of inflammatory reactions.

There are two distinct physical forms of mucus: a thin layer of stable, water-insoluble gel firmly adhering to the mucosal surface, and a soluble mucus component mixed with luminal juices that overlies the gel. Both the adherent gel and the soluble mucus are composed of mucus glycoproteins (or mucins) which confer on the secretion its viscous and gel-forming properties. Other gastrointestinal secretions such as enzymes, plasma proteins, secretory IgA, bile, microorganisms, intrinsic factor, sloughed cells and ingested matter at various stages of digestion may also be present in the mucus layer, particularly in the soluble mucus component (Horowitz 1977, Allen 1981, Allen *et al.* 1984). The mucus gel is formed by the non-covalent interactions of the large, highly hydrated glycoprotein molecules. The soluble mucus gel contains 90-95% water by weight, and the concentration of mucus glycoprotein in the gel varies according to the source of the mucus. For example, human and pig gastric mucus contain approximately 50 mg/ml of mucin, while pig small intestinal and colonic mucus contain 20 mg/ml and 30 mg/ml of mucin, respectively (Mantle and Allen 1989).

Purified undegraded mucus glycoproteins from the gastrointestinal tract are comprised of 60-80% carbohydrate, 12-46% protein and approximately 5% ester sulphate by weight (Allen 1981, Mantle and Allen 1989). They are very large molecules ranging in molecular weight from 2×10^5 - 44×10^6 Da. The mucus glycoproteins are polymeric molecules comprised of a number of glycoprotein subunits, each consisting of a central peptide core surrounded by carbohydrate side chains attached covalently such that its structure resembles that of a bottlebrush. The protein moiety of many glycoproteins has two distinct regions: a glycosylated segment to which the carbohydrate is attached, and a naked, non-glycosylated segment that is susceptible to digestion by proteolytic enzymes. In a large number of mucins, the glycoprotein subunits are bound together by covalent disulphide

bridges between cysteine residues located in non-glycosylated regions of the mucin protein. Some gastrointestinal mucins possess a further protein component integrated into their polymeric structures. These protein components are compositionally quite distinct from the glycoprotein subunits and are bound into the mucin polymers by disulphide bridges.

Mucus glycoproteins from different areas of the gastrointestinal tract and from different species have strikingly similar protein compositions that are characterised by their high content (40-50%) of serine, threonine and proline (Nemoto and Yosizawa 1969, Bella and Kim 1972, Allen 1981, Allen *et al.* 1984, Forstner and Forstner 1986, Mantle and Allen 1989). Serine and threonine provide the sites of attachment for the oligosaccharide side chains, and the high proline content is thought to provide the required conformation for extensive glycolysation and close packing of oligosaccharide side chains along the protein core by preventing the protein from folding into a compact globular structure (Allen 1981). The amino acid compositions of mucus glycoproteins from the gastrointestinal tract of pigs have been given by Allen (1981), Forstner and Forstner (1986), and Mantle and Allen (1989).

The adherent mucus gel provides a protective buffer zone between the mucosa and the intraluminal environment throughout the gastrointestinal tract (Smithson *et al.* 1981, Allen 1981, Allen *et al.* 1984, Forstner *et al.* 1984, Forstner and Forstner 1986). The gel is permeable to low molecular weight solutes (M.W.<1000), but not to large molecules such as proteins. The rate of diffusion of solutes through the mucus gel is quite slow and some ions become bound into the mucus layer. Indigenous bacteria colonising the gastrointestinal tract are embedded in the mucus layer and are thereby physically separated from the mucosa, limiting their potential to cause tissue damage. Similarly, invasive pathogens (bacteria, viruses and parasites) must penetrate the mucus barrier to gain access to the mucosa (Kim *et al.* 1984). Subtle differences in mucin oligosaccharide chains from different species and different regions of the gastrointestinal tract lead to important variations in immune recognition, receptor binding and the adhesion and penetration of microorganisms (Mantle and Allen 1989). The adherent gel and the viscous luminal mucus also protect the delicate underlying epithelium from mechanical damage by the motile forces of digestion and the passage of food and faecal material. Mucus function is best understood in the stomach and duodenum where the gel is particularly important for protection of the mucosa from acid and pepsin degradation (Fromm 1981, Mantle and Allen 1989, Low 1990).

Purified mucins from the stomach, intestine and colon of the pig, rat and human are all susceptible to proteolytic digestion by pepsin, trypsin, chymotrypsin

and bacterial proteases such as pronase at least *in vitro* (Hashimoto *et al.* 1963, Hoskins 1978, Allen *et al.* 1984). Proteolysis results in destruction of non-glycosylated regions of the mucin polymer and release of degraded glycoprotein subunits (Mantle and Allen 1989). Erosion of the mucus barrier in the intestine and colon probably occurs as a consequence of similar attack by pancreatic digestive enzymes and bacterial proteases. Besides proteolysis, a further contributing factor to the loss of mucus gel *in vivo* is mechanical erosion due to the passage of solid food and the motile forces of digestion. Thus, in normal gastrointestinal mucosa, a dynamic balance exists between erosion of the adherent mucus layer and secretion of new gel by the mucosa to maintain the continuity of the mucus barrier.

Salivary mucin secretion occurs primarily in response to sympathetic activation, although the response can be augmented by parasympathetic activation. Gastric mucin secretion is stimulated via hormonal mechanisms. The two best documented hormonal stimulants of gastric mucus production are secretin and the prostaglandins. Vagus nerve stimulation and acetylcholine also increase the soluble mucus secretion from the mucous neck cells. In the intestine and colon, control of mucus secretion not only varies among the different regions but also depends on the location of the goblet cells, whether they are in crypts or in the surface epithelium. Secretion from deep mucus cells in the crypts is regulated by parasympathetic stimulation, whereas secretion from superficial mucus cells is controlled by surface active luminal substances. In this manner, surface goblet cells provide immediate protection for the mucosa from noxious materials in the lumen, while goblet cells within the crypts are able to respond to systemic variables. The decrease of gastrointestinal mucus production has been shown to occur by antiinflammatory drugs and cytotoxic agents.

There is little information on the effect of diet composition on mucus secretion with the exception of that for fibre. Vahouny *et al.* (1985) reported evidence for increased goblet cell secretory activity in rats in response to feeding diets containing wheat bran and cellulose compared to fibre-free diets. Satchithanandam *et al.* (1990) also found mucin production increased following feeding of diets containing guar gum or citrus fibre and suggested that the increased mucin production is responsible for more rapid transit times and delayed or impaired nutrient absorption also resulting from fibre consumption. Galactosamine flow from the small intestine of the pig given a protein-free diet with 15% wheat bran was significantly higher at high dietary intake levels than that measured following feeding of a protein-free diet without added fibre (Fuller and Cadenhead 1991).

1.4. TOTAL QUANTITIES OF ENDOGENOUS NITROGEN AND AMINO ACIDS SECRETED INTO THE GASTROINTESTINAL TRACT

The quantities of nitrogen and amino acids supplied by the gastrointestinal secretions and their degree of digestion are of importance in the direct determination of net endogenous amino acid excretion at the end of the ileum. The amount of nitrogen reported entering the digestive tract of the pig from these secretions varies greatly among studies. Table 1.1 summarises the data available regarding the amounts of nitrogen (N) secreted by each part of the porcine gastrointestinal tract. This variation is due largely to experimental factors such as liveweight, diet composition, level of feeding, surgical and experimental techniques. It would appear, however, that in the pig the secretion from the small intestinal mucosa makes the greatest contribution (59.8% of total N) to the total amount of N secreted into the gastrointestinal tract per day. The next greatest contribution to total N would appear to be from the stomach (25.6% of total N). This is supported by endogenous nitrogen secretion data presented by Souffrant (1991) who showed that the total endogenous nitrogen secretion to the end of the ileum was 38-60% of total nitrogen intake, with secretion from the small intestine accounting for 22-27% of total nitrogen intake. The nitrogen from salivary plus gastric secretion was 5-8%, pancreatic nitrogen secretion 4-16%, bile nitrogen secretion 4-6% and sloughed cells 2-3% of total nitrogen intake. Sloughed cells were reported to add 1.4-2.0 g of nitrogen per day to total endogenous nitrogen secretion. In contrast, Fauconneau and Michel (1970) estimated that mucosal shedding contributed to 75% of total endogenous nitrogen secretion.

Data pertaining to the daily amino acid outputs from the pancreatic, biliary and intestinal secretions, the only ones of the gastrointestinal tract referred to in the literature, are shown in Table 1.2. The estimate of mean daily total amino acid output at the end of the small intestine from summing the individual amino acids from each of these secretions is 83 g (70-92 g). The greatest contribution by weight is provided by glycine followed by glutamic acid with histidine, cystine and methionine contributing the least.

The amino acid compositions of salivary and gastric secretion have not been reported in the literature, but the amino acid compositions of most of their components are published (Nasset 1957, Bovey and Yanari 1960, Desnuelle 1960, Taylor 1968, Allen 1978, Foltmann 1986). In contrast, the amino acid composition of pancreatic secretion has been well studied and the relative proportions of amino acids have been reported by Corring and Jung (1972), Corring (1975, 1980b),

Table 1.1 Amounts of nitrogen (g day^{-1}) secreted into the gastrointestinal tract of the 50 kg liveweight pig

Secretion	Nitrogen	Reference
Salivary	7-14	Buraczewska (1979)
	0.4	Corring (1980b)
	0.4	Juste (1982)
	0.2	Souffrant (1991)
mean	**2.7**	
Salivary + Gastric	0.3-0.6	Zebrowska *et al.* (1983)
	2.0-3.3	Souffrant (1991)
mean	**1.6**	
Gastric	1.0-1.3	Zebrowska *et al.* (1975)
	3.0-5.8	Juste (1982)
	9.6	Souffrant (1991)
mean	**5.1**	
Pancreatic	3.0	Corring (1975)
	2.9	Corring (1980b)
	2.5-6.7	Juste (1982)
	1.3-2.3	Partridge *et al.* (1982)
	2.0-2.1	Zebrowska *et al.* (1983)
	2.5-2.6	Imbeah *et al.* (1988)
	1.9	Corring *et al.* (1990)
	0.8-4.6	Makkink & Verstegen (1990)
mean	**2.8**	
Bile	0.2-1.6	Horowitz (1977)
	2.0	Sambrook (1981)
	1.7	Corring *et al.* (1990)
	1.8-3.0	Juste (1982)
mean	**1.8**	
Small Intestine	8-12	Horszczaruk *et al.* (1974)
	14.4	Juste (1982)
	9.5	Low (1982a)
	14.6	Buraczewska (1979)
	10-12	Souffrant (1991)
mean	**11.9**	
Total to end of small intestine	22.4-28.8	Buraczewska (1979)
	10.1	Low (1982a)
	2-15	Zebrowska (1982)
	22	Low and Zebrowska (1989)
	22.1-29.4	Souffrant (1991)
mean	**18.4**	

Table 1.2 Endogenous amino acid secretions (g day^{-1}) into the gastrointestinal tract of the pig

Amino Acid	Pancreas[1]	Bile[2]	Intestine[3]	Total
Lysine	0.8	0.05	4.7	5.5
Histidine	0.4	0.03	1.4	1.8
Arginine	0.7	0.04	3.5	4.2
Aspartic Acid	1.6	0.06	5.5	7.2
Threonine	0.8	0.03	3.4	4.2
Serine	1.1	0.04	3.2	4.3
Glutamic Acid	1.4	0.14	7.9	9.4
Proline	0.7	0.03	3.2	3.9
Glycine	0.8	13.01	3.0	16.8
Alanine	0.8	-	3.1	3.9
Cystine	0.5	0.08	-	0.6
Valine	0.9	0.04	3.4	4.3
Methionine	0.2	0.01	0.8	1.0
Isoleucine	0.7	0.03	2.6	3.3
Leucine	1.1	0.05	5.3	6.4
Tyrosine	0.8	0.02	1.8	2.6
Phenylalanine	0.6	0.03	2.7	3.3
Total	13.9	13.7	55.5	82.7

1 Corring (1975)
2 Juste (1982)
3 Buraczewska (1979)

Partridge *et al.* (1982), Corring *et al.* (1984), Ozimek *et al.* (1985), and Zebrowska (1985). The predominant amino acids in pancreatic secretion are leucine, serine, aspartic and glutamic acids, while histidine and methionine are found in low proportions. The effect of diet composition on the relative proportions of the amino acids in pancreatic secretion is not known. The amino acid composition of bile has been reported by Juste (1982). Glycine is the major contributor (95%) to amino acid nitrogen in the bile of the pig, and contributes 20% of the total amino acid excretion to the end of the small intestine. Buraczewska (1979) published the amino acid composition of the secretion from the small intestine. This secretion contains high proportions of leucine, aspartic and glutamic acids and low proportions of histidine

and methionine like the pancreatic secretion. Endogenous nitrogen secretions, however, do not remain unchanged in the digesta but are digested and absorbed along with the dietary nitrogen (Snook and Meyer 1964b, Fauconneau and Michel 1970, Buraczewski 1980).

1.5 DIGESTION OF ENDOGENOUS PROTEIN SECRETIONS

Digestion of the endogenous protein secretions occurs along the entire length of the intestine and is probably a continuous process. Twombly and Meyer (1961) estimated that approximately 90% of endogenous protein secreted into the gastrointestinal tract of the rat was digested and absorbed. Fauconneau and Michel (1970) and Zebrowska and Buraczewska (1972), however, considered the digestion of endogenous protein to be much slower than that of exogenous protein, with the majority of endogenous protein digestion occurring in the ileum and caecum by the joint action of bacterial and endogenous enzymes. Ochoa-Solano and Gitler (1968) labelled endogenous protein with ^{35}S-methionine and fed ovalbumen labelled with ^{75}Se-selenomethionine and determined the relative rates of disappearance of the two labels from the small intestine of the rat. They concluded that dietary proteins were digested and absorbed more rapidly than endogenous proteins. A study with pigs which were given diets containing casein or gluten and were intravenously infused with ^{14}C-leucine or ^{3}H-lysine also showed that the endogenous amino acids were absorbed more slowly than the dietary amino acids (Low 1982a).

In contrast, other workers have shown endogenous and exogenous proteins to be digested and absorbed at similar rates. Nasset *et al.* (1973) in a study with rats demonstrated that pancreatic juice protein when given orally was readily digested and absorbed, while Romero and Canolty (1979) reported that labelled (^{14}C- and ^{3}H-histidine) endogenous proteins disappeared from the intestinal contents at the same rate as exogenous proteins following their infusion into the duodenum of cannulated rats. Relatively low proportions of protein with molecular weights corresponding to those of digestive enzymes in the duodenum of pigs given a protein-free diet was interpreted by Asche *et al.* (1989) to be the result of the rapid hydrolysis of these enzymes in the intestine. Mucin, however, has been shown to be resistant to enzymic digestion (Hashimoto *et al.* 1963, Hoskins 1978). The gastrointestinal proteases are readily able to degrade the polymeric mucin into fragments, but further breakdown is impeded by the presence of the carbohydrate chains attached to the protein core. The next digestion step appears to require the action of bacterial glycosidases to remove the oligosaccharides before the mucin

Table 1.3 Endogenous amino acid secretion (g day^{-1}) remaining undigested and unabsorbed at the end of the ileum of the pig

Amino Acid	Total Secreted[1]	Total Remaining at the End of the Ileum	
		46%[2]	15%[3]
Lysine	5.5	2.5	0.8
Histidine	1.8	0.8	0.3
Arginine	4.2	1.9	0.6
Aspartic Acid	7.2	3.3	1.1
Threonine	4.2	1.9	0.6
Serine	4.3	2.0	0.6
Glutamic Acid	9.4	4.3	1.4
Proline	3.9	1.8	0.6
Glycine	16.8	7.7	2.5
Alanine	3.9	1.8	0.6
Cystine	0.6	0.3	0.1
Valine	4.3	2.0	0.6
Methionine	1.0	0.4	0.2
Isoleucine	3.3	1.5	0.5
Leucine	6.4	2.9	1.0
Tyrosine	2.6	1.2	0.4
Phenylalanine	3.3	1.5	0.5
Total	82.7	37.8	12.4

1 from Table 1.2
2 Rérat *et al.* (1976) and Low (1982a)
3 Rérat (1990)

becomes susceptible to further attack by proteases. There is an obvious advantage for some of the endogenous proteins to be more resistant to digestion than exogenous proteins. In particular, mucoproteins are thought to protect the mucosal cells against proteolytic attack, which would require some resistance to rapid hydrolysis.

Estimates of the quantity of endogenous protein digested and reabsorbed reported in the literature vary markedly. Thus, Rérat *et al.* (1976) reported a mean of 50 g of amino acids and 8 g of nitrogen having been absorbed per day as measured by their appearance in the hepatic portal vein of growing pigs fed a

protein-free diet, while Low (1982a) estimated that 10-14 g of endogenous N is absorbed per day over the entire gastrointestinal tract of the pig. Buraczewski (1980) estimated that 90-120 g of endogenous amino acids are absorbed daily up to the end of the small intestine of the pig. Rérat (1990) using a number of different techniques calculated that 46 g of endogenous amino acids were digested and absorbed from a total secretion of 54 g (i.e. 85% of total N secreted is recycled). The estimates of Rérat *et al.* (1976) and Low (1982a) indicate that approximately half (54%) of the total endogenous nitrogen secreted (Table 1.1) is digested and absorbed after secretion into the gastrointestinal tract. The estimate of the amount of endogenous nitrogen reabsorbed by Buraczewski (1980), however, is much higher than that estimated to have been secreted. Using the estimates of Rérat *et al.* (1976) and Low (1982a), it can be calculated that approximately 8 g of endogenous nitrogen and 38 g of endogenous amino acids secreted into the gastrointestinal tract remain undigested and unabsorbed at the end of the ileum, while that using the estimate of Rérat (1990), gave 3 g of endogenous nitrogen and 12 g of endogenous amino acids remain undigested and unabsorbed at the end of the ileum. The values of 3-6 and 0.7-7.3 g of nitrogen per day leaving the terminal ileum of the pig reported by Low and Zebrowska (1989) and Souffrant (1991), respectively, would appear to be in agreement with the above estimates.

The amounts of individual amino acids remaining undigested and unabsorbed at the end of the ileum were calculated from the total amino acid secretion data (Table 1.2) and the mean estimates of the proportion of secreted N remaining undigested and unabsorbed at the end of the small intestine (Rérat *et al.* 1976, Low 1982a, Rérat 1990). These values (Table 1.3) can be used as estimates of net endogenous amino acid excretions at the terminal ileum of the pig, and can be compared with those determined by the indirect methods of determining endogenous amino acid excretion. The final section of this review critically reviews the indirect methods which have been used to quantify the net nitrogen and amino acid excretions at the terminal ileum of the pig and rat.

1.6 MEASUREMENT OF ENDOGENOUS NITROGEN AND AMINO ACID EXCRETION IN ILEAL DIGESTA

The most commonly used approaches for the determination of net endogenous nitrogen and amino acid excretions in ileal digesta have been the protein-free and regression methods. Recently approaches involving synthetic amino acid-based diets, guanidinated proteins and radioactive isotopes have been developed and

evaluated. All these methods are described and discussed below.

1.6.1 Protein-Free Method

The traditional method for the determination of endogenous nitrogen and amino acids involves feeding the animal a nitrogen-free diet and then measuring the nitrogen and amino acids in digesta collected at the terminal ileum. The exclusion of such an important nutrient as protein from the diet, however, may create a physiologically abnormal metabolism in the animal (Low 1980). There is evidence that the amount of protein secreted into the gastrointestinal tract is reduced when an animal is fed a protein-free diet (Snook and Meyer 1964b, Fauconneau and Michel 1970, Corring and Saucier 1972, Schneeman *et al.* 1977, Buraczewska 1979, Buraczewski 1980, Rodriguez *et al.* 1982, 1983). Cell replication and cell protein turnover may be reduced in the gastrointestinal tract in the absence of dietary protein (Munro and Goldberg 1964, Millward *et al.* 1976, Simon 1989, Muramatsu 1990). Also, the breakdown and re-utilisation of secreted enzymes may be greater in the protein-free state (Snook and Meyer 1964b, Fauconneau and Michel 1970). It is also possible, that the reduced enzyme synthesis and activity in the protein-free state may lead to a lowered digestibility of endogenous protein leading in turn to a greater accumulation of endogenous nitrogen at the end of the ileum.

The protein-free method has been widely used to determine endogenous ileal amino acid and nitrogen excretion (Zebrowska and Buraczewska 1972, Holmes *et al.* 1974, Buraczewska *et al.* 1975, Sauer *et al.* 1977, Van Weerden *et al.* 1980, Taverner *et al.* 1981, Wilson and Leibholz 1981, Darcy *et al.* 1982, Leibholz 1982, Zebrowska 1982, Picard *et al.* (1983, 1984), Green *et al.* 1987, Leibholz and Mollah 1988, De Lange *et al.* (1989a, b), Furuya and Kaji 1989, Wang and Fuller 1989, Leterme *et al.* 1990, Furuya and Kaji 1991, Hennig *et al.* 1991). Literature values for the endogenous nitrogen and amino acid excretion (expressed as mg/kg dry matter intake) from the terminal ileum of the growing pig are given in Table 1.4. The values presented in this table are from studies with growing pigs given protein-free diets containing 3-6% dietary fibre. The endogenous amino acid excretions are highly variable between studies, particularly for proline. Serine, threonine, proline, glycine, aspartic and glutamic acids are predominant in endogenous ileal amino acid excretions determined under protein-free alimentation. These amino acids constitute a large proportion of mucus glycoproteins (Hashimoto *et al.* 1963, Nemoto and Yosizawa 1969, Bella and Kim 1972, Cetta *et al.* 1972). Aspartic and glutamic acids are present in relatively high proportions in pancreatic and intestinal secretions (Table 1.2). Glycine is the predominant amino acid in bile secretion (Juste 1982), but proline is not present in relatively high proportions in any of the gastrointestinal

Table 1.4 Summary of literature values for endogenous ileal amino acid excretion (g kg^{-1} dry matter intake) in the pig determined under protein-free alimentation

	Reference											
Amino Acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**
Essential													
Arginine	.49	.43	.48	.41	.25	.34	.73	.62	.37	.45	.39	.30	.44
Histidine	.14	.26	-	.18	.12	.18	.22	.26	.15	.41	.21	.13	.21
Isoleucine	.21	.79	.19	.34	.24	.60	.36	.47	.23	.28	.31	.33	.36
Leucine	.39	.76	.34	.64	.40	.72	.60	.77	.41	.62	.62	.49	.56
Lysine	.27	.53	.25	.47	.27	.48	.53	.63	.26	.38	.23	.46	.40
Methionine	.06	.17	.10	.10	.06	.18	.16	.22	.14	.11	.13	.12	.13
Phenylalanine	.23	.40	-	.38	.23	.45	.60	.79	.25	.36	.57	.30	.41
Threonine	.39	.97	.53	.48	.35	.43	.65	.91	.46	.87	.69	.34	.59
Valine	.31	.94	.33	.51	.35	.67	.48	.65	.34	.45	.45	.37	.49
Cystine	-	.30	-	.19	.13	-	-	-	-	.19	.12	.14	.18
Non-essential													
Alanine	.42	.69	.44	.46	.36	-	.59	.73	.42	-	1.01	.50	.56
Aspartic Acid	.56	1.17	.70	.79	.48	-	1.01	1.24	.50	-	.90	.62	.80
Glutamic Acid	.71	2.26	.72	.89	.64	-	1.16	1.39	.61	-	1.11	.82	1.03
Glycine	1.39	1.02	1.51	.62	.46	-	1.94	1.44	1.23	-	.57	.45	1.06
Proline	4.74	1.65	2.29	.41	.35	-	6.22	3.64	-	-	.55	.79	2.29
Serine	.38	1.56	.50	.42	.30	-	.70	.85	.46	-	.58	.30	.61
Tyrosine	.13	.38	-	.33	.21	.36	.41	.47	.30	.35	.35	.19	.32
Total amino acids	10.95	14.47	8.38	7.58	5.20	-	16.36	15.08	6.13	-	9.28	-	10.38
Nitrogen	2.05	2.97	1.81	1.36	-	1.12	3.17	2.96	1.71	-	1.38	1.35	1.91

1. Sauer *et al.* (1977), n=6, 45-70 kg
2. Van Weerden *et al.* (1980), n=8, 45 kg
3. Taverner *et al.* (1981), n=5, 86 kg
4. Darcy *et al.* (1982), n=5, 59 kg
5. Green *et al.* (1987), n=4, 20-25 kg
6. Leibholz & Mollah (1988), n=6, 25 kg
7. De Lange *et al.* (1989a), n=7, 60 kg
8. De Lange *et al.* (1989b), n=4, 55 kg
9. Furuya & Kaji (1989), n=4, 42 kg
10. Wang and Fuller (1989), n=8
11. Leterme *et al.* (1990), n=6, 50 kg
12. Hennig *et al.* (1991), n=8, 144 kg

secretions (Table 1.2) other than mucus. De Lange *et al.* (1989b) cited evidence for the high proline secretion under protein-free alimentation being the result of large quantities of glutamine from muscle breakdown being metabolised to proline in the intestinal tract. This may be enhanced by the reduction of intestinal transport of proline and other amino acids under protein-free alimentation (Nagchaudri and Sharma 1972, Karasov *et al.* 1986). Taverner *et al.* (1981) suggested that the high levels of proline and glycine in endogenous ileal excretion following protein-free feeding were the result of their absorption as constituents of small peptides and their reflux back into the lumen as free amino acids following intracellular digestion. Sauer (1982) interpreted the true digestibility coefficients determined using a protein-free diet that were greater than unity, in particular proline and glycine, to be due to an overestimation of these amino acids under protein-free alimentation.

The composition of the protein-free diet influences the determined amino acid excretions at the terminal ileum. Increased fibre in the protein-free diet has been shown to increase the endogenous loss of amino acids from the terminal ileum of the pig (Sauer *et al.* 1977, Van Weerden *et al.* 1980, Taverner *et al.* 1981, Green *et al.* 1987, De Lange *et al.* 1989a). Dietary fibre has been shown to increase cell sloughing and increase mucus and pancreatic secretions (Sections 1.3.6, 1.3.9, and 1.3.3.2.c). De Lange *et al.* (1989a), however, found no effect on endogenous loss following feeding of a protein-free diet containing a higher level of fat.

There is evidence that the presence of dietary peptides in the intestinal lumen is important in maintaining physiologically normal levels of secretion of digestive enzymes, particularly the pancreatic enzymes (Snook 1965, Fauconneau and Michel 1970, Corring and Saucier 1972, Schneeman 1982). Schneeman (1982) and Temler *et al.* (1983) found that dietary peptides and proteins were more potent stimulators of pancreatic secretion than free amino acids, but Green and Nasset (1983) found no difference in pancreatic enzyme activity between rats fed intact casein- or synthetic amino acid-based diets. Also, Corring *et al.* (1984) did not observe a reduction in pancreatic output in pigs fed a protein-free diet over an 8-day period. Protein-free feeding may lead to lowered secretion of mucus protein and a decreased rate of turnover of epithelial cells (Munro and Goldberg 1964, Fauconneau and Michel 1970, Snook 1973, Buraczewska 1979). Moughan and Rutherfurd (1990) suggested the lowered endogenous amino acid excretion following feeding of a protein-free diet is probably the result of a reduced rate of cellular loss, a lowered secretion of digestive enzymes and mucoproteins, as well as a higher level of enzyme reutilisation. Dietary protein and its digestion products have been shown to strongly influence the secretion of gastric, pancreatic, bile and intestinal secretions (refer Sections 1.3.2.2, 1.3.3.2.a, 1.3.4, 1.3.5).

1.6.2 Synthetic Amino Acid Diet

A further approach to the determination of endogenous ileal amino acid excretion which has been used in rats, involves feeding them a diet containing synthetic amino acids as the sole nitrogen source but devoid of the amino acid under consideration. Skilton *et al.* (1988) compared amino acid flows from rats given synthetic amino acid diets devoid of either aspartic acid and serine, glutamic acid and proline, glycine and alanine, or glutamic acid and lysine with those determined following protein-free alimentation, and found no significant differences in ileal endogenous amino acid excretion except for proline, glycine and alanine. The excretions of proline, glycine and alanine were higher under protein-free feeding than after the consumption of a synthetic amino acid diet. Darragh *et al.* (1990) also reported no significant differences between endogenous ileal amino acid excretions for rats given either a synthetic amino acid based diet that was devoid of specific amino acids or a protein-free diet. Both of these studies found the absorption of the dietary synthetic amino acids to be virtually complete and used this finding to calculate endogenous amino acids flows for all the amino acids. The synthetic amino acid fed rats endogenous ileal amino acids flows were similar to those determined after protein-free feeding.

De Lange *et al.* (1989b) determined the effect of protein status on endogenous ileal amino acid loss by intravenously administering a balanced amino acid mixture or a saline solution to pigs given a protein-free diet, an approach somewhat analagous to the above mentioned synthetic amino acid method. They reported a decrease in endogenous protein and proline excretion for the pigs parenterally given the amino acid mixture compared to those given saline parenterally. This implies that any alterations in metabolism due to the absence of dietary amino acids does not significantly affect endogenous amino acid excretions, with the possible exception of proline. From the studies reported above, the oral or parenteral administration of free amino acids to animals given an otherwise protein-free diet does not markedly influence the endogenous ileal amino acid excretion with the exceptions of proline, glycine and alanine. Endogenous ileal amino acid loss, therefore, is not significantly affected by the altered metabolism of the protein-free state.

1.6.3 Regression Method

Another traditionally used approach to determining endogenous amino acid excretion is the regression method. In this method, the animal is fed increasing levels of dietary protein, the terminal ileal amino acid flows are measured, and endogenous loss is determined by extrapolation to zero protein intake. The increase

in amino acid flow with increasing dietary protein intake is attributed entirely to increased amounts of undigested food protein, it being assumed that there is no change in the amounts of the endogenous amino acid secretions. There is evidence, however, that the rate of secretion into the intestine does vary with the amount of protein given (Snook and Meyer 1964a, b, Corring and Saucier 1972, Lavau *et al.* 1974, Temler *et al.* 1983, Ozimek *et al.* 1984). Consequently, some of the increase in amino acid flow with increased dietary protein intake is probably the result of enhanced secretion of endogenous proteins. Also, there may not be a linear relationship between feed intake and amounts of endogenous nitrogen or amino acids in digesta, and any increase in protein level in the feed is always associated with other changes in dietary composition which hinders the interpretation of the results.

Literature values for endogenous amino acid excretions at the terminal ileum for pigs determined by the regression method are shown in Table 1.5. There is some variation across the different studies which is probably due to differences in digesta collection methods and animal weights. These values determined by the regression method are similar to those found by feeding the pigs a protein-free diet. This can be seen clearly in those studies where both methods have been used to determine the flow of amino acids at the terminal ileum of the pig (Taverner *et al.* 1981, Leibholz and Mollah 1988, Furuya and Kaji 1989). Also, different proteins do not appear to significantly affect the determined (regression) endogenous amino acid flows (Taverner *et al.* 1981, Leibholz and Mollah 1988).

The regression method would appear philosophically to be a better method than the protein-free method for calculating endogenous nitrogen and amino acids in ileal digesta. There may not, however, be a linear relationship between level of feed intake and amounts of endogenous nitrogen or amino acids in digesta. Also, any increase in protein level in the feed is always associated with other changes in dietary composition which complicates the interpretation of results.

1.6.4 Homoarginine Method

Hagemeister and Erbersdobler (1985) proposed the homoarginine method for the determination of endogenous ileal nitrogen. This technique enables the discrimination between endogenous and dietary protein. The dietary protein is guanidinated such that its protein-bound lysine is transformed to homoarginine. Homoarginine is found only in trace amounts in animals and unlike other markers (^{15}N, ^{13}C, ^{14}C) does not appear to be used in the formation of endogenous protein. The homoarginine is absorbed and subsequently partly transformed back to lysine in the liver by the enzyme arginase. It must be assumed, however, that the

Table 1.5 Summary of the literature values for endogenous ileal amino acid excretion (g kg^{-1} dry matter intake) in the pig determined by the regression method

Amino Acid	Reference 1 wheat	1 barley	1 overall	2 milk	2 cottonseed meal	3 casein	4 milk proteins	5 barley	Mean
Essential									
Arginine	.62	.58	.53	.29	.40	.37	1.44	.25	.56
Histidine	-	-	-	.15	.13	.14	.33	-	.19
Isoleucine	.15	.14	.15	.61	.59	.23	.80	.28	.37
Leucine	.33	.43	.33	.64	.67	.41	1.14	.58	.57
Lysine	.33	.28	.32	.37	.37	.26	1.05	.32	.41
Methionine	.14	.16	.13	.16	.12	.14	.30	.08	.49
Phenylalanine	-	-	-	.36	.40	.25	.54	.39	.39
Threonine	.58	.51	.60	.45	.39	.42	1.06	.33	.54
Valine	.55	.28	.42	.56	.69	.33	1.41	.35	.57
Cystine	-	-	-	-	-	-	.30	-	.30
Non-essential									
Alanine	.62	.59	.58	-	-	.41	1.22	.28	.62
Aspartic Acid	.72	.83	.82	-	-	.47	1.81	.47	.85
Glutamic Acid	.82	.91	.85	-	-	.55	2.24	1.07	1.07
Glycine	2.27	2.12	1.71	-	-	1.23	2.76	.25	1.72
Proline	3.67	4.26	1.73	-	-	-	-	-	3.22
Serine	.62	.60	.63	-	-	.46	1.60	.33	.71
Tyrosine	-	-	-	.34	.36	.29	.46	.29	.35
Total amino acids	11.42	11.69	8.80	-	-	5.96	18.46	5.27	10.30
Nitrogen	2.51	3.00	2.29	.94	.89	1.67	3.44	-	2.11

1. Taverner *et al.* (1981), n=2,2,7, 86 kg
2. Leibholz & Mollah (1988), n=6,6, 25 kg
3. Furuya & Kaji (1989), n=4, 42 kg
4. Leibholz (1982), n=20, 4 kg
5. Moughan *et al.* (1987), n=11, 26 kg

guanidinated protein is digested and absorbed at similar rates and in the same manner as other dietary protein, that homoarginine does not cause altered protein metabolism in the animal, and that there is no significant arginase activity within the gastrointestinal tract. With regard to the latter assumption, Schuttert *et al.* (1991) in an *in vitro* experiment did not detect any arginase activity in the small intestine of the growing rat. Rutherfurd and Moughan (1990) who studied the effects of pH and protein level on the extent of guanidination of casein, gelatin and soya protein isolate, reported that lysine was unable to be completely converted into homoarginine in any of these proteins, with the highest rate of conversion being for gelatin (95%). In a subsequent experiment (Moughan and Rutherfurd 1990) it was shown that the degree of guanidination of gelatin had no significant effect on the lysine flows determined at the distal ileum of the rat.

The homoarginine method has been applied to the estimation of endogenous nitrogen and amino acid excretion by several workers (Siriwan and Bryden 1987, Siriwan *et al.* 1987, Moughan and Rutherfurd 1990, 1991). Siriwan and Bryden (1987) and Siriwan *et al.* (1987), who used the homoarginine technique with chickens fed guanidinated casein, reported that 90% of the amino acids appearing in the ileum were of endogenous origin. The endogenous flow of lysine for rats fed an enzymically hydrolysed casein-based diet was not significantly different from that determined using guanidinated gelatin, and these values were significantly greater than those determined for rats given a protein-free diet (Moughan and Rutherfurd 1990). This indicates an effect of dietary protein and peptides on endogenous ileal amino acid excretions.

Moughan and Rutherfurd (1991) investigated the effect of protein source on endogenous lysine flow at the distal ileum of the rat using the homoarginine and ^{14}C-acetylated lysine methods. They compared guanidinated gelatin and isolated soya bean protein, and acetylated gelatin, isolated soya bean protein and casein, and found no effect of protein source on endogenous ileal lysine flow in the rat when these proteins were fed over a short time (8 hours). The major disadvantage of the method is that it provides direct information only for endogenous lysine flow. Further disadvantages include the possible accumulation of homoarginine in the body over time due to the slow rate of conversion of homoarginine to lysine. Again, homoarginine may interfere with the urea cycle leading to an accumulation of ammonia in the body. This may have occurred in the studies of Moughan and Rutherfurd (1990, 1991) where food intake declined with time over the experimental period.

1.6.5 Tracer Method

Another method which distinguishes between the exogenous and endogenous nitrogen in digesta after feeding the animal a protein-containing diet is the tracer (^{14}C, ^{15}N, ^{13}C, ^{35}S, ^{75}Se) technique (Nasset and Ju 1961, Ochoa-Solano and Gitler 1968, Kohler *et al.* 1978, Buraczewska *et al.* 1979, Bergner *et al.* 1980, 1983, 1984, Gebhardt *et al.* 1978, Simon *et al.* 1978, De Lange *et al.* 1990). The most successful application to date has been the ^{15}N tracer (Kohler *et al.* 1978, Bergner *et al.* 1983, 1984, De Lange *et al.* 1990, Krawielitzki *et al.* 1990). Two approaches are possible with this technique, in that either the food protein or the animals' nitrogen pool is labelled. When the labelled nitrogen is fed orally it is assumed that the labelled and unlabelled amino acids are equally absorbed, and that the endogenous nitrogen secretion does not become labelled to a significant extent during the course of the experiment. These assumptions are contestable. Also, labelled feedstuffs are difficult to obtain so the labelling of the animal's nitrogen pool has been used most often. The proportion of endogenous nitrogen in digesta is calculated from the dilution of the isotope. Souffrant *et al.* (1982) and Souffrant (1991) have reviewed the use of the ^{15}N dilution technique and outlined some of the difficulties with this method. The practical aspects of the technique which require further critical analysis include the method of labelling the animal's nitrogen pool, and the selection of the pool with a labelling level equal to that of total endogenous nitrogen. Choice of precursor pool has a significant effect on the dilution factor (Moughan *et al.* 1991). The ^{15}N method can determine the endogenous nitrogen in digesta and faeces, but not the proportions of endogenous amino acids. The latter means that a constant endogenous amino acid composition is used to determine true amino acid digestibility coefficients. This constant amino acid composition is usually that determined following feeding of the animal a protein-free diet which as discussed earlier has its own inherent difficulties. The cost of ^{15}N-labelled substances also detracts from the routine use of this method.

De Lange *et al.* (1990) used the ^{15}N dilution technique to determine "real" ileal protein and amino acid digestibilities for feedstuffs in growing pigs. They employed intravenous infusion of ^{15}N-leucine, the TCA-soluble fraction of blood as their precursor pool, and the endogenous amino acid composition for pigs fed a protein-free diet with intravenous infusion of amino acids (De Lange *et al.* 1989b). A limitation of this approach is that the TCA-soluble fraction of blood contains free amino acids that can be used for the synthesis of endogenous protein, however amino acids absorbed from the intestinal lumen can be used for protein synthesis in the intestinal wall without entering the blood (Alpers 1972). The contribution of endogenous protein, therefore, could be underestimated. Roos *et al.* (1990) who

compared true digestibility values determined by the homoarginine and ^{15}N tracer methods, found that the ^{15}N method gave values around 5% lower than the homoarginine technique. De Lange *et al.* (1990), however, found that endogenous protein in ileal digesta determined by ^{15}N dilution was 25.5-30.5 g/kg dry matter intake in diets based on barley, wheat, canola meal or soybean meal, values which are much higher than those reported by the same workers for pigs fed a protein-free diet (De Lange *et al.* 1989a, 1989b). Souffrant *et al.* (1982), De Lange *et al.* (1990) and Makkink and Heinz (1991) using the ^{15}N technique reported that different proteins elicited different endogenous ileal nitrogen flows in the growing pig which is in contrast to the results of Moughan and Rutherfurd (1991) using acetylated and guanidinated proteins. Van der Poel *et al.* (1991) using the ^{15}N technique showed an increase in endogenous protein excretion (6.6 to 10.7 g per 100 g dry matter intake) following the feeding of piglets with common beans (*Phaseolus vulgaris* L.), which contain antinutritional factors, compared with those fed a soybean isolate. The variations in the excretions of the individual amino acids at the end of the ileum with dietary factors are not known.

1.6.6 Peptide Alimentation

A further approach to the determination of endogenous ileal amino acid excretions which involves feeding the animal a diet containing peptides as the sole nitrogen source has recently been reported (Darragh *et al.* 1990, Moughan *et al.* 1990b, Moughan and Rutherfurd 1990). Darragh *et al.* (1990), who fed rats an enzyme hydrolysed casein-based diet in which the nitrogen was present as a mixture of free amino acids and oligopeptides, found that the endogenous amino acid flows of the enzymically hydrolysed casein (EHC) fed rats were greater than those given a protein-free or a synthetic amino acid diet. Moughan and Rutherfurd (1990) also used this approach in rats and showed the EHC fed rats had endogenous ileal amino acid flows that were significantly higher than those for the protein-free fed animals. This latter study which also compared endogenous lysine flows in rats, determined following the feeding of a guanidinated gelatin-based diet, reported no significant difference between the lysine flows of the rats fed the EHC (522 μg/g freeze dry matter intake) and the gelatin (488 μg/g freeze dry matter intake) diets, but the lysine flows for the protein-free fed rats were significantly lower (238 μg/g freeze dry matter intake). The diets used in these studies contained the same levels of fibre and, where applicable, levels of nitrogen-containing compounds. It would thus appear that endogenous amino acid flow at the terminal ileum is underestimated when determined using the traditional protein-free method. These studies also provided evidence for a direct effect of dietary protein and peptides on the net loss

of endogenous lysine and other amino acids from the small intestine of the growing rat.

The approach used by Darragh *et al.* (1990) and Moughan and Rutherfurd (1990) for the determination of endogenous amino acid excretion assumes that the dietary peptides and amino acids are completely absorbed by the end of the ileum. The presence of dietary peptides and free amino acids in the ileal digesta would result in the overestimation of endogenous ileal amino acid loss. Moughan *et al.* (1990b) attempted to overcome this limitation by separating endogenous protein from the dietary peptides and free amino acids in the ileal digesta of rats using trichloroacetic and perchloric acids. The resulting endogenous ileal amino acid flows, however, were low while analysis of the supernate indicated that there was incomplete precipitation of the endogenous amino acid excretions. Moughan *et al.* (1990b) concluded that further research was required regarding the separation of endogenous protein and unabsorbed dietary peptides and free amino acids in ileal digesta before this technique could be used routinely for the determination of endogenous ileal amino acid loss.

1.7 CONCLUSION

The direct method for determining net endogenous ileal amino acid excretion, in which the total amino acids secreted less those digested and absorbed are calculated (Table 1.3), gives a much higher estimate of endogenous loss than that determined by any of the indirect methods. The traditional protein-free and regression methods appear to underestimate the endogenous amino acid and nitrogen losses at the terminal ileum of the rat and pig when compared to the homoarginine, tracer and peptide alimentation techniques. The homoarginine technique has provided evidence for an increase in net endogenous amino acid excretions for animals given protein-containing diets compared with a protein-free one, but provides data only for lysine. The ^{15}N isotope dilution technique has also shown an influence of dietary protein on endogenous excretions but requires further evaluation and validation. A new approach whereby the animal is fed peptides as the sole nitrogen source shows considerable promise if the unabsorbed dietary peptides and amino acids can be separated from the endogenous protein. The research reported here aimed to further develop and evaluate the peptide alimentation method for the determination of net endogenous ileal amino acid excretions using the rat and pig as the experimental animals, and to further elucidate the role of peptides and protein in enhancing ileal endogenous amino acid loss in

simple-stomached animals.

In the present work, preliminary studies evaluated the filtration efficiency of ultrafiltration devices and examined three pre-filtration treatments for ileal digesta (Chapter 2). The peptide alimentation method for the determination of endogenous ileal amino acid excretions was then applied in the rat and the resulting values compared to those determined using the traditional protein-free method (Chapter 2). The composition of endogenous protein-, peptide- and free amino acid-nitrogen in digesta nitrogen was determined from the distal ileum of the rat following different storage and collection conditions (Chapters 3 and 4). The peptide alimentation method was then applied to the determination of endogenous ileal amino acid excretions of the pig, and the resulting values compared with those from pigs under protein, synthetic amino acid and protein-free alimentation (Chapter 5). Finally, the relationships between feed intake and individual ileal amino acid excretions were determined in the pig following peptide alimentation and under a defined feeding regime.

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Chapter 2

Endogenous Amino Acid Flow at the Terminal Ileum of the Rat Determined Under Conditions of Peptide Alimentation

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2.1 ABSTRACT

The study aimed to determine endogenous ileal amino acid excretion in the growing rat fed an enzymically hydrolysed casein (EHC) based diet with subsequent treatment of the digesta using ultrafiltration technology. Comparison was made with endogenous excretions obtained from rats fed an EHC-based or a protein-free diet and without any treatment of the ileal digesta. Preliminary investigations were made to determine the filtration efficiency of the ultrafiltration devices and to examine three alternative pre-filtration treatments, trichloroacetic acid (TCA) and perchloric acid (PCA) precipitation, and centrifugation (SPIN). In the preliminary work, fifteen purified protein, peptide and amino acid solutions were ultrafiltered using a molecular weight (M.W.) exclusion limit of 10,000 Daltons and the recovery of nitrogen indicated an effective filtration (generally >90% separation) on nominal molecular weight. Further, fresh rat ileal digesta samples were treated with either TCA, PCA or SPIN and the resulting supernatants ultrafiltered with the fractions being analysed for nitrogen and amino acids. The precipitates contained 26, 57 and 39% of the total nitrogenous material for the TCA, PCA and SPIN treatments, respectively, indicating that PCA was the most effective precipitant. In the main study, twelve 100 g male rats were fed either an EHC-based diet or a protein-free diet and samples of ileal digesta were collected after slaughter. The digesta from the 6 EHC fed rats were ultrafiltered after centrifugation and the high molecular weight fraction added to the precipitate. Endogenous ileal amino acid flows were determined for the total digesta and for the digesta following the centrifugation plus ultrafiltration treatment. The endogenous amino acid flows were generally higher for the total digesta than for the digesta following centrifugation plus ultrafiltration and significantly so ($P<0.05$) for lysine, glutamic acid and proline. The protein-free fed rats had significantly ($P<0.05$) lower amino acid flows than those rats fed the EHC-based diet (ultrafiltration treatment) with the greatest differences occurring for isoleucine, serine, glutamic acid, valine, leucine, alanine and threonine.

2.2 INTRODUCTION

The measurement of endogenous amino acid flow at the terminal ileum of monogastric animals is of basic importance in the study of their nutrition. Traditionally, the endogenous loss of nitrogen and amino acids from the small intestine of animals has been determined after feeding them a protein-free diet. This

method may be criticised, however, for creating a physiologically abnormal state (Low 1980).

Recent studies (Darragh *et al.* 1990; Moughan and Rutherfurd 1990) have shown that the endogenous amino acid loss from the rat small intestine is higher under peptide alimentation than under protein-free or synthetic free amino acid feeding. Further, Moughan and Rutherfurd (1990) have shown that the endogenous flow of lysine at the terminal ileum of rats fed a diet containing guanidinated protein was significantly higher than that of rats fed a protein-free diet.

The approach of Darragh *et al.* (1990), however, in which endogenous ileal amino acid excretion was determined after feeding rats an hydrolysed casein based diet, relied on the assumption that the dietary amino acids were completely absorbed. Although the latter seems likely, it is difficult to establish unequivocally. Consequently, a method for determining endogenous loss has been proposed (Moughan *et al.* 1990) which removes the need to make assumptions concerning the completeness of absorption of the dietary amino acids. In this method the animal is fed a semi-synthetic diet containing enzymically hydrolysed casein as its sole nitrogen source. Ileal digesta are collected and the nitrogenous fraction separated physically using large volume disposable Centriprep-10 ultrafiltration devices (Amicon, W.R. Grace and Co., Danvers, Massachusetts). The high molecular weight (M.W.>10,000 Daltons) fraction resulting from the ultrafiltration provides a measure of endogenous amino acid flow. If some of the dietary amino acids and small peptides are not absorbed, they will be removed in the low molecular weight fraction. In addition to the unabsorbed dietary amino acids and peptides, the low molecular weight fraction will contain non-protein nitrogen and may contain endogenous free amino acids and small peptides. The latter if present are expected to be at a low concentration. Nevertheless, their removal in the low molecular weight fraction may lead to some underestimation of the actual endogenous loss of amino acids.

The present study aimed to determine endogenous ileal amino acid flows in the growing rat using the ultrafiltration technique and to compare these flows with those obtained when the treatments were either an enzyme-hydrolysed casein diet but without treatment of digesta or a protein-free diet. Prior to the conduct of this study two preliminary investigations were undertaken. The first aimed to determine the filtration efficiency of the devices when used with purified solutions. The direct ultrafiltration of ileal digesta may be inefficient and possibly unreliable due to blockage of the membrane by solid material and a pre-treatment of the digesta to remove the majority of solid material prior to ultrafiltration would appear desirable. Thus in a second preliminary investigation, three pre-filtration treatments of the ileal

digesta, namely trichloroacetic acid or perchloric acid precipitation and centrifugation were compared.

2.3 EXPERIMENTAL

2.3.1 Validation of Ultrafiltration Technology

Centriprep-10 Concentrators (M.W. exclusion limit 10,000 Daltons, Amicon, W.R. Grace and Co., Danvers, Massachusetts) were used for ultrafiltration. Duplicate 7 ml aliquots of each of 15 purified protein, peptide or amino acid solutions were ultrafiltered using the concentrators, following the manufacturer's instructions (Amicon 1987). The solutions consisted of a known weight (mg) of crystalline solid dissolved in 25 ml of deionised water or phosphate buffer saline (0.01 M) to give 0.12-0.65 mg nitrogen ml^{-1}. The weight of solid depended on the solubility of the substance. The solubility of some substances was increased by adjusting the pH of the solution with small additions of 0.01 M HCl or NaOH. The solutions were individually centrifuged in Centriprep concentrators at 1350 g for 40 minutes. The filtrate (M.W.<10,000) was poured off and the sample centrifuged at 1350 g for a further 20 minutes. The second filtrate was then poured off and added to the first. The nitrogen content of the resultant filtrate (M.W.<10,000) and retentate (M.W.>10,000) fractions were determined.

2.3.2 Evaluation of Pre-filtration Treatments

2.3.2.1 Collection of Ileal Digesta

Eighteen 100 g Sprague-Dawley male rats were selected at random from a group of rats, which had been weaned at 4 weeks of age and reared on a high quality diet, at the Small Animal Production Unit, Massey University. The animals were kept individually in raised stainless steel cages with wire mesh floors at 20±2°C, and with a 12 h light/dark cycle. The rats were fed a casein-based (10% crude protein) diet for a 12 day preliminary period followed by an enzymically hydrolysed casein-based diet (EHC) (Table 2.1) for 4 days. The EHC comprised free amino acids and small (M.W.<10,000) peptides. The diets were offered *ad libitum* for a 3 h period (0830-1130 h) each day and water was available at all times.

On the 16th day the rats were asphyxiated with carbon dioxide gas and decapitated 3h±15 minutes after the start of feeding. The final 20 cm of ileum was immediately dissected from the body, the outer intestinal surface carefully cleaned using absorbent tissue paper and the contents slowly flushed out using exactly 10 ml of physiological saline (0.9% NaCl) from a plastic syringe. The digesta from the 18 rats were kept separate and packed in ice immediately after collection. The digesta samples were equally and randomly assigned to one of three pre-filtration

Table 2.1 Ingredient composition (g kg^{-1} air dry weight) of the enzymically hydrolysed casein (EHC) and protein-free diets

Ingredient	Diet Composition	
	EHC	Protein-free
Wheaten cornflour	629	729
Purified cellulose	50	50
Corn oil	65	65
Sucrose	100	100
Vitamin/mineral mix[1]	50	50
Chromic oxide	6	6
Enzymically hydrolysed casein[2]	100	-

[1] Supplied (mg kg^{-1} diet): vitamin E 750, vitamin K 3, riboflavin 8, thiamin 9, pyridoxine 8, pantothenic acid 38, nicotinic acid 38, folic acid 2, Fe 105, Zn 75, Mn 75, Cu 9, Co 1; (µg kg^{-1} diet): vitamin A 648, vitamin D_3 113, vitamin B_{12} 30, I 750, Se 230; (g kg^{-1} diet): choline 1.5, Na 2.0, K 2.9, Cl 4.9, Mg 0.7, S 0.5, Ca 9.3, P 4.8

[2] Sigma Chemical Company, St Louis, U.S.A. Type I from bovine milk. Total nitrogen = 12.7%. Amino nitrogen = 6.3%; M.W.<5,000 Da.

treatments: trichloroacetic acid (TCA), perchloric acid (PCA) and centrifugation (SPIN).

2.3.2.2 Treatment of Digesta

The ileal digesta of each rat were treated separately within 30 minutes of collection. The digesta were weighed and their volumes measured. They were then transferred to separate centrifuge tubes and reacted with either trichloroacetic acid (TCA) or perchloric acid (PCA) or were not treated chemically but simply centrifuged (SPIN). The TCA and PCA treatments were as described by Moughan *et al.* (1990). The precipitates and supernatants were separated by centrifugation at 1350 g at 0°C for 10 minutes. In the SPIN treatment the digesta were centrifuged at 1400 g for 45 minutes at 0°C. The precipitates were freeze-dried, finely ground and analysed for nitrogen and amino acids. The liquid supernatants were analysed for nitrogen and amino acids.

2.3.2.3 Fractionation of the Supernatants

The supernatants were fractionated to determine possible changes in molecular size due to the chemical treatments. Duplicate aliquots (8-10 ml) of each supernatant were ultrafiltered using a membrane with a 10,000 Dalton M.W.

exclusion limit. The nitrogen and amino acid contents of the subsequent fractions were determined.

Duplicate 2.5 ml samples of digesta supernatant of 3 randomly selected rats from each of the three pre-filtration treatments (TCA, PCA, or SPIN) were subjected to gel chromatography using Sephadex G-10 (Pharmacia, Uppsala, Sweden) (approximate M.W. exclusion limit for globular proteins of 700 Daltons). The column was 60 cm long and 1 cm in diameter and the eluent was phosphate buffer saline (0.01 M) flowing at a rate of 14.44 ml hr^{-1}. The column was calibrated with bovine serum albumin (2 mg/ml), riboflavin (0.35 mg/ml) and phenylalanine (2 mg/ml). Fractions were collected every 16 minutes and analysed for nitrogen. Proteins and large peptides were eluted in fractions 11-14, and small peptides and free amino acids in fractions 18-23.

2.3.3 Determination of Endogenous Amino Acid Flows

Six rats were treated as described for the preliminary studies. Terminal ileal digesta were collected and centrifuged at 1400 g for 45 minutes at 0°C. The supernatants were subjected to ultrafiltration using Centriprep-10 Concentrators (M.W. exclusion limit 10,000 Daltons, Amicon, W.R. Grace and Co, Danvers, Massachusetts) according to the manufacturer's instructions (Amicon, 1987). The retentate was added back to the precipitate and this material was freeze-dried, finely ground and analysed for amino acids and chromium. The filtrate was analysed for amino acids. A further six rats were given a standard casein-based diet for 10 days followed by a protein-free diet for 6 days. The composition of the latter diet is given in Table 2.1. In all other respects the rats were treated as described in the above section on the evaluation of pre-filtration treatments. The terminal ileal digesta were not subjected to any treatment or filtration prior to being freeze-dried, finely ground and analysed for amino acids and chromium. The diets were analysed for amino acids and chromium.

2.3.4 Chemical Analysis

Amino acids were determined after acid hydrolysis using a Beckman 119 B.L. amino acid analyser. Duplicate samples (5-7 mg of digesta; 500-1000 µl of supernatant) were hydrolysed in 500 µl of 6 M HCl plus 1% phenol for 24 h at 110±2°C in glass tubes sealed under a vacuum of 1 Pa. Cystine, methionine and tryptophan, being partly destroyed under acid hydrolysis, were not determined.

Nitrogen was determined in duplicate using a micro-Kjeldahl technique. The material was digested with concentrated sulphuric acid with potassium sulphate added to increase the digestion temperature, and the free ammonia released was determined by the Berthelot reaction (Chaney and Marbach 1962).

The chromium contents of samples (8-10 mg) of diet, ileal digesta precipitates

and total ileal digesta were determined in duplicate on an Instrumentation Laboratory Atomic Absorption Spectrophotometer using the method of Costigan and Ellis (1987).

2.3.5 Data Analysis

The proportions of nitrogen and total amino acids in the supernatant and precipitate, and the high and low molecular weight fractions of the supernatant after ultrafiltration for the treatments TCA, PCA and SPIN were subjected to a one-way analysis of variance. The significance of the differences between the treatment means were determined by orthogonal contrasts (Sokal and Rohlf 1981).

Endogenous flows of amino acids at the terminal ileum relating to the ingestion of 1 g of freeze dry matter (FDM) were calculated using the following equation:

$$\text{amino acid flow } (\mu g\ g^{-1}\ FDM) = \text{amino acid concentration in ileal digesta } (\mu g\ g^{-1}\ FDM) \times \frac{\text{diet chromium (mg } g^{-1}\ FDM)}{\text{ileal chromium (mg } g^{-1}\ FDM)}$$

Endogenous amino acid flows for the 6 EHC fed rats were calculated based on the amino acid content of the precipitate plus high molecular weight fraction following centrifugation plus ultrafiltration treatments (Treated Digesta) and for the total digesta by including the amino acids in the supernatant ultrafiltrate (Untreated Digesta). The endogenous amino acid flows for the EHC fed rats were subjected to a paired t-test for each amino acid singly (Sokal and Rohlf 1981). The endogenous amino acid flows for the EHC fed rats (after centrifugation plus ultrafiltration of the digesta) and the protein-free fed rats were subjected to a one-way analysis of variance for each amino acid.

2.4 RESULTS

2.4.1 Validation of Ultrafiltration Method

The recoveries of nitrogen (N) from the fractions of the purified protein, peptide and amino acid solutions after ultrafiltration are shown in Table 2.2. Effective filtration (generally >90% separation) on nominal molecular weight was found for the solutions.

2.4.2 Pre-filtration Treatments

The rats were healthy and readily consumed the experimental diets. Food intakes on the last day were 11±0.5 g (Mean±SE). At slaughter, faeces were not detected in the gastric contents, indicating that coprophagy had not occurred.

Table 2.2 The recovery[1] of nitrogen (N) from purified protein, peptide and amino acid solutions in the retentate and filtrate after ultrafiltration[2].

Solute	Initial Solute Concentration	Nominal M.W. (Daltons)	% N in Filtrate (MW<10,000)	% N in Retentate (MW>10,000)
Thyroglobulin	0.85	669,000	3	97
Bovine serum albumin	4.04	67,000	6	94
Ovalbumin	2.01	45,000	10	90
Trypsin	2.05	24,000	8	92
Myoglobin	2.05	16,950	0.5	99.5
Lysozyme	2.15	14,300	0.8	99.2
Porcine insulin	2.37	6000	91	9
Bovine insulin	1.83	6000	87	13
Coenzyme A	2.04	767.5	88	12
β-NAD	2.42	663.5	92	8
Riboflavin	0.82	376.4	94	6
Tryptophan	4.02	222.8	92	8
Phenylalanine	4.32	165.2	88	12
Histidine	4.11	155.2	94	6
Lysine	4.07	146.2	93	7

[1] Mean of duplicate determinations
[2] Centriprep-10 Concentrators (Amicon, W.R. Grace Company, Danver, Massachusetts).

The amounts of nitrogen from ileal digesta remaining in solution after precipitation by treatments TCA and SPIN (expressed as proportions of the total nitrogen within each of the treated samples) were 74 and 61%, respectively, whereas with PCA the corresponding mean value was 30% ($P<0.01$). The proportions of amino acids remaining in solution for the various treatments were similar to those obtained for nitrogen. This is not unexpected as the level of non-amino nitrogen in rat ileal digesta is very low (Moughan *et al.* 1990). Consideration of the data overall, and assuming that non-amino nitrogen is a negligible proportion of total nitrogen, significantly ($P<0.01$) more of the total nitrogenous material was found in the precipitate for the PCA treatment (70%) compared with the TCA (26%) or SPIN (39%) treatments, making PCA the most effective pre-filtration treatment.

Pre-filtration treatment of the ileal digesta did not significantly (P>0.05) influence the proportions of the supernatant amino acids in the high molecular weight (M.W.>10,000) and low molecular weight fractions which were around 60 and 40%, respectively. It appears that at least half of the nitrogenous material remaining in the supernatant was soluble protein. It was noted that the amino acids threonine, serine, glutamic acid, proline, alanine, valine, isoleucine and leucine were particularly abundant (>70% of total amino acids) in the high M.W. supernatant fraction. Data for glycine were not available as there was inadequate resolution of this amino acid during analysis. Taking the results of the gel filtration and ultrafiltration (for the comparable digesta samples) together it was found that the amounts of supernatant nitrogen in the high M.W. (>10,000) fraction, expressed as proportions of the total digesta nitrogen, were similar at 37, 30 and 33% for the TCA, PCA and SPIN treatments, respectively. These results were similar to those found for the amino acids. The proportions of supernatant nitrogen in the large peptide fraction (700<M.W.<10,000), expressed as proportions of total digesta nitrogen, varied across treatments being 15, 4 and 19% for the TCA, PCA and SPIN, respectively. The proportions of supernatant nitrogen in the lowest M.W. fraction (M.W.<700) were 22, 9 and 19% for TCA, PCA and SPIN, respectively. There appeared to be more large peptides (700<M.W.<10,000) in the TCA and SPIN supernatants. There also appeared to be more small peptides and free amino acids (M.W.<700) in the TCA and SPIN supernatants than for PCA.

Although PCA was the most effective precipitant in absolute terms, it also gave the most variable data (Coefficient of Variation (CV) = 53%). There was also an unexplained discrepancy for PCA, between the data based on measurements of nitrogen and those on total amino acids. The simple centrifugation treatment gave consistent results (CV = 14%) and removed around 40% of the digesta nitrogen in the precipitate. Overall, the latter method was considered to be the most suitable for routine treatment of digesta.

2.4.3 Endogenous Amino Acid Flows

The rats remained healthy throughout the 16 day study. The food intakes (mean±SE) of the animals on the final day were 11±0.6 g and 9±0.3 g for the enzymically hydrolysed casein and protein-free treatments, respectively. At slaughter, faeces were not found in the gastric contents of rats on either treatment.

The mean endogenous ileal amino acid flows (precipitate + >10,000 M.W. fraction) for the centrifugation (SPIN) plus ultrafiltration treatment are compared in Table 2.3 with the corresponding total digesta amino acid flows. The mean endogenous ileal amino acid flows for the EHC fed rats following centrifugation and ultrafiltration treatment of digesta are compared with those for the rats fed the

Table 2.3 Mean (±SE) endogenous amino acid flows[1] at the terminal ileum of rats (n=6) fed an enzymically hydrolysed casein-based diet determined on the total untreated digesta or digesta subjected to centrifugation plus ultrafiltration

Amino acid	Untreated Digesta	Treated Digesta[2]	Level of Significance[3]
Lysine	435±25	293±24	**
Histidine	167±8	159±16	NS
Arginine	228±25	178±32	NS
Aspartic acid	901±63	719±63	NS
Threonine	613±31	612±64	NS
Serine	1044±90	963±71	NS
Glutamic acid	2413±115	1455±118	***
Proline	630±28	498±42	*
Alanine	352±19	300±22	NS
Valine	580±39	498±37	NS
Isoleucine	491±42	437±32	NS
Leucine	542±31	450±31	NS
Tyrosine	240±24	184±13	NS
Phenylalanine	219±21	168±16	NS

1 $\mu g\ g^{-1}$ freeze dry matter intake

2 Centrifuged and ultrafiltered; flow was based on amino acids in the precipitate plus retentate (M.W.>10,000)

3 NS = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001.

protein-free diet in Table 2.4.

The amino acid flows for the total digesta were generally higher than those after the centrifugation plus ultrafiltration treatment for the EHC fed rats and significantly so (P<0.05) for lysine, glutamic acid and proline. The protein-free and EHC-fed rats (centrifugation plus ultrafiltration treatment) had significantly (P<0.05) different flows for most of the amino acids, except histidine, arginine, aspartic acid, tyrosine and phenylalanine.

Table 2.4 Mean (±SE) endogenous amino acid flows[1] at the terminal ileum of rats fed an enzymically hydrolysed casein (EHC) based diet determined on digesta samples subjected to centrifugation plus ultrafiltration and flows for rats fed a protein-free diet

	Endogenous Flow		
Amino Acid	**EHC[2] (n=6)**	**Protein-Free (n=6)**	**Level of Significance[3]**
Lysine	293±24	219±16	*
Histidine	159±16	140±7	NS
Arginine	178±32	219±13	NS
Aspartic Acid	719±63	655±36	NS
Threonine	612±64	393±25	**
Serine	963±71	355±25	***
Glutamic Acid	1455±118	637±34	***
Proline	498±42	389±23	*
Alanine	300±22	198±14	**
Valine	498±42	238±14	***
Isoleucine	437±32	152±11	***
Leucine	450±31	261±19	***
Tyrosine	184±13	163±11	NS
Phenylalanine	168±16	148±11	NS

1 $\mu g\ g^{-1}$ freeze dry matter intake
2 Digesta were centrifuged and ultrafiltered; flows were based on amino acids in the precipitate plus retentate (M.W.>10,000)
3 NS = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001.

2.5 DISCUSSION

2.5.1 Validation of Ultrafiltration Technology

The efficiencies of ultrafiltration of the purified protein, peptide and amino acid solutions using the Centriprep-10 Concentrators were high and similar to those reported by the manufacturer (Amicon, 1987). The nominal M.W. cut-off used to characterise the membranes in the concentrators is a convenient parameter to work with but is only an estimate of the size of molecules that will pass through the membrane pores, and so may not be valid for all solutes (Gasper 1984; Amicon

1987). There is a potential inaccuracy of separation for molecules with weights around the exclusion limit. The M.W. exclusion limit of 10,000 Daltons was chosen here because, historically, peptides and proteins were differentiated by molecular weight, with proteins being defined as having molecular weights greater than 10,000 Daltons (approximately 80-90 amino acids), (Jakubke and Jeschkeit 1977). The chemical differences between polypeptides and proteins, however, are indistinct. In the present work, the aim was to separate dietary free amino acids and small peptides from the large undigested endogenous proteins in ileal digesta, so the potential inaccuracies of separation for entities with molecular weights around 10,000 Daltons was not critical. It was concluded that the Centriprep devices were suitable for the present purposes.

2.5.2 Pre-filtration Treatment of Digesta

The nitrogen containing compounds in the ileal digesta of rats fed an enzymically hydrolysed casein-based diet were incompletely precipitated by the reagents TCA and PCA, and by centrifugation (SPIN). PCA, however, appeared to be the most efficient of the three treatments. The composition of the supernatant fractions indicated that a considerable amount of intact protein remained in solution following precipitation, which agrees with the findings of Moughan *et al.* (1990) for the PCA and TCA treatment of ileal digesta from rats fed a protein-free diet.

Munro and Fleck (1969) noted that in spite of the widespread use of TCA and PCA for precipitation of tissue proteins there is little information on the conditions required for efficient and safe precipitation. Bhatty (1972) found that the complete precipitation of rapeseed meal proteins required a six fold increase in TCA concentrations over that required to precipitate casein and haemoglobin. Jansen *et al.* (1952) reported that the human mucoprotein plasma cholinesterase was soluble in 10% TCA at room temperature and Anderson and Maclagan (1955) found that urine mucoproteins were soluble in 20% TCA. Further, Mandel and Ellison (1963) showed that a considerable portion of the protein in human parotid and submaxillary saliva was TCA-soluble mucoprotein. Bell (1963) compared 20 methods of protein removal from biological materials and found that the results obtained by different methods on the same solution and the same method on different solutions were often quite dissimilar.

It is of note that in the present study threonine, serine, glutamic acid, proline, alanine, valine, isoleucine and leucine were found in relatively high concentrations in the supernatants following treatment of digesta with TCA, PCA and SPIN. Buraczewska (1979) found high proportions of lysine and threonine in the nitrogenous secretions of the pig intestine. Moughan *et al.* (1990) also noted high proportions of aspartic acid, threonine, serine, glycine, alanine, valine and isoleucine

in the supernatants of ileal digesta of rats fed a protein-free diet. Serine, threonine, glycine and proline are predominant components of rat small intestinal mucin (Bella and Kim 1972), bovine submaxillary mucin (Hashimoto *et al.* 1963), bovine duodenal mucosa glycoproteins (Cetta *et al.* 1972), and rabbit intestinal mucosa glycoproteins (Nemoto and Yosizawa 1969).

The data obtained in the present study from ultrafiltration and gel chromatography provide estimates of the proportions of protein (M.W.>10,000), large peptides (700<M.W.<10,000), and small peptides plus free amino acids plus non-protein nitrogen containing compounds (M.W.<700) in the supernatant. All the treatments resulted in a similar proportion (30-37% of total digesta nitrogen) of soluble protein. Higher proportions of the digesta total nitrogen appeared to be present as large peptides or small peptides + free amino acids, for the TCA and SPIN treatments in comparison with the PCA treatment. This implies that there is some breakdown of proteins and peptides either by chemical hydrolysis or residual protease activity occurring during the TCA and SPIN treatments. Bell (1963) found an increase in diffusible nitrogen present in solution after precipitation with TCA and other acid precipitants which she suggested was the result of hydrolysis of large nitrogen-containing molecules. From the data presented it seems that around 20% of the total digesta amino acids were present either as free amino acids or peptides with molecular weights less than 10,000 Daltons. These amino acids may be of unabsorbed dietary origin or of endogenous origin. It is also possible that they have originated during processing from intact endogenous protein. Experimental conditions which would eliminate possible chemical and enzyme hydrolysis are required to accurately determine the endogenous levels of free amino acids and small peptides in terminal ileal digesta.

In this study, PCA appeared to be the most efficient protein precipitant under the conditions adopted. However, discrepancies between the proportions of nitrogen and amino acids in the supernatants and precipitates caused some concern. The PCA treatment was the most complicated and laborious treatment. The need to neutralise any unreacted perchloric acid in the digesta precipitate and supernatant resulted in dilution of the supernatant and probably added extra salts which appear to have reduced the sensitivity of the nitrogen and amino acid analyses. The SPIN treatment required no addition of chemicals, or time for a reaction to occur, and provided less variable data.

2.5.3 Endogenous Amino Acid Flow

The endogenous ileal amino acid flows obtained for rats fed a diet containing enzymically hydrolysed casein as the sole amino acid source and after the centrifugation plus ultrafiltration treatment were significantly ($P<0.05$) lower for

lysine, glutamic acid and proline than the corresponding flows based on the total digesta. Flows were generally lower but not significantly ($P<0.05$) so for all the other amino acids except threonine. The endogenous flows for the total digesta were slightly lower than previously reported values for rats fed a similar diet but with no treatment of the digesta (Darragh *et al.* 1990; Moughan and Rutherfurd 1990). The estimation of endogenous amino acid flow based on the total ileal digesta may overestimate the amino acid flow because of the presence of unabsorbed dietary free amino acids and small peptides. Similarly, the estimates of endogenous flow following precipitation and ultrafiltration may be an underestimate because there may be endogenous free amino acids and small peptides present in the digesta which are discarded in the low molecular weight ultrafiltration fraction. Further research is required to quantitate these base levels of endogenous free amino acids plus small peptides at the terminal ileum of the rat. It appears from the present study that the levels can be no greater than 20% of the total amino acid flow.

The endogenous ileal amino acids flows for rats given the protein-free diet (Table 2.4) agreed very closely with other values reported in the literature (Skilton *et al.* 1988; Darragh *et al.* 1990; Moughan and Rutherfurd 1990). The protein-free flows were generally significantly ($P<0.05$) lower compared to those for the EHC (centrifugation + ultrafiltration) rats. The greatest differences between the flows occurred for isoleucine, serine, glutamic acid, valine, leucine, alanine and threonine. Where statistically significant differences were detected, the protein-free values were lower by an overall average of 45% units (differences expressed as a proportion of EHC mean value). The estimated differences may be conservative, however, depending upon the actual levels of endogenous free amino acids plus small peptides present at the terminal ileum.

The finding that the EHC (centrifugation + ultrafiltration) amino acid flows were significantly higher than the corresponding protein-free values, is further evidence that dietary peptides in the gut lumen affect the endogenous loss of amino acids at the terminal ileum of the rat. The result is in agreement with the recent findings of Moughan and Rutherfurd (1990) who reported that the endogenous ileal lysine flow for rats fed a guanidinated gelatin based diet was similar to that for rats receiving an iso-fibrous EHC diet and significantly greater than for protein-free fed rats. Skilton *et al.* (1988) and Darragh *et al.* (1990) did not find an increase in endogenous ileal amino acid loss above that determined under protein-free alimentation, when rats were given a synthetic amino acid-based diet devoid of several non-essential amino acids. This further suggests that the effect on endogenous amino acid loss found here and in the studies by Darragh *et al.* (1990) and Moughan and Rutherfurd (1990) is mediated by dietary peptides.

In conclusion, the present results indicate that the recently available Centriprep ultrafiltration devices are accurate and reliable. Further evidence was obtained regarding the inefficiency of PCA and TCA as protein precipitants for ileal digesta. The most rapid, reliable and least laborious pre-treatment of the ileal digesta prior to ultrafiltration was found to be centrifugation. The refined method for determining endogenous amino acid loss applied here, provides further evidence that the traditional protein-free method leads to considerable underestimation of the endogenous flow of amino acids at the terminal ileum of the growing rat.

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Chapter 3

Protein-, Peptide- and Free Amino Acid-Nitrogen in Endogenous Digesta Nitrogen at the Terminal Ileum of the Rat

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3.1 ABSTRACT

The proportions of endogenous protein-, peptide- and free amino acid-nitrogen (N) in digesta N from the distal ileum of the rat were determined immediately after collection or following storage frozen (-20°C and -196°C). Eighteen growing rats were given a protein-free diet for 6 days, euthanased and samples of digesta were collected from the terminal 20 cm of ileum. The digesta samples were centrifuged and the supernate ultrafiltered. Total nitrogen and total amino acids were determined in the precipitate plus retentate (MW>10,000 Da) and ultrafiltrate (MW<10,000 Da) fractions, and urea, creatinine, ammonium and free amino acids in the ultrafiltrate. The storage of digesta did not significantly affect the proportions of N-containing substances in the precipitate plus retentate or ultrafiltrate excepting that ammonium levels in the latter fraction were significantly lower for digesta processed immediately. On average, 67% of the total digesta N was in the precipitate plus retentate fraction and 61% of the total N was protein N. Essential and non-essential amino acid N comprised 26 and 36%, respectively, of total digesta N in the precipitate plus retentate. On average, the ultrafiltrate fraction contained 33% of the total digesta N, and the total digesta N contained 10.4, 10.6, 1.8, 1.5 and 0.5% free amino acid N, peptide N, urea N, ammonium N and creatinine N, respectively. Non-amino N (urea N + ammonium N + creatinine N) contributed 4% of the total N in the whole digesta. The N from essential and non-essential amino acids in the ultrafiltrate (free + peptide), expressed as percentages of the total N in the whole digesta, were 8 and 13%, respectively. On average, 21% of the total digesta N was present as free amino acid-N and peptide-N. These findings give support to the application of a proposed method for the determination of total endogenous ileal amino acid excretion under conditions of peptide alimentation.

3.2 INTRODUCTION

A considerable quantity of endogenous nitrogen enters the mammalian gut (Fauconneau and Michel 1970; Snook 1973; Buraczewska 1979; Low 1982). This nitrogen derives mainly from enzymes, mucoproteins, desquamated cells, serum albumin, peptides, free amino acids, amines and urea (Fauconneau and Michel 1970). Other non-dietary sources of nitrogen are microorganisms and ingested body hair. These latter sources are usually included in the endogenous nitrogen measured at the distal ileum, even though they are not strictly endogenous. Endogenous

compounds are continuously secreted, digested and absorbed during the passage of digesta through the digestive tract (Snook and Meyer 1964, Buraczewski 1980) and endogenous nitrogen measured at the terminal ileum is the net result of these processes. The gross amino acid composition of endogenous material leaving the small intestine is known, but the molecular form in which the endogenous amino acids are excreted is not well characterised. Information on the form of the endogenous nitrogen excretion, apart from being of basic interest, is important to the evaluation of a recently developed method (Moughan *et al.* 1990, Butts *et al.* 1991) for the determination of total endogenous ileal amino acid excretion under conditions of peptide alimentation.

In the latter method for determining endogenous loss, the animal is fed a semi-synthetic diet containing enzymically hydrolysed casein (free amino acids and peptides; MW<10,000 Daltons (Da)) as the sole nitrogen source, and ileal digesta are collected from the terminal ileum of the animal following slaughter. The digesta are separated by centrifugation, and the resulting supernate is ultrafiltered using a molecular weight exclusion limit of 10,000 Da. The resulting high molecular weight fraction (MW>10,000 Da) following ultrafiltration is added to the digesta precipitate, and the low molecular weight fraction (MW<10,000 Da) is discarded. Consequently, any unabsorbed dietary amino acids and small peptides will be removed in the low molecular weight fraction (MW<10,000 Da). The latter, however, may also contain endogenous free amino acids and small peptides, which could lead to the underestimation of the actual endogenous loss of amino acids.

Findings to date on the molecular composition of ileal amino acid excretion are discrepant. Buraczewska *et al.* (1975) reported 60% of the total nitrogen in the non-protein fraction of ileal digesta for pigs fitted with re-entrant cannulas and given a protein-free diet. In perfused isolated loops of ileum from pigs previously fed a protein-free diet, Buraczewska (1979) determined that around 30% of the total effluent nitrogen comprised free amino acid plus peptide nitrogen. Asche *et al.* (1989) reported that approximately 50% of the total digesta nitrogen in the ileum of protein-free fed pigs was in the form of free amino acids and peptides. For the growing rat, Moughan *et al.* (1990) estimated that less than 16% of the total digesta nitrogen from the terminal ileum was free amino acid plus peptide nitrogen. All the above studies may be criticised, however, in that no precautions were taken to avoid auto-digestion in the samples after collection and during processing. Also, Asche *et al.* (1989) and Moughan *et al.* (1990) used distilled water rather than physiological saline during the collection and processing of the digesta which may cause intestinal cells to lyse and release their cell contents into the digesta. There is also concern

that storing digesta frozen may affect the composition of its nitrogenous component, either by lysing the desquamated cells in the digesta during freezing, or through proteolytic activity of residual digestive and microbial enzymes in the digesta during freezing and thawing. Freezing digesta to -20°C provides more time during freezing and thawing for residual proteolytic breakdown to occur as well as leading to possible lysis of cells, while rapid freezing to -196°C may also cause lysis of cells but considerably reduces the residual enzymic breakdown during freezing but not that during thawing. The immediate processing of digesta is not affected by the freezing process, and also minimises the effects of residual proteolytic enzyme activity.

The objectives of the present study were firstly to determine the composition of endogenous nitrogen-containing material in rat ileal digesta, and secondly to examine the effect on compositions of storing digesta frozen to either -20°C or more rapidly to -196°C.

3.3 EXPERIMENTAL

Eighteen 120 g Sprague-Dawley male rats were selected at random from a group of rats, which had been weaned at 4 weeks of age and reared on a high quality diet at the Small Animal Production Unit, Massey University. The animals were kept individually in raised stainless steel cages with wire mesh floors at 20±2°C, and with a 12 h light/dark cycle. The rats were fed a casein-based (10% crude protein) diet for a 10 day preliminary period followed by a protein-free diet (Table 3.1) for 6 days. The diets were offered *ad libitum* for a 3 h period (0830-1130 h) each day, and water was available at all times. Chromic oxide was included in the protein-free diet as an indigestible marker.

On the 16th day the rats were equally and randomly assigned to three digesta processing treatments, and were then asphyxiated with carbon dioxide gas and decapitated 3 h ± 15 minutes after the start of feeding. The terminal 20 cm of ileum (immediately anterior to the ileo-caecal junction) was immediately dissected from the body, the outer intestinal surface cleaned using absorbent tissue paper and the contents slowly flushed out with 10 ml of physiological saline (0.9% NaCl; w:v). The digesta samples were kept separate and processed according to treatment. For Treatment I, the digesta were frozen immediately in a standard commercial freezer to -20°C and stored for 7 days; for Treatment II, the digesta were frozen immediately in liquid air at -196°C and stored for 2 days; and for Treatment III, the

Table 3.1 Ingredient composition (g kg^{-1} air dry weight) of a protein-free diet fed to growing rats

Ingredient	Composition
Wheaten cornflour	729
Purified cellulose	50
Maize oil	65
Sucrose	100
Vitamin/Mineral Premix[1]	50
Chromic oxide (Cr_2O_3)	6

[1] Supplied (mg kg^{-1} diet): vitamin E 750, vitamin K 3, riboflavin 8, thiamin 9, pyridoxine 8, pantothenic acid 38, nicotinic acid 38, folic acid 2, Fe 105, Zn 75, Mn 75, Cu 9, Co 1; (µg kg^{-1} diet): vitamin A 648, vitamin D_3 113, vitamin B_{12} 30, I 750, Se 230; (g kg^{-1} diet): choline 1.5, Na 2.0, K 2.9, Cl 4.9, Mg 0.7, S 0.5, Ca 9.3, P 4.8.

digesta were processed immediately.

The samples from rats assigned to Treatments I and II were rapidly thawed to 4°C prior to laboratory processing. For all the treatments, the digesta samples were centrifuged at 1450 xg for 45 minutes at 0°C, the supernate was decanted and retained, and the precipitate washed with 5 ml of physiological saline. The digesta were centrifuged again for 30 minutes at 1450 xg at 0°C. The second supernate was added to the first and the total weight recorded. The precipitate was also weighed and then immediately frozen (-20°C).

The supernate was immediately ultrafiltered using Centriprep-10 Concentrators (Amicon, W.R. Grace and Co. Ltd, Danvers, Massachusetts) according to the manufacturer's instructions (Amicon 1987). The concentrators have been shown to provide effective separation of nitrogen-containing compounds based on molecular weight (Butts *et al.* 1991). The ultrafiltrate (low molecular weight fraction, MW<10,000 Da) was weighed and stored frozen (-20°C) and subsequently analysed for total nitrogen, free amino acids, total amino acids, urea, creatinine and ammonium. The retentate (high molecular weight fraction, MW>10,000 Da) was added to the frozen precipitate, and the material freeze-dried,

weighed, finely ground and stored at -20°C for subsequent analysis of nitrogen, chromium and total amino acids. The diet was analysed for chromium and nitrogen.

Total nitrogen (N) was determined using a micro-Kjeldahl method. The material was digested with concentrated sulphuric acid and potassium sulphate, and the released ammonium was determined using the Berthelot reaction (Chaney and Marbach 1962). Free ammonium was determined in the undigested ultrafiltrates by the same reaction. Urea was determined using the diacetyl monoxime reaction (Marsh *et al.* 1965), and measured on a Technicon auto-analyser (Technicon Instruments Ltd, Tarrytown, U.S.A.). Creatinine was determined by the Jaffé method using a Cobas Fara II auto analyser (Roche Products Ltd, Basle, Switzerland). The amino acid composition of the dried material was determined in 5 mg samples by ion exchange chromatography using a Beckman 119 BL amino acid analyser. The samples were hydrolysed in 500 µl 6 M HCl with 1% added phenol, for 24 h at 110±2°C in glass tubes sealed under vacuum. Cystine, methionine and tryptophan, being partly destroyed under acid hydrolysis, were not determined. The total amino acid compositions of the ultrafiltrates were determined on 500 µl of sample which was freeze-dried in the tube prior to hydrolysis in 500 µl of 6 M HCl with 1% added phenol, and determined as above. The free amino acid contents of the ultrafiltrates were determined on 500 µl of unhydrolysed sample. Chromium analysis was carried out on 8-10 mg samples of diet and ileal digesta precipitate plus retentate according to the method of Costigan and Ellis (1987) using an Instrumentation Laboratory Absorption Spectrophotometer. All chemical analyses were determined in duplicate.

The total amounts of each N-containing substance determined were converted to amounts of N based on the percentage (by weight) of N present in the compound. Endogenous flows of total amino acids and N (precipitate + retentate + supernate) at the terminal ileum relating to the ingestion of 1 g of freeze dry matter (FDM) were calculated using the following equation:

$$\text{amino acid flow } (\mu g\ g^{-1}\ FDM) = \text{amino acid concentration in whole ileal digesta } (\mu g\ g^{-1}\ FDM) \times \frac{\text{diet chromium } (mg\ g^{-1}\ FDM)}{\text{ileal chromium } (mg\ g^{-1}\ FDM)}$$

The data were tested for homogeneity of variance across treatments using Bartlett's test, and were subjected to a one-way analysis of variance. Orthogonal contrasts were used to determine the significance of the differences between treatment means (Sokal and Rohlf 1981).

3.4 RESULTS

The rats consumed the protein-free diet readily and remained healthy throughout the study. Food intakes on the last day of the study were 9±0.3 g (mean±SE). Faeces were not detected in the gastric contents at slaughter, indicating that coprophagy had not occurred. The variances for all data were found to be homogenous.

The mean amounts of the respective N-containing compounds in the precipitate plus retentate and ultrafiltrate fractions expressed as proportions of N in the whole ileal digesta are given in Table 3.2. The proportions of total N found in the precipitate plus retentate (MW>10,000 Da) were not significantly different across treatments. Also, there were no significant differences in the proportions of N-containing compounds in the ultrafiltrate (MW<10,000 Da) across treatments except for ammonium ($P<0.05$) in the digesta of rats following immediate processing (Treatment III). Overall, the precipitate plus retentate contained 67% of the total digesta N, the remainder (33%) being in the ultrafiltrate. The ultrafiltrate N contained mainly peptides and free amino acids (10.6 and 10.4% of total digesta N, respectively) with minor contributions from urea (1.8%), ammonium (1.5%), and creatinine (0.5%). The mean levels of undetermined N in the precipitate plus retentate and ultrafiltrate were 5.8 and 8.0% of total ileal digesta N, respectively.

The mean amounts of precipitate plus retentate and ultrafiltrate total amino acid N for individual amino acids expressed as proportions of whole ileal digesta N are given in Table 3.3. There were no significant differences in the amounts of amino acid N in the precipitate plus retentate of rat digesta stored under different conditions. Therefore, the data were pooled across the three treatments. The nitrogen from glycine (8.0% of total digesta N), arginine (6.9%) and aspartic acid (6.7%) made the greatest individual contributions to the precipitate plus retentate N for all treatments. Further, there were no significant differences in the amounts of total amino acid N in the ultrafiltrates of rat digesta stored under different conditions. Therefore, the data were pooled across the three treatments. The nitrogen from glycine (6.9% of total digesta N) made the greatest individual contribution to the ultrafiltrate N for all the treatments. The ultrafiltrate contained 7.9% of total digesta N as essential amino acid N and 13.1% as non-essential amino acid N.

Table 3.2 Mean proportions[1] of nitrogen-containing compounds in the precipitate[2] and ultrafiltrate of ileal digesta for rats given a protein-free diet and after different treatments[3] of the digesta

	Digesta Treatment					
Source of N	**I**	**II**	**III**	**Overall SE**	**Level of Significance**	**Overall Mean**
Precipitate						
Total	65.3	67.4	68.9	1.87	NS	67.2
Protein[4]	58.4	61.1	64.8	2.11	NS	61.4
Undetermined[5]	6.9	6.3	4.1	0.78	-	5.8
Ultrafiltrate						
Total	34.7	32.6	31.1	1.86	NS	32.8
Free Amino Acid	9.7	9.7	11.8	0.74	NS	10.4
Peptide[6]	12.0	11.0	8.9	0.91	NS	10.6
Urea	1.9	1.9	1.7	0.22	NS	1.8
Ammonium	2.3^{a}	1.7^{a}	0.3^{b}	0.23	*	1.5
Creatinine	0.5	0.5	0.4	0.14	NS	0.5
Undetermined[5]	8.3	7.8	8.0	1.21	-	8.0

1 Proportion = amount of N from the N-containing compound expressed as a percentage of the total N in the whole ileal digesta; n=6 for all treatments; Means within rows with different superscripts were significantly different; NS not significant; * $P<0.05$; Mean ileal total nitrogen for Treatment I-III, respectively were 756, 699, 715 mg/100 g ileal freeze dry matter and 33,32, 25 mg/$kg^{0.75}$/day.

2 Precipitate fraction = precipitate plus retentate (MW>10,000 Da).

3 Treatment I: digesta stored at -20°C; Treatment II: digesta stored at -196°C; Treatment III: digesta processed immediately after collection.

4 Sum of amino acid nitrogen.

5 Undetermined N = residual N (found by difference).

6 Peptide N = Total amino acid N - free amino acid N (total amino acids were determined following acid hydrolysis).

Table 3.3 Mean proportions[1] of total amino acid nitrogen in the precipitate plus retentate (MW>10,000 Da) and the ultrafiltrate (MW<10,000 Da) of ileal digesta for rats given a protein-free diet[2]

	Precipitate plus Retentate		Ultrafiltrate	
Amino Acid	**Mean**	**SE**	**Mean**	**SE**
Lysine	3.8	0.08	1.5	0.43
Histidine	3.6	0.09	2.0	0.20
Arginine	6.9	0.16	1.7	0.25
Aspartic acid	6.7	0.08	1.9	0.19
Threonine	4.6	0.06	0.7	0.07
Serine	4.3	0.06	1.0	0.11
Glutamic acid	5.5	0.07	1.0	0.20
Proline	5.4	0.07	1.0	0.10
Glycine	8.0	0.15	6.9	0.82
Alanine	3.2	0.05	1.0	0.13
Valine	2.8	0.03	0.7	0.09
Isoleucine	1.5	0.03	0.3	0.04
Leucine	2.7	0.05	0.7	0.06
Tyrosine	1.2	0.01	0.3	0.03
Phenylalanine	1.2	0.02	0.3	0.03

[1] Proportion = amount of N from the N-containing compound expressed as a percentage of the total N in the whole ileal digesta.

[2] There was no significant effect (P>0.05) of treatment, therefore the data were pooled over treatments; n=18; Treatment I: digesta stored at -20°C; Treatment II: digesta stored at -196°C; Treatment III: digesta processed immediately following collection.

Table 3.4 gives the amounts of free amino acid N and peptide N present in the ultrafiltrate, expressed as proportions of N in the whole ileal digesta. Generally, there was no effect of treatment and the data were pooled across treatments. Arginine (1.6% of total digesta N) and glycine (1.4%) made the major contributions to free amino acid N in the ultrafiltrates. Glycine was the major contributor to peptide N in the digesta ultrafiltrates. The essential amino acids expressed as percentages of total digesta N were the larger proportion (5.4%) of free amino acid N, but the lower proportion (2.5%) of peptide N in digesta ultrafiltrates.

The mean amounts of N from individual amino acids in the free amino acid

Table 3.4 Mean proportions[1] of free amino acid nitrogen and peptide nitrogen in the ultrafiltrate (MW<10,000 Da) of ileal digesta for rats given a protein-free diet[2]

	Free Amino Acid N		Peptide N	
Amino Acid	**Mean**	**SE**	**Mean**	**SE**
Lysine	1.0	0.14	0.5	0.08
Histidine	0.7	0.07	1.3	0.15
Arginine	1.6	0.18	0.1	0.10
Aspartic Acid	0.4	0.09	1.5	0.12
Threonine	0.6	0.08	0.1	0.08
Serine	0.9	0.09	0.1	0.02
Glutamic Acid	0.8	0.10	0.2	0.09
Proline	0.5	0.07	0.5	0.02
Glycine	1.4	0.33	5.5	0.61
Alanine	0.8	0.08	0.2	0.03
Valine	0.5	0.06	0.2	0.01
Isoleucine	0.2	0.04	0.1	0.01
Leucine	0.6	0.08	0.1	0.02
Tyrosine	0.2	0.03	0.1	0.01
Phenylalanine	0.2	0.03	0.1	0.01

[1] Proportion = amount of N from the N-containing compound expressed as a percentage of the total N in whole ileal digesta.

[2] There was no significant effect (P>0.05) of treatment, therefore the data were pooled over treatments; n=18; Treatment I: digesta stored at -20°C; Treatment II: digesta stored at -196°C; Treatment III: digesta processed immediately following collection.

and peptide fractions of the ultrafiltrate (MW<10,000 Da) expressed as percentages of the total amount of nitrogen for the respective amino acids in the whole ileal digesta are given in Table 3.5. The highest proportion of amino acid N found in the ultrafiltrate was for glycine (46% of total glycine N in digesta), most of which was present in the form of peptides (37% of total digesta glycine N). Histidine, lysine and alanine were also present in relatively high proportions in the ultrafiltrate at 36, 28 and 24% of total individual amino acid N in whole ileal digesta. In general,

peptide N made up a small proportion (<10 % of total individual amino acid N in digesta) of each amino acid's total digesta N except for glycine (37%), histidine (23%) and aspartic acid (17%). Free amino acid N contributed from 5% of total individual amino acid N in the digesta for aspartic acid to 19% for lysine, arginine and alanine.

There were no significant storage treatment differences in the flows of endogenous amino acids or nitrogen in the rat whole ileal digesta, therefore, the data were pooled. Overall mean endogenous amino acid and nitrogen flows are presented in Table 3.6.

3.5 DISCUSSION

The present results indicate that the conditions used for the storage of digesta had no significant effect on total protein, peptide or free amino acid N levels in the precipitate plus retentate (MW>10,000 Da) or ultrafiltrate (MW<10,000 Da) fractions of ileal digesta collected from rats given a protein-free diet. The processing of digesta immediately following collection (Treatment III) resulted in a significantly lower amount of ammonium N present in the ultrafiltrate, which may indicate a lower degree of microbial activity. Ammonium, however, was present in only small quantities (1.5% of total digesta N) in the ultrafiltrate and makes only a minor contribution to overall digesta N. The undetermined nitrogen component of the digesta was a relatively small proportion of total N in both the precipitate plus retentate and ultrafiltrate (6 and 8%, respectively), and may comprise undetermined amino acids, amines, amides and nucleic acids.

The endogenous ileal amino acid flows found here were similar to those determined in other studies using growing rats given a protein-free diet (Skilton *et al.* 1988, Darragh *et al.* 1990, Moughan and Rutherfurd 1990, Butts *et al.* 1991). The high levels of glycine, proline, threonine, arginine, glutamic and aspartic acids found in ileal endogenous dry matter in the present study have also been found by other workers with both pigs (Taverner *et al.* 1981, De Lange *et al.* 1989a and b, Furuya and Kaji 1989, Moughan and Schuttert 1991) and rats (Skilton *et al.* 1988, Darragh *et al.* 1990, Moughan and Rutherfurd 1990, Moughan *et al.* 1990, Butts *et al.* 1991) given protein-free diets. In the present study, glycine was also the major contributor to peptide N. Glycine, proline, serine, threonine, glutamic and aspartic acids are the predominant components of intestinal and salivary mucus (Hashimoto *et al.* 1963, Nemoto and Yosizawa 1969, Bella and Kim 1972, Cetta *et al.* 1972,

Table 3.5 Mean (SE) amino acid nitrogen in the free amino acid and peptide fractions of the ultrafiltrate (MW<10,000 Da) expressed as percentages of the total amount of nitrogen for the respective amino acid in whole ileal digesta of rats given a protein-free diet

Amino Acid	Free Amino Acid N[1]	Peptide N[2]	Total Amino Acid N in Ultrafiltrate[3]
Lysine	19 (1.2)	9 (0.9)	28 (1.2)
Histidine	13 (1.0)	23 (1.4)	36 (1.4)
Arginine	19 (1.1)	1 (0.2)	20 (1.1)
Aspartic Acid	5 (0.6)	17 (1.2)	22 (1.3)
Threonine	11 (0.9)	2 (0.4)	13 (1.0)
Serine	17 (1.2)	2 (0.3)	19 (0.9)
Glutamic Acid	12 (1.0)	3 (0.4)	15 (1.0)
Proline	8 (1.0)	8 (0.7)	16 (1.1)
Glycine	9 (0.9)	37 (1.3)	46 (1.6)
Alanine	19 (1.3)	5 (0.6)	24 (1.1)
Valine	14 (1.1)	6 (0.5)	20 (1.0)
Isoleucine	11 (1.0)	6 (0.7)	17 (0.9)
Leucine	18 (1.4)	3 (0.2)	21 (1.2)
Tyrosine	13 (0.9)	7 (0.4)	20 (0.9)
Phenylalanine	13 (0.8)	7 (0.4)	20 (0.9)

1 Free amino acid N in the ultrafiltrate expressed as a percentage of the total amount of amino N for the respective amino acid in the whole ileal digesta.

2 Peptide amino acid N in the ultrafiltrate expressed as a percentage of the total amount of amino N for the respective amino acid in the whole ileal digesta.

3 Amino acid N in the ultrafiltrate expressed as a percentage of the total amount of amino N for the respective amino acid in the whole ileal digesta.

Table 3.6 Overall mean endogenous amino acid flows[1] in the whole ileal digesta[2] for rats given a protein-free diet

Amino Acid	Mean (SE)
Lysine	196 (4.6)
Histidine	136 (2.9)
Arginine	214 (3.4)
Aspartic acid	641 (11.1)
Threonine	388 (5.6)
Serine	314 (4.6)
Glutamic acid	586 (10.9)
Proline	446 (5.4)
Glycine	427 (5.1)
Alanine	204 (4.1)
Valine	230 (3.5)
Isoleucine	145 (2.9)
Leucine	252 (5.1)
Tyrosine	150 (1.8)
Phenylalanine	142 (2.5)
Nitrogen	913 (48.9)

[1] $\mu g\ g^{-1}$ freeze dry matter intake; Data pooled over treatments; n=18.
[2] Precipitate plus Retentate plus Ultrafiltrate.

Allen 1981), and it has been shown that mucus glycoproteins are particularly resistant to acid and enzymic digestion (Hashimoto *et al.* 1963, Hoskins 1978), which could explain their predominance in ileal digesta.

The present results confirm earlier findings of Moughan *et al.* (1990) that the major components of endogenous nitrogen excretion at the terminal ileum of the growing rat are amino acids, and that non-amino nitrogen-containing compounds make up only a small proportion of digesta total nitrogen. The amino acids are found mainly as protein with free and peptide bound amino acids making up only a small proportion of total endogenous nitrogen excretion at the distal ileum. This supports the suggestion by Snook (1973) that the major sources of endogenous nitrogen include mucins, plasma and cellular proteins. In the present study it was

found that 21% of the total digesta N was present in the form of free amino acids plus peptides. A recent experiment at this institute, similar to the presently reported work but with the growing pig (Moughan and Schuttert 1991), reported a somewhat lower value (11% of total digesta N) for free amino acid plus peptide N.

The low overall level of amino N in the supernate (21% of total digesta N) is important in the evaluation of a recently developed method (Moughan *et al.* 1990; Butts *et al.* 1991) involving an ultrafiltration step for the determination of endogenous ileal amino acid loss under conditions of peptide (enzymically hydrolysed casein) alimentation. In using this approach, there may be a loss of endogenous free amino acids and small peptides when the ultrafiltrate fraction of ileal digesta is discarded, which would lead to an underestimation of the actual total endogenous amino acid excretion. Of significance, therefore, are the present findings and those of Moughan *et al.* (1990) which indicate that the consequent underestimation of the flows of most of the dietary essential amino acids would be low.

In the present study, 46% of the total glycine, 36% of the total histidine and 28% of the total lysine in ileal digesta were in the ultrafiltrate (MW<10,000 Da) with other amino acids making up 13-24% of each amino acid total in ileal digesta. Lysine was present in the ultrafiltrate predominantly in the free form while histidine and glycine were present mainly in the peptide form. This would imply that the endogenous flows for these amino acids determined in the growing rat using the newly proposed method would be underestimated to a greater extent. In a previous study (Butts *et al.* 1991) which compared the endogenous ileal amino acid flows for the growing rat following protein-free and peptide (enzymically hydrolysed casein) alimentation, lysine and histidine flows were higher (significantly so for lysine) under peptide alimentation and following ultrafiltration of digesta, than after protein-free feeding. Based on the present results, it would appear that the actual flows of lysine and histidine for the hydrolysed casein fed rats would be higher still, which provides further support that protein-free alimentation leads to lower estimates of endogenous amino acid loss. The lysine, histidine and glycine endogenous ileal flows determined after feeding the animals hydrolysed casein followed by ultrafiltration of the digesta can be corrected using the following estimates of ileal amino acid flow (calculated from the present results) 55, 49 and 196 $\mu g\ g^{-1}$ freeze dry matter intake, respectively.

In the present study, it was necessary to feed the animals a protein-free diet to determine the base levels of free amino acid plus peptide N in ileal digesta. It may be that when dietary protein is consumed, the composition as well as the level of

endogenous excretion may be altered. The results of the present study must be interpreted in light of this reservation, and emphasise a need to determine the influence of protein-feeding on the composition of endogenous N loss.

It is concluded that the methods of digesta storage used here had no significant effect on the amounts of nitrogen-containing compounds determined in ileal digesta precipitate plus retentate and ultrafiltrate fractions for rats fed a protein-free diet. Also, the endogenous nitrogen-containing material at the terminal ileum of rats fed a protein-free diet mainly comprised protein (MW>10,000 Da), with non-amino nitrogen, free amino acid nitrogen and peptide nitrogen constituting only small fractions of the total nitrogen excretion. The present findings give support to the practical application of a recently proposed method for the determination of total endogenous ileal amino acid excretion.

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Chapter 4

Composition of Endogenous Ileal Digesta Nitrogen for the Rat - The Use of Distilled Water for Digesta Collection

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4.1 ABSTRACT

The composition of endogenous nitrogen (N) in digesta from the distal ileum of the growing rat was determined following the collection of digesta using different flushing media. Twelve growing rats were given a protein-free diet for 6 days and samples of digesta were collected from the terminal 20 cm of ileum of the euthanased animal using either distilled water or physiological saline as the flushing medium. The digesta samples were centrifuged and the supernate ultrafiltered. Nitrogen and amino acid contents were determined in the precipitate plus retentate (M.W.>10,000 Da) and ultrafiltrate (M.W.<10,000 Da) fractions, as well as urea, creatinine, ammonium and free amino acids in the ultrafiltrates. There was no significant effect of collection method on the levels of N-containing substances in rat endogenous ileal digesta.

4.2 INTRODUCTION

In an earlier study to determine the molecular form of nitrogenous material in the terminal ileal digesta of the growing rat (Chapter 3), physiological saline was used as the flushing medium to remove digesta from the terminal small intestine of the euthanased animal. The use of saline, however, increases the mineral content of the dried digesta sample. An alternative flushing medium is distilled water, but use of the latter may cause intestinal cells to lyse releasing cytoplasmic contents into the digesta. This, in turn, may affect the molecular composition of the digesta nitrogenous material. The molecular form of nitrogenous material in the terminal ileal digesta is important to the application of a new method for the determination of total endogenous ileal amino acid excretion under conditions of peptide alimentation (Butts *et al.* 1991). The objective of the present study was to determine whether the replacement of physiological saline by distilled water in the collection of rat ileal digesta altered the molecular composition of the ileal digesta nitrogen-containing material.

4.3 EXPERIMENTAL

Twelve 120 g Sprague-Dawley male rats were given a protein-free diet for 6 days and digesta from the terminal 20 cm of ileum collected from the euthanased

animal. The diet, animals, housing and experimental procedure were as described in Chapter 3. At slaughter, the animals were randomly and equally allocated to two methods of digesta collection. Digesta were collected by flushing material from the terminal small intestine with either 10 ml distilled water or 10 ml of physiological saline (0.9% NaCl; w:v). After collection, the digesta samples were kept separate and immediately frozen to -20°C. An earlier study (Chapter 3) established that storage at this temperature did not influence the proportions of N-containing compounds in rat ileal digesta. Prior to laboratory analysis, the digesta samples were rapidly thawed to 4°C and then centrifuged at 1450 xg for 45 minutes at 0°C. The supernate was decanted and retained. The precipitate was washed with 5 ml of distilled water or physiological saline according to treatment. The digesta were centrifuged for a further 30 minutes at 1450 xg at 0°C. The combined supernates were subjected to ultrafiltration using Centriprep-10 concentrators (molecular weight (MW) exclusion limit 10,000 Daltons (Da); Amicon, W.R Grace and Co., Danvers, U.S.A.). This ultrafiltration process provides molecular separation of proteins (M.W.>10,000 Da) from peptides and free amino acids (M.W.<10,000 Da). The large molecular weight fraction (retentate; MW>10,000 Da) was added to the precipitate, and the total precipitate plus retentate weighed prior to freeze drying. The low molecular weight fraction (ultrafiltrate; MW<10,000 Da) was weighed and frozen for subsequent analysis. Total nitrogen (N) and amino acids were determined in the precipitate plus retentate and ultrafiltrate fractions. Free amino acids, ammonium, creatinine and urea were also determined in the ultrafiltrate fraction.

4.4 RESULTS

The mean amounts of N from the respective N-containing compounds in ileal digesta for the distilled water and physiological saline treatments are given in Table 4.1. There was no significant (P>0.05) effect of the flushing medium on the composition of the nitrogenous material in digesta. Protein was the major component of the digesta N. The mean endogenous nitrogen and amino acid flows in the whole rat digesta for the distilled water and physiological saline treatments are presented in Table 4.2. There were no significant differences (P>0.05) between treatments for the flows of individual amino acids or N at the terminal ileum of the growing rat.

Table 4.1 The effect of flushing medium for the collection of digesta on the mean[1] protein nitrogen (N), peptide N, free amino acid N, and non-amino acid-N in endogenous ileal digesta for the growing rat

	Flushing Medium[2]		
Source of N	**Physiological Saline**	**Distilled Water**	**Overall SE**
Protein[3]	442	446	11.2
Peptide[4]	91	94	9.6
Free Amino Acid	73	82	5.9
Ammonium	17	14	1.1
Creatinine	4	4	0.8
Urea	14	14	5.6
Undetermined[5]	115	91	4.2
Total	756	745	39.0

1 mg N/100 g ileal digesta freeze dry matter; n=6.
2 There was no significant (P>0.05) effect of flushing medium on digesta N composition.
3 Sum of amino acid N (amino acids were determined by acid hydrolysis).
4 Peptide N = Total amino acid N - free amino acid N.
5 Undetermined N = residual N found by difference.

4.5 DISCUSSION

The levels of N-containing substances in the precipitate plus retentate and ultrafiltrate fractions were similar to those found in an earlier study (Chapter 3). The ultrafiltrate N comprised mostly peptides and free amino acids, with minor contributions from urea, ammonium and creatinine. The endogenous ileal amino acid and nitrogen flows for the growing rat determined in the present study were lower than those reported by Taverner (1979) and Bolton and Miller (1985), but similar to those found by Skilton *et al.* (1988), Darragh *et al.* (1990), Moughan and Rutherfurd (1990), Butts *et al.* (1991).

It does not appear that there was a significant lysing of intestinal cells following collection of rat ileal digesta with distilled water. The lysis of intestinal cells would be expected to cause increased amounts of free amino acids and peptides in the ileal digesta. It is concluded that the use of distilled water as the flushing

medium had no significant effect on the relative amounts of nitrogen-containing compounds present in digesta for the growing rat. Physiological saline increases the ion content of the digesta sample which results in hygroscopic dried digesta samples and could potentially interfere with the binding of amino acids to the ion-exchange column during amino acid analysis (Ambler 1981). In this case, distilled water would be preferred for the collection of ileal digesta.

Table 4.2 Mean (n=6) endogenous amino acid flows[1] in the whole ileal digesta[2] for growing rats given a protein-free diet and after collection of the digesta with distilled water or physiological saline

	Flushing Medium[3]		
Amino Acid	**Physiological Saline**	**Distilled Water**	**Overall SE**
Lysine	197	209	5.7
Histidine	132	142	3.0
Arginine	220	222	4.5
Aspartic acid	660	674	12.1
Threonine	410	402	6.5
Serine	324	330	5.5
Glutamic acid	618	640	11.8
Proline	450	474	6.3
Glycine	431	433	6.0
Alanine	211	213	5.1
Valine	237	241	4.4
Isoleucine	148	154	3.8
Leucine	257	265	6.0
Tyrosine	157	151	2.7
Phenylalanine	143	149	2.6
Nitrogen	982	996	49.7

[1] amino acid flow ($\mu g\ g^{-1}$ FDMI) = amino acid concentration in whole ileal digesta ($\mu g\ g^{-1}$ FDM) $\times$ diet chromium (mg g^{-1} FDM) / ileal chromium (mg g^{-1} FDM)

[2] Precipitate plus Retentate plus Ultrafiltrate fractions.

[3] There was no significant effect ($P>0.05$) of flushing medium on endogenous ileal amino acid flows.

4.6 REFERENCES

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Chapter 5

Endogenous Lysine and Amino Acid Flows at the Terminal Ileum of the Growing Pig (20 kg bodyweight) - The Effect of Protein-Free, Synthetic Amino Acid, Peptide and Protein Alimentation

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5.1 ABSTRACT

The study aimed to determine the effects of state of body nitrogen balance and the presence of dietary peptides and protein in the digestive tract on the excretion of endogenous amino acids from the ileum of the pig. Endogenous lysine excretion was determined for pigs given a protein-free diet, an enzymically hydrolysed casein (EHC)-, a zein- or synthetic amino acid-based diet. Digesta from the EHC-fed animals were centrifuged and ultrafiltered after collection and the precipitate plus retentate fraction was used to determine the endogenous flows. Such processing excludes unabsorbed dietary amino acids from the measure of endogenous loss. Zein is naturally deficient in both lysine and tryptophan and these two amino acids were omitted from the synthetic amino acid-based diet to allow direct measurement of endogenous lysine flow. Pigs given the zein and synthetic amino acid diets received free lysine and tryptophan orally throughout the study but for the last 2 days of the study, when these amino acids were infused intravenously. Endogenous flows for amino acids other than lysine were determined for pigs on the protein-free and EHC diets. Six entire male pigs (15 kg liveweight) were allocated to each of the four diets and received the diet for 10 days. On the tenth day the pigs were given their daily dietary allowance hourly and euthanased ten hours after the start of feeding. Digesta were collected from the terminal 20 cm of ileum. The mean endogenous ileal lysine flows for the zein and EHC fed pigs were not significantly different (overall mean = 419 mg kg^{-1} dry matter intake) but were higher ($P<0.05$) than those for the protein-free and synthetic amino acid fed pigs (overall mean = 268 mg kg^{-1} dry matter intake) whose mean flows were not significantly different from each other. The mean endogenous ileal flows for amino acids other than lysine were higher ($P<0.05$) for the EHC fed pigs compared to the animals on the protein-free diet, except for proline, glycine and arginine. The similar endogenous ileal lysine excretion for pigs receiving a synthetic amino acid-based diet and in positive body nitrogen balance, and protein-free fed pigs in negative body nitrogen balance, indicates that negative body nitrogen balance *per se* does not lead to a lowered endogenous ileal excretion. The present results indicate that the presence of dietary peptides or protein in the gut increases amino acid excretion at the terminal ileum above that found with protein-free or synthetic amino acid alimentation. Endogenous ileal amino acid flow in the pig may be underestimated when determined by the traditional protein-free method.

5.2 INTRODUCTION

An understanding of the factors influencing endogenous amino acid flow at the terminal ileum of animals is important in the study of digestion and nutrition. Traditionally, the endogenous loss of nitrogen and amino acids from the small intestine has been determined after feeding a protein-free diet. This method has been criticised, however, for creating a physiologically abnormal state (Low 1980), which may lead to a decreased rate of whole body protein synthesis (Millward *et al.* 1976, Muramatsu 1990) and thus affect the amount of protein entering the gut.

Skilton *et al.* (1988) and Darragh *et al.* (1990) determined the endogenous excretions of dietary non-essential amino acids at the terminal ileum of rats fed a diet containing a mixture of synthetic amino acids as the sole nitrogen source, but devoid of the amino acid under consideration. The animals were supplied with a balanced array of dietary amino acids for protein metabolism, thus overcoming the problem of a possible decreased rate of whole body protein synthesis consequent upon feeding a protein-free diet. In both studies, the endogenous amino acid flows were not different to those for rats fed a protein-free diet. De Lange *et al.* (1989) reported lower endogenous ileal protein excretion for pigs given a protein-free diet but with parenteral administration of amino acids compared with pigs on a protein-free diet infused with saline. It does not appear, therefore, that negative body nitrogen retention associated with feeding animals a protein-free diet leads to a lowered ileal endogenous loss. It is possible, however, that dietary protein or the products of protein digestion have a direct effect on endogenous amino acid excretion. Recent studies with the growing rat (Darragh *et al.* 1990, Moughan and Rutherfurd 1990) have shown that endogenous amino acid loss from the small intestine is higher under peptide alimentation than under protein-free or synthetic amino acid alimentation. Also, Moughan and Rutherfurd (1990) reported that the endogenous flow of lysine at the terminal ileum of rats given a diet containing guanidinated protein was substantially higher than that for rats given a protein-free diet.

Darragh *et al.* (1990) determined endogenous ileal amino acid excretions after feeding rats an hydrolysed casein diet. This method relied on the assumption that the dietary amino acids were completely absorbed. Moughan *et al.* (1990) proposed a new method for routinely determining ileal amino acid excretion under peptide alimentation, that removed the need to make assumptions concerning the completeness of absorption of the dietary amino acids. In this method, the animal is fed a semi-synthetic diet containing enzymically hydrolysed casein (EHC; M.W.<5,000 Da) as the sole nitrogen source. Ileal digesta are collected from the

animal, and protein (M.W.>10,000 Da) immediately separated by centrifugation and ultrafiltration. Any dietary amino acids and small peptides not absorbed will be removed in the low molecular weight fraction (M.W.<10,000 Da). The low molecular weight fraction may also contain non-protein nitrogen and endogenous free amino acids and small peptides, which may lead to some underestimation of the actual endogenous loss of amino acids with this method, though it is considered (Moughan and Schuttert 1991, Butts *et al.* 1991) that free amino acid nitrogen plus peptide nitrogen is only a small fraction of the total endogenous ileal nitrogen (also refer Chapter 3). Butts *et al.* (1991) used the above described method with growing rats, and found significantly higher endogenous ileal amino acid flows in comparison with animals given a protein-free diet.

Another approach to the estimation of endogenous amino acid flow, which has not yet been used experimentally, is to feed an animal natural proteins devoid of specific amino acids, and to measure the flows of those amino acids at the ileum. There are, however, few naturally occurring proteins which are devoid of specific amino acids. One such protein is zein, a protein from maize (*Zea mays* L., Graminae), which contains very low levels of lysine and tryptophan. The levels of these amino acids in zein are reported to range from zero to 0.2% for lysine and zero to 0.1% for tryptophan (Block and Mitchell 1946, Block and Bolling 1947, Mossé *et al.* 1966, Paulis and Wall 1975, Budaveri 1989).

In the present study, all of the above discussed approaches to estimating endogenous lysine and other amino acid flows at the terminal ileum, some of which have previously been used only with rats, were applied to the growing pig. Endogenous amino acid losses were determined at the terminal ileum of the pig after feeding a protein-free diet or a synthetic amino acid-, an enzymically hydrolysed casein-, or a zein-based diet. The overall aims of the study were to examine the effect of state of body nitrogen balance (protein-free versus synthetic amino acid dietary treatments) and the presence of dietary peptides and protein in the digestive tract (hydrolysed casein and zein versus protein-free dietary treatments), on the endogenous loss of lysine and other amino acids from the ileum of the growing pig.

5.3 EXPERIMENTAL

5.3.1 Animals

Twenty-four Landrace x (Landrace x Large White) entire-male pigs of mean (±SE) bodyweight 13 (±1.2) kg were obtained from the Pig Research Unit, Massey University. The animals were kept at 24±2°C in individual metabolism crates,

which allowed complete and separate collection of urine and faeces. Twelve of the pigs underwent surgery for the implantation of indwelling jugular catheters to allow infusion of amino acid solutions. The pigs were sedated and placed in dorsal recumbency under halothane (Fluothane, Imperial Chemical Industries Ltd, England) anaesthesia administered via a face mask. A 3 cm incision was made in the ventral cervical region and the external jugular vein exteriorized via blunt dissection. A purse string suture was made into the vein around a 2-3 mm incision for the insertion of the catheter (Silastic; 1.5 mm internal diameter). The catheter was secured by tightening the purse string suture. The catheter was exteriorized by tunnelling dorsally through the subcutaneous fat to a stab wound between the scapulae. The catheter was then further secured by sutures to the skin and a band of medical adhesive tape wrapped around the catheter. The initial incision was closed off and the catheter was further secured with supportive netting. The pigs regained consciousness within 60 minutes following the completion of surgery and had regained normal appetite within 12 hours.

5.3.2 Diets

Four experimental diets, the ingredient compositions of which are given in Table 5.1, were prepared. The synthetic amino acid diet was formulated so the essential amino acids were present in the same proportions as those in zein. Chromic oxide was included in each diet as an indigestible marker. The zein and synthetic amino acid diets were virtually devoid of lysine and tryptophan. A small amount of lysine (0.17%) was found to be present in the zein, but it can be shown from first principles that such an amount would have only a minor effect on the determination of endogenous lysine flow. The determined levels of total nitrogen were 0.04, 1.43, 1.31, 1.55% for the essentially protein-free, synthetic amino acid, enzymically hydrolysed casein and zein diets, respectively.

5.3.3 Experimental Procedure

The experiment was conducted as a completely randomised design with 3 replicates each of 8 pigs. The pigs within each replicate were equally and randomly assigned to the four dietary treatments. The pigs were fed the diets for 9 days of a 10 day experimental period at 0.08 of metabolic bodyweight ($W^{0.75}$). They were fed the diets twice daily (0830 and 1630 h) in equal portions. The food was mixed with water (1:1; w:v) immediately prior to feeding and fresh water was freely available for 30 minutes after each meal. On the tenth day the level of feed intake was increased (0.10 $W^{0.75}$) to ensure collection of an adequate digesta sample. The feeding frequency was also increased on the tenth day to ensure a constant flow of digesta at the terminal ileum.

The pigs fed the zein and synthetic amino acid diets received daily dietary

Table 5.1 Ingredient composition (g kg^{-1} air dry weight) of the experimental diets

Ingredient	Diet			
	Protein-free	Enzymically Hydrolysed Casein	Synthetic Amino Acid[4]	Zein[4]
Wheaten cornflour	822.0	722.0	712.0	722.0
Maize oil	35.0	35.0	35.0	35.0
Purified cellulose	30.0	30.0	30.0	30.0
Sucrose	70.0	70.0	70.0	70.0
Vitamin/mineral mix[1]	38.0	38.0	38.0	38.0
Sodium bicarbonate	-	-	10.0	-
Chromic oxide	5.0	5.0	5.0	5.0
Enzymically hydrolysed casein[2]	-	100.0	-	-
Zein[3]	-	-	-	93.9
Synthetic Amino Acids				
Arginine	-	-	1.8	-
Histidine	-	-	1.3	-
Isoleucine	-	-	4.4	-
Leucine	-	-	7.5	-
Methionine	-	-	1.5	-
Cystine	-	-	2.2	-
Phenylalanine	-	-	7.5	-
Tyrosine	-	-	5.8	-
Threonine	-	-	2.8	-
Tryptophan	-	-	-	-
Valine	-	-	3.7	-
Lysine	-	-	-	-
Aspartic acid	-	-	5.2	-
Glutamic acid	-	-	31.2	-
Serine	-	-	1.0	-
Proline	-	-	10.6	-
Glycine	-	-	1.2	-
Alanine	-	-	6.2	-

[1] A mixture of Pfistart-10, Pfizer Laboratories Ltd, Auckland, New Zealand, dicalcium phosphate and salts supplied (mg kg^{-1} diet): vitamin E 24, riboflavin 3, vitamin K 2, niacin 18, calcium pantothenate 9, thiamine 1, pyridoxine 2, folic acid 1, betaine 24, Mn 24, Zn 24, Fe 66, Cu 60, Co 120, I 0.3, choline 30; (µg kg^{-1} diet): vitamin A 270, vitamin D_3 30, vitamin B_{12} 15, biotin 60, Se 180; (g kg^{-1} diet): Na 1.2, Cl 1.8, Mg 0.4, S 0.5, K 2.8, Ca 9.7, P 5.0.

[2] Prepared by New Zealand Dairy Research Institute, Palmerston North, New Zealand (M.W.<5,000 Da) as described by Darragh *et al.* (1990)

[3] Sigma Chemical Company, St. Louis, U.S.A.; 100% protein. Supplied (% of protein (99.9% dry matter): lysine 0.17, histidine 1.45, arginine 1.89, aspartic acid 5.57, threonine 2.97, serine 4.99, glutamic acid 29.76, proline 11.41, glycine 1.28, alanine 6.72, valine 4.03, isoleucine 4.77, leucine 8.03, tyrosine 6.29, phenylalanine 8.06.

[4] The diet was supplemented with lysine (5.35 g kg^{-1} of diet) and tryptophan (0.75 g kg^{-1} of diet).

supplements of lysine (5.36 g kg^{-1} of diet) and tryptophan (0.75 g kg^{-1} of diet). Over the final two days of the study, the latter diets were not supplemented with lysine or tryptophan but the pigs received a discontinuous intravenous infusion of these amino acids at the same rate as for the dietary supplementation. The amino acids were dissolved in sterile physiological saline and were infused via venous catheters in two equal portions administered at 30 and 60 minutes after the completion of each meal. Pigs receiving the zein and synthetic amino acid diets underwent surgery for the insertion of indwelling jugular catheters on Day 7. For all pigs, total urine voided was quantitatively collected on the 6th and 9th days of trial. Urine was collected over acid (10% H_2SO_4 at 0.025 urine volume) for 16 hours (1600 h to 0800 h) on each collection day. The rates of urinary metabolite excretion for the zein and synthetic amino acid fed pigs following oral and parenteral supplementation with lysine and tryptophan, were determined to monitor any possible effects on nitrogen metabolism of method of amino acid administration. On the tenth day of the study, pairs of pigs were fed equal portions of their daily intake at hourly intervals. The pigs fed the zein and synthetic amino acid diets received the parenteral infusions of lysine and tryptophan via their venous catheters 20 minutes after the consumption of each hourly meal. Fresh faecal samples were collected during the tenth day for the determination of digestible energy contents of the diets and nitrogen retention, and each pair of pigs was killed 10 hours after the commencement of hourly feeding.

The pigs were killed by a 10 ml intracardial injection of sodium pentobarbitone (Euthanal: 300 mg/ml), following anaesthesia with halothane. There is evidence that administration of barbiturate minimises the shedding of mucosal cells which may occur at death (Badawy 1964). The small intestine was dissected out and the final 20-25 cm of ileum was immediately removed. The ileal contents were flushed out with 10 ml of distilled water and frozen. It has been shown with rats (Chapter 3) that there is no difference in total endogenous amino acid excretions between digesta collected in distilled water or physiological saline.

5.3.4 Chemical Analysis

The digesta from each pig receiving the enzymically hydrolysed casein diet were homogenised and divided into two equal portions. One portion was freeze-dried while the other was centrifuged for 45 minutes at 1450 xg and then subjected to ultrafiltration according to the method described by Butts *et al.* (1991). The high molecular weight fraction (M.W.>10,000 Da) was added to the precipitate and subsequently freeze-dried. The ileal digesta and faeces were freeze-dried and finely ground. Total nitrogen, amino acids and chromium were determined in the diets, faeces and ileal digesta. Urinary total nitrogen, urea and creatinine were also

determined. Gross energy was determined in the diets and faeces samples.

Amino acids were determined after acid hydrolysis using a Beckman 119 B.L. amino acid analyser. Samples of 5-7 mg were hydrolysed in 500 µl of 6 M HCl plus 1% added phenol for 24 hours at 110±2°C in glass tubes sealed under vacuum. Cystine, methionine and tryptophan, being partly destroyed under acid hydrolysis, were not determined. Nitrogen (N) was determined by the Kjeldahl method using a Kjeltec Auto 1030 Analyser (Tecator, Sweden). Gross energy was determined by bomb calorimetry with an automatic adiabatic bomb calorimeter (Gallenkamp Autobomb, U.K.). Urea and creatinine were determined using a Technicon Autoanalyser (Technicon Instruments Ltd, U.S.A.). Urea was determined on a modified manifold based on the diacetyl monoxime reaction (Marsh *et al.* 1965). Creatinine was determined using the method of Palmer and Peters (1969) based on trinitrobenzene sulphonate and calibrated with citrulline standards. Chromic oxide concentrations were determined by the method of Fenton and Fenton (1979). All chemical analyses were performed in duplicate.

5.3.5 Data Analysis

The 16 hour urinary N excretion data and estimated (digestibility coefficient) daily faecal N excretion data were used to calculate estimates of body nitrogen retention for pigs on each dietary treatment. The urinary urea N:creatinine N and total N:creatinine N ratios were calculated for the pigs fed the synthetic amino acid and zein diets, after oral and parenteral administration of lysine and tryptophan, from the 16 hour total collections of urine. Endogenous lysine and amino acid flows at the terminal ileum relative to the ingestion of 1 g of food dry matter (DM) were calculated using the equation:

$$\text{amino acid flow (mg kg}^{-1}\text{ DM)} = \text{amino acid concentration in ileal digesta (mg kg}^{-1}\text{ DM)} \times \frac{\text{diet chromium (mg g}^{-1}\text{ DM)}}{\text{ileal chromium (mg g}^{-1}\text{ DM)}}$$

Endogenous ileal amino acid flows for pigs fed the enzymically hydrolysed casein diet were determined for the whole digesta and for the portion of digesta remaining after centrifugation and ultrafiltration. Apparent and true amino acid digestibility coefficients for the pigs fed the zein diet were determined using the following equations:

$$\text{apparent amino acid (AA) digestibility (\%)} = \frac{\text{Dietary AA (mg g}^{-1}\text{) - ileal AA flow (mg g}^{-1}\text{)}}{\text{Dietary AA (mg g}^{-1}\text{)}} \times \frac{100}{1}$$

true amino acid (AA) digestibility (%)

$$= \frac{\text{Dietary AA (mg g}^{-1}\text{) - (ileal AA - endogenous AA)(mg g}^{-1}\text{)}}{\text{Dietary AA (mg g}^{-1}\text{)}} \times \frac{100}{1}$$

The amino acid flow data were tested for homogeneity of variance using Bartlett's test (Sokal and Rohlf 1981). The urinary metabolite excretion data for the pigs fed the synthetic amino acid- and zein-based diets and after oral and parenteral amino acid administration were each subjected to a paired t-test. The whole ileal digesta and processed (centrifuged plus ultrafiltered) ileal digesta amino acid flows for pigs fed the enzymically hydrolysed casein-based diet were subjected to a paired t-test for each amino acid singly. The effects of diet, replicate and diet x replicate on endogenous flow were examined for each amino acid singly by analysis of variance (GLM Procedure, SAS Institute Inc., U.S.A.). The apparent and true ileal digestibility coefficients for the pigs fed the zein diet were also subjected to analysis of variance. The respective treatment means were compared where appropriate using orthogonal contrasts (Sokal and Rohlf 1981).

5.4 RESULTS

The pigs remained healthy and readily consumed the experimental diets. Adequate samples of ileal digesta were collected from all pigs except one animal on the synthetic amino acid diet. The determined digestible energy contents of the protein-free, enzymically hydrolysed casein, synthetic amino acid and zein diets, were 15.8, 16.6, 16.4 and 16.5 MJ kg^{-1}, respectively. The variances for the amino acid flows were homogeneous across the dietary treatments.

The mean excretion rates for urinary urea N and total N, and the urinary ratios of urea N:creatinine N and total N:creatinine N for the pigs fed the synthetic amino acid and zein diets with oral and parenteral administration of lysine and tryptophan are given in Table 5.2. There were no significant differences ($P>0.05$) in the urinary metabolite excretions or their ratios between oral and parenteral administration of lysine and tryptophan for the synthetic amino acid and zein diets. Within dietary treatment the urinary creatinine N excretion rates were not significantly different ($P>0.05$) between oral and parenteral supplementation with lysine and tryptophan. Urinary creatinine excretion values were similar for all pigs on all diets, and were 32, 45, 43 and 35 $mg/kg^{0.75}$ day^{-1} for the pigs given the protein-free, enzymically hydrolysed casein-, synthetic amino acid- and zein-based diets, respectively.

Table 5.2 Excretion rates[1] for urinary total nitrogen (N), urea N, urea N:creatinine N, and total N:creatinine N for growing pigs given a synthetic amino acid or zein diet with oral or parenteral supplementation of lysine and tryptophan

	Synthetic Amino Acid Diet[2]		Zein Diet		
Urinary Metabolite	Oral	Parenteral	Oral	Parenteral	Overall SE
Total N	11	11	20	18	2.2
Urea N	8	9	13	12	1.5
Urea N:Creatinine N	12	14	26	25	1.4
Total N:Creatinine N	17	17	40	37	1.5

[1] Mean; $mg/kg^{0.75}\ hr^{-1}$; None of the oral parenteral differences were significant ($P>0.05$)
[2] n=5 for the synthetic amino acid diet, n=6 for zein diet

The estimated mean (±SE) daily nitrogen retentions for growing pigs given the protein-free, enzymically hydrolysed casein-, synthetic amino acid- and zein-based diets were -3 (±0.5), 6 (±0.6), 8 (±0.5) and 5 (±6) g day^{-1}, respectively. The pigs fed the synthetic amino acid-, enzymically hydrolysed casein- and zein-based diets were clearly in positive nitrogen balance while those fed the protein-free diet were in negative nitrogen balance.

The mean endogenous amino acid flows at the terminal ileum for growing pigs given the EHC-based diet, with or without subsequent treatment of the digesta, are presented in Table 5.3. The amino acid flows for the processed digesta (centrifugation plus ultrafiltration) were generally lower than those for digesta without treatment, but statistically significant differences ($P<0.05$) were found only for histidine, arginine, glutamic acid and proline.

There was no effect ($P>0.05$) of replicate and diet x replicate on the endogenous ileal amino acid flows. The mean endogenous lysine flows at the terminal ileum are presented in Table 5.4. The lysine flows for the zein and enzymically hydrolysed casein (EHC, processed digesta) fed pigs were significantly higher ($P<0.05$) than those for the protein-free and synthetic amino acid fed pigs, whose lysine flows were not significantly ($P>0.05$) different from each other. There

Table 5.3 Endogenous amino acid flows[1] at the terminal ileum of the growing pig given an enzymically hydrolysed casein-based diet and determined on the total unprocessed digesta or digesta subjected to centrifugation plus ultrafiltration

	Endogenous Flow			
Amino Acid	**Total Digesta**	**Processed Digesta[2]**	**Overall SE**	**Level of Significance[3]**
Lysine	609	448	74.0	NS
Histidine	536	359	72.4	*
Arginine	605	510	79.6	*
Aspartic Acid	1535	1276	166.0	NS
Threonine	1063	993	125.7	NS
Serine	1477	1378	221.0	NS
Glutamic Acid	3927	2580	611.3	*
Proline	3620	2227	634.2	*
Glycine	1715	1261	222.0	NS
Alanine	781	637	90.0	NS
Valine	778	687	120.4	NS
Isoleucine	597	516	116.5	NS
Leucine	888	744	119.2	NS
Tyrosine	408	359	59.2	NS
Phenylalanine	385	386	44.4	NS

1 Mean; mg kg^{-1} dry matter intake; n = 6
2 Digesta were centrifuged and ultrafiltered prior to analysis
3 NS not significant; * $P<0.05$

was no significant difference between the mean lysine flow of the EHC fed pigs and that of the pigs fed zein with parenteral administration of lysine and tryptophan. The mean endogenous flows for amino acids other than lysine for the protein-free and EHC (processed digesta) fed pigs are given in Table 5.5. The endogenous amino acid flows for the EHC fed pigs were significantly higher ($P<0.05$) than those for the protein-free fed pigs, except for arginine, proline and glycine.

Table 5.6 gives the mean apparent and true ileal amino acid and nitrogen digestibility coefficients for the pigs fed the zein diet. The true digestibilities were calculated using the endogenous amino acid flows from both the protein-free and

Table 5.4 Endogenous lysine flows[1] at the terminal ileum of the growing pig given protein-free, synthetic amino acid-, enzymically hydrolysed casein- or zein-based diets

	Dietary Treatment[2]					
	Protein-Free	Synthetic Amino Acid	Enzymically Hydrolysed Casein[3]	Zein	Overall SE	Level of Significance[4]
Lysine flow	252[a]	284[a]	448[b]	389[b]	36.5	*

1 Mean; mg kg^{-1} dry matter intake
2 n=6, except for the synthetic amino acid diet where n=5
3 Digesta were centrifuged and ultrafiltered prior to analysis
4 Means with different superscripts were significantly different, * = P<0.05

EHC (processed digesta) fed pigs. The mean endogenous ileal nitrogen flows for the protein-free and EHC fed pigs were 7.5 and 14.5 g kg^{-1} dry matter intake, respectively. The true nitrogen and amino acid digestibilities calculated by both methods were significantly higher (P<0.05) than the apparent digestibilities except for glutamic acid, alanine, leucine, tyrosine and phenylalanine. The true digestibilities of histidine, aspartic acid, threonine and serine based on the respective endogenous flows for the pigs fed the EHC diet were significantly greater (P<0.05) than those based on the respective protein-free endogenous flows.

5.5 DISCUSSION

The synthetic amino acid- and zein-based diets used in the present study were virtually devoid of lysine and tryptophan. It was thus necessary to parenterally infuse these essential amino acids in the pigs receiving the latter diets. It was assumed that parenteral infusion as opposed to oral ingestion of the amino acids did not affect the metabolism of the absorbed dietary amino acids and thus the endogenous amino acid flows at the terminal ileum. In support of this assumption were the similar rates of excretion of urinary metabolites for pigs, regardless of the

Table 5.5 Endogenous amino acid flows[1] at the terminal ileum of the growing pig under protein-free or peptide alimentation

	Endogenous Flow			
Amino Acid	**Protein-Free Diet**	**Enzymically Hydrolysed Casein Diet[2]**	**Overall SE**	**Level of Significance[3]**
Histidine	179	359	39.6	*
Arginine	396	510	120.4	NS
Aspartic Acid	637	1276	115.2	*
Threonine	474	993	91.2	*
Serine	541	1378	156.0	*
Glutamic Acid	946	2580	344.8	*
Proline	1223	2227	435.2	NS
Glycine	1205	1261	237.3	NS
Alanine	362	637	66.5	*
Valine	306	687	47.7	*
Isoleucine	215	516	67.6	*
Leucine	359	744	66.0	*
Tyrosine	176	359	28.1	*
Phenylalanine	182	386	30.4	*

1 Mean; mg kg^{-1} dry matter intake; n = 6
2 Digesta were centrifuged and ultrafiltered prior to analysis
3 NS not significant; * $P<0.05$

Table 5.6 Mean[1] apparent and true ileal amino acid digestibility for growing pigs given a zein-based diet

	Amino Acid Digestibility (%)				
Amino Acid	**Apparent**	**True[2]**	**True[3]**	**Overall SE**	**Level of Significance[4]**
Histidine	51[a]	64[b]	77[c]	2.6	**
Arginine	46[a]	69[b]	77[b]	4.2	**
Aspartic Acid	52[a]	62[b]	73[c]	2.7	**
Threonine	42[a]	59[b]	78[c]	3.3	**
Serine	53[a]	63[b]	79[c]	3.2	**
Glutamic Acid	60	63	70	3.7	NS
Proline	49[a]	61[b]	72[b]	4.9	*
Alanine	60	64	67	3.9	NS
Valine	56[a]	59[a]	74[b]	4.0	*
Isoleucine	57[a]	62[a]	69[b]	3.7	*
Leucine	62	63	65	4.1	NS
Tyrosine	59	63	67	3.3	NS
Phenylalanine	59	62	65	3.6	NS
Nitrogen	53[a]	63[b]	68[b]	4.1	*

1 n=6
2 Calculated using the mean amino acid flows for pigs given the protein-free diet
3 Calculated using the mean amino acid flows for pigs given the enzymically hydrolysed casein diet with centrifugation and ultrafiltration of digesta
4 Means within rows with different superscripts were significantly different; NS not significant, * = $P<0.05$, ** = $P<0.01$.

route of amino acid administration. It appears that delayed discontinuous intravenous infusion of the synthetic amino acids as opposed to ingestion with the food had little overall effect on body protein metabolism. A second assumption in the present study involved the use of the protein zein. For the zein-fed pigs all the ileal lysine was assumed to be endogenous, as the zein contained only a small amount of lysine. The determined amount of lysine from zein, consumed by the animals on the final day of study ranged from 40-170 mg. If it is accepted (based on the mean determined apparent digestibility coefficient for total nitrogen in zein of 0.53) that half of the dietary lysine was absorbed anterior to the terminal ileum, the amount of lysine from zein remaining unabsorbed at the end of the small intestine would range from 1.3 to 3.8% of the determined endogenous lysine flow. It is concluded, therefore, that the low level of lysine present naturally in zein would have had only a negligible influence on the determination of endogenous lysine loss.

A recently developed method (Moughan *et al.* 1990, Butts *et al.* 1991), involving feeding pigs an enzymically hydrolysed casein (EHC) based diet, was used in the present study. The latter method involves processing (centrifugation and ultrafiltration) of digesta following collection, to remove any unabsorbed dietary peptides and free amino acids. A difficulty with this approach is that endogenous peptides and free amino acids present in the digesta are also removed in the low molecular weight ultrafiltrate fraction. The resulting endogenous amino acid flows, therefore, will be underestimates of the actual flows. The determined flows based on the processed digesta following EHC feeding were lower, but generally not significantly ($P>0.05$) so, compared to those based on the unprocessed digesta. The inter-animal variation was high and the differences may have been statistically significant if there had been more animals per treatment. On average, the processed digesta flows were 18.4% lower than the total digesta flows, and at least part of this difference would likely be due to the loss of endogenous amino acids during sample processing. Such a degree of underestimation is consistent with recent findings (Moughan *et al.* 1990, Moughan and Schuttert 1991, Chapter 3) on the relatively low proportion (ca. 11-21% of total digesta nitrogen) of endogenous peptides and free amino acids in endogenous total amino acids, and indicates that the hydrolysed casein was well absorbed by the growing pigs in the present study. The EHC processed digesta amino acid flows can be considered as slight underestimates of the actual flows.

The present results for endogenous lysine flow at the terminal ileum provide evidence that the presence of dietary protein or peptides (M.W.<5,000 Da) in the digestive tract results in an increased loss of endogenous lysine from the small intestine of the growing pig. This confirms earlier findings with the growing rat

(Darragh *et al.* 1990, Moughan and Rutherfurd 1990, Butts *et al.* 1991) and pig (De Lange *et al.* 1990). The result, however, is in contrast to the recently reported finding of Wang and Fuller (1989) of no difference between the endogenous ileal amino acid flows for pigs fed a protein-free diet or a diet containing synthetic amino acids (44 g kg^{-1}) and casein (70 g kg^{-1}). The present observation is in accordance with earlier findings with the rat (Skilton *et al.* 1988) that free amino acid alimentation leads to a similar ileal endogenous lysine flow to that for pigs on a protein-free diet. The animals on the protein-free diet were in negative body nitrogen balance while those receiving the synthetic amino acid diet devoid of lysine but with intravenous lysine infusion were in positive nitrogen balance. It does not appear, therefore, that negative body nitrogen balance *per se* leads to a lowered endogenous lysine loss with protein-free alimentation. De Lange *et al.* (1989) determined ileal amino acid recoveries for pigs fed a protein-free diet but with parenteral infusion of a balanced amino acid mixture or saline. They found no significant differences between the ileal amino acid recoveries for the pigs parenterally infused with amino acids and those infused with saline. It appears, however, that there is a direct effect of the products of protein breakdown during digestion on endogenous protein loss. Dietary peptides and protein in the gut have been reported to be more effective than free amino acids in stimulating pancreatic protease synthesis and secretion (Schneeman 1982, Temler *et al.* 1983, Puigserver *et al.* 1986) and intestinal protease activities (Maze *et al.* 1979, Poullain *et al.* 1989).

There was no significant difference ($P>0.05$) in the present study between the mean endogenous lysine flows for the EHC- or zein-fed pigs. Moughan and Rutherfurd (1990, 1991) who fed guanidinated proteins and an EHC-based diet to rats, also reported similar ileal endogenous lysine flows between the EHC rats and animals given either guanidinated gelatin, soyabean or casein. This implies that there is little influence of the primary or tertiary structures of protein *per se* on endogenous lysine loss from the ileum.

The mean endogenous amino acid flows for the protein-free fed pigs in the present study were similar to those reported by Sauer *et al.* (1977), Taverner *et al.* (1981), Green *et al.* (1987) and Furuya and Kaji (1989). Further, comparison of the ileal flows of amino acids for the EHC-fed pigs with those for the protein-free fed animals (Table 5.5) indicated that the above discussed effect of peptide alimentation on endogenous lysine flow was general for the amino acids. The apparent and true digestibility coefficients for amino acids in the synthetic amino acid diet were determined for the pigs in the present study and are given in Table 5.7. The dietary synthetic amino acids were almost completely absorbed by the pigs as indicated by the high coefficients of apparent and true digestibility. On this basis, the determined

Table 5.7 Mean[1] apparent and true ileal amino acid digestibility for growing pigs given a diet containing synthetic amino acids as the sole nitrogen source

	Amino Acid Digestibility (%)		
Amino Acid	**Apparent**	**True[2]**	**Overall SE**
Histidine	90	101	1.1
Arginine	82	100	2.2
Aspartic Acid	86	98	2.6
Threonine	78	94	4.6
Serine	98	100	2.5
Glutamic acid	95	100	0.6
Alanine	90	99	1.6
Valine	97	97	1.8
Isoleucine	96	99	0.7
Leucine	93	99	0.9
Tyrosine	94	98	0.9
Phenylalanine	96	99	0.5

[1] n=5
[2] Calculated using the endogenous amino acid flow data obtained in this study for pigs given a protein-free diet

ileal flows for the amino acids other than lysine were assumed to be endogenous flows. The latter flows are compared with the protein-free estimates in Table 5.8. As was the case for lysine, the ileal endogenous amino acid flows for the pigs given the synthetic amino acid-based diet were not significantly different to those for pigs given the protein-free diet, with the exception of proline which was significantly greater ($P<0.05$) for the protein-free fed pigs. In work with the growing rat, Skilton *et al.* (1988) and Darragh *et al.* (1990) also found similar ileal endogenous amino acid flows for protein-free versus synthetic amino acid alimentation. The negative body nitrogen retention resulting from the consumption of a protein-free diet has been thought to markedly reduce the endogenous ileal nitrogen excretion. The present results and those of Skilton *et al.* (1988), Darragh *et al.* (1990) and De Lange *et al.* (1989), however, would suggest that the state of body nitrogen retention

Table 5.8 Mean endogenous amino acid flows[1] at the terminal ileum for the growing pig given either a protein-free or a synthetic amino acid-based diet

	Endogenous Flow[2]			
Amino Acid	**Protein-Free Diet**	**Synthetic Amino Acid Diet[3]**	**Overall SE**	**Level of Significance[4]**
Histidine	179	145	25.5	NS
Arginine	396	266	106.0	NS
Aspartic acid	637	712	84.2	NS
Threonine	474	629	73.3	NS
Serine	541	470	71.0	NS
Glutamic acid	946	728	128.1	NS
Proline	1223	480	347.4	*
Glycine	1205	1498	244.3	NS
Alanine	362	402	182.9	NS
Valine	306	387	33.3	NS
Isoleucine	215	246	24.5	NS
Leucine	359	458	37.8	NS
Tyrosine	176	258	28.6	NS
Phenylalanine	182	260	28.6	NS

1 mg kg^{-1} dry matter intake
2 n=6 for the protein-free diet, n=5 for the synthetic amino acid diet
3 Estimated assuming complete absorption of the dietary synthetic amino acids
4 NS not significant; * $P<0.05$

does not influence endogenous ileal amino acid excretion. Proline secretion in both the rat and pig has been reported to be enhanced by protein-free alimentation (Taverner *et al.* 1981, Skilton *et al.* 1988, De Lange *et al.* 1989, Moughan and Rutherfurd 1990), and this is regarded as an artefact of the protein-free state (Taverner 1979).

It would appear, therefore, that protein or peptide alimentation increases amino acid excretion at the end of the ileum over that found with protein-free or synthetic amino acid feeding. This would imply that the presence of dietary peptides and protein or their digestion products in the gut lumen may stimulate the

secretion of protein into the gastrointestinal tract, or may inhibit the digestion and absorption of endogenous protein along the gut. Free amino acid alimentation, however, does not appear to increase ileal amino acid excretions, indicating that it is not free amino acids but protein and peptides that influence endogenous ileal excretion. Other dietary factors such as the crude fibre content (Low 1985), cellulose content (Sauer *et al.* 1977, Taverner *et al.* 1981), dry matter intake (Wilson and Leibholz 1981), level of protein (Twombly and Meyer 1961), and the presence of digestive enzyme inhibitors (Snook 1973, Schneeman 1982, Van der Poel *et al.* 1991) have also been shown to affect the endogenous ileal amino acid excretions.

The apparent digestibility of zein in the present study with pigs was found to be low. Studies using the rat have also found zein to be poorly digested (Gupta *et al.* 1958, Rogers *et al.* 1960, Chen *et al.* 1962, Peraino and Harper 1963, De Muelenaere *et al.* 1967, Porter and Rolls 1971). Rogers and Harper (1966) suggested that the insolubility of zein in aqueous solutions was a major factor in its reduced rate of digestion. The true amino acid and nitrogen digestibility coefficients for zein, however, were markedly higher than the respective apparent digestibility coefficients. The method for determining ileal endogenous amino acid flow significantly affected the estimation of true digestibility. The protein-free endogenous ileal flows gave lower true amino acid digestibility coefficients than did the EHC endogenous flows. This was particularly marked for threonine for which the mean true digestibility coefficient determined using the protein-free endogenous threonine flow was 59% and the mean true (EHC) digestibility coefficient was 78%. It appears that the choice of method for determining endogenous amino acid flow may strongly influence the resultant true amino acid digestibility coefficients for feedstuffs given to the growing pig.

It is concluded that the traditional protein-free method for determining endogenous ileal amino acid excretion in the growing pig leads to considerable underestimation of endogenous loss. Further, and given that the ileal endogenous excretion was equally low for pigs in positive body nitrogen balance receiving synthetic amino acids as their sole source of dietary nitrogen, indicates that negative body nitrogen balance is not the primary cause of the lowered excretion of the protein-free fed animals. The higher endogenous amino acid flows observed after feeding enzymically hydrolysed casein and zein provide evidence that the presence of dietary peptides and protein in the gut increases amino acid excretion at the terminal ileum of the pig. The feeding of an enzymically hydrolysed casein-based diet followed by centrifugation plus ultrafiltration of the digesta is a superior method for determining endogenous amino acid flow at the terminal ileum of the growing pig and for determining true coefficients of dietary amino acid digestibility.

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Chapter 6

The Effect of Food Dry Matter Intake on Endogenous Ileal Amino Acid Excretion Determined Under Peptide Alimentation in the 50 kg liveweight Pig

6.2 ABSTRACT

The study aimed to determine the total daily ileal excretion of amino acids at different food dry matter intakes and the relationships between endogenous ileal amino acid excretion and food dry matter intake for the 50 kg liveweight pig under conditions of peptide alimentation. Sixteen male pigs (40-60 kg liveweight), each fitted with a simple T-cannula at the terminal ileum, were fed an hydrolysed casein-based semi-synthetic diet at 8 different levels of intake. The experiment involved two trials of 8 pigs each, each comprising a cross over design. Each trial involved 4 pairs of pigs with each pair receiving one of 4 sequences of treatments. Each sequence comprised 4 levels of food dry matter intake arranged in a Latin square. The food dry matter intakes were 0.06, 0.08, 0.10 and 0.12, and 0.05, 0.07, 0.09 and 0.11 of metabolic liveweight ($W^{0.75}$) day^{-1} for the first and second trials, respectively. Chromic oxide was included in the diet (0.6%) as an indigestible marker to allow correction of the ileal flows to complete digesta collection. The diet was fed to the pigs at each level of intake for 8 day periods. On the 5th and 8th day of each period, ileal digesta were collected via the cannula continuously for 24 hours. Pooled digesta from each pig at each level of food dry matter intake were sub-sampled and freeze-dried, and the amino acid, nitrogen, dry matter and chromium contents determined. There was an increase in ileal excretion of amino acids and nitrogen with increasing food dry matter intake. There were significant ($P<0.05$) linear relationships between endogenous ileal amino acid, nitrogen and dry matter excretion (mg day^{-1}) and food dry matter intake (g day^{-1}) except for lysine, glutamic acid and phenylalanine which increased in a curvilinear manner ($P<0.05$). There was a significant ($P<0.05$) negative curvilinear relationship between endogenous ileal nitrogen per ileal dry matter (mg N/g ileal dry matter) and food dry matter intake. The results show that dietary dry matter intake strongly influences endogenous excretion from the ileum. The relationships determined, under more physiologically normal conditions than feeding a protein-free diet, provide preliminary data on the magnitude of small intestinal amino acid losses in the pig.

6.2 INTRODUCTION

The measurement of endogenous amino acid loss at the terminal ileum is of practical importance in the determination of amino acid requirements by the factorial method and of true amino acid digestibilities of feedstuffs. In the factorial approach to estimating amino acid requirements for growth and maintenance, the

daily requirement for an amino acid is calculated as the sum of amino acid loss at nitrogen equilibrium and tissue amino acid gain during growth, corrected for the efficiency of utilisation of the dietary amino acid for growth plus maintenance (Agricultural Research Council 1981). The accuracy of this method is directly dependent upon how accurately the parameters and the form of the various relationships between parameters are specified. The maintenance component of the factorial method comprises the loss of catabolised body amino acids in the urine resulting from the inefficiency of whole body protein synthesis, the loss of amino acids through the continual shedding of skin and hair, and the loss of body amino acids from the gastrointestinal tract. Moughan (1989) concluded that the loss of catabolised amino acids and the loss of endogenous amino acids from the gastrointestinal tract made the greatest contribution to predicted maintenance amino acid requirements for the 50 kg liveweight pig.

Traditionally, the endogenous loss of nitrogen and amino acids from the gastrointestinal tract of animals has been determined after feeding a protein-free diet. Recent studies with the growing rat (Darragh *et al.* 1990; Moughan and Rutherfurd 1990; Butts *et al.* 1991) and the growing pig (Chapter 5), however, have shown that the endogenous loss of amino acids from the small intestine is higher following the feeding of diets containing peptides than under protein-free or synthetic amino acid alimentation. Further, the endogenous ileal amino acid excretion determined under peptide alimentation is not significantly different to that determined following feeding of a diet containing protein (Moughan and Rutherfurd 1990; Chapter 5). In the method for determining endogenous loss under peptide alimentation, the animal is fed a semi-synthetic diet containing enzymically hydrolysed casein (EHC) as the sole nitrogen source. Ileal digesta are collected from the animal, and protein (M.W.>10,000 Da) immediately separated by centrifugation and ultrafiltration. Any dietary amino acids and small peptides not absorbed will be removed in the low molecular weight fraction. The low molecular weight fraction may also contain endogenous free amino acids and peptides which could lead to an underestimation of endogenous amino acid loss when using this approach. Recent studies (Moughan *et al.* 1990, Moughan and Schuttert 1991, Chapter 3), however, have shown the levels of endogenous free amino acids and peptides to be low.

In the present study relationships between endogenous ileal amino acid excretion and food dry matter intake were derived for growing pigs given a semi-synthetic diet containing oligopeptides. The overall aim was to provide data on ileal endogenous amino acid loss for the 50 kg liveweight pig, determined under conditions physiologically more normal than those found with the protein-free

method. Such data may be used in practice for estimating amino acid requirements factorially and for correcting ileal amino acid flows to derive true estimates of amino acid digestibility.

6.3 EXPERIMENTAL

6.3.1 Animals and Housing

Two groups each of 8 Large White x (Large White x Landrace) entire male pigs with an overall mean liveweight of 32±0.8 kg (mean±SE) were obtained from the Pig Research Unit, Massey University. The animals were kept in individual smooth-walled steel metabolism crates at 21±2^{o}C .

6.3.2 Surgical Implantation of Simple T-Cannulas in the Terminal Ileum

The pigs underwent surgery for the implantation of a simple T-cannula in the terminal ileum (10-15 cm from the ileo-caecal valve) to allow collection of ileal digesta. The cannula was constructed of medical grade Silastic tubing (i.d. 10 mm; o.d. 16 mm; Silkomed, Rusch, West Germany). The length of the cannula barrel was 85 mm and the foot measured 75 mm wide by 6 mm deep. The pigs were sedated and placed in left lateral recumbency under halothane (Fluothane, Imperial Chemical Industries Ltd, England) anaesthesia administered via a face mask. A 5-6 cm vertical incision was made into the body wall 3-4 cm behind the last rib just above the midline. The small intestine was exteriorised via blunt dissection. A 2-2.5 cm incision was made along the anti-mesenteric side of the small intestine 10-15 cm anterior to the ileo-caecal junction. A Murphy's purse string suture was made around the incision and the cannula inserted through this incision. The free ends of the purse string suture were gently pulled and secured tightly around the barrel of the cannula. The cannula was further secured by a purse string suture placed 2-3 mm below the Murphy's suture. The cannula was exteriorised via a stab wound approximately 1 cm in diameter, 3-4 cm anterior to the initial incision. The intestine was secured to the peritoneum and fascia with discontinuous sutures. The initial incision was closed with continuous sutures in the deep muscle layers and peritoneum, discontinuous sutures in the subcutaneous muscle, and discontinuous mattress sutures in the skin. All pigs were given antibiotic injections daily for 3-5 days following surgery. The pigs regained consciousness within 4-5 hours of surgery and had normal appetites within 48 hours of surgery.

6.3.3 Diets and Feeding

During a 14 day post-operative recovery period, the pigs were fed a semi-synthetic casein-based preliminary diet (100 g kg^{-1} crude protein) to appetite. This

was given in equal portions twice daily for the first 5 days of the recovery period, and thereafter the feeding frequency was increased to 6 equal feeds per day at 0800, 1000, 1200, 1400, 1600 and 1800 h. Following the recovery period, an enzymically hydrolysed casein-based diet (Table 6.1) was given to the pigs in 6 equal portions at the times given above. This feeding regime was chosen to reflect, as far as was practical, the feeding pattern of pigs on *ad libitum* feeding which has been shown to predominantly occur during daylight hours and to have 3-6 peaks of feeding activity (Ingram *et al.* 1980, Wangsness *et al.* 1980). The diet was mixed with water (1:1, ml:g) immediately prior to feeding. Fresh water was provided at all times. Feed refusals were collected where appropriate and oven dry matters determined. Chromic oxide was added to the diet of the pigs at the start (0800 h) of each 24 hour digesta collection period. Chromic oxide was included in the diet (0.6%) as an indigestible marker to allow correction of the determined amounts of digesta to those consistent with complete digesta collection.

6.3.4 Experimental Procedure

The study was conducted as two consecutive trials each comprising a cross over design. Each trial involved 4 pairs of pigs, with each pair receiving one of four sequences of treatment. Each sequence comprised four levels of food dry matter intake arranged in a Latin square. The Latin square pattern was chosen so that, within a square, each treatment (food dry matter intake) followed all other treatments only once. In each Latin square there were two pigs and one food dry matter intake level per cell. The pigs were equally and randomly assigned to one of the four food dry matter intake sequences. The food dry matter intakes for the first trial were 0.06, 0.08, 0.10 and 0.12 metabolic liveweight ($W^{0.75}$) day^{-1}, and for the second were 0.05, 0.07, 0.09 and 0.11 $W^{0.75}$ day^{-1}. The pigs were fed at each food intake level for 8 day periods. On the 5th and 8th days of each period, ileal digesta were collected via the cannula for 24 hours each day. For the pigs in the first trial, the ileal digesta were collected via a rubber tube fitted to the cannula, which emptied into a container held on ice underneath the metabolism crate. For the pigs in the second trial, the digesta were collected from the open cannula directly into ostomy bags (E.R. Squibb & Sons, Inc., U.S.A.) which were attached to a base plate secured firmly to the pig's flank. The ostomy bags were emptied every 15 minutes into a container held on ice. In both trials, the digesta for each pig were kept separate and the pH of the digesta adjusted hourly to pH 3.5 by the addition of 6M H_2SO_4, to reduce bacterial and enzyme activity. At the end of the 24 hour collection period, the digesta from each pig were pooled, weighed and then frozen for subsequent analysis.

Table 6.1 Ingredient composition (g kg^{-1} air dry weight) of the experimental diets

Ingredient	Experimental Diet
Wheaten cornflour	705
Maize Oil	35
Purified cellulose	50
Sucrose	70
Vitamin/Mineral Mix[1]	40
Enzymically hydrolysed casein[2]	100

[1] Tasmix Grower/Finisher Premix, Pfizer Laboratories, Auckland, New Zealand. Supplied (mg kg^{-1} diet): vitamin E 24, riboflavin 4, vitamin K, niacin 17, calcium pantothenate 10, thiamine 2, pyridoxine 2, folic acid 2, betaine 25, Mn 25, Zn 23, Fe 66, Cu 62, Co 130, I 0.5, choline 35; (µg kg^{-1} diet): vitamin A 265, vitamin D_3 30, vitamin B_{12} 16, biotin 63, Se 178; (g kg^{-1} diet): Na 1.4, Cl 1.8, Mg 0.4, S 0.5, K 2.8, Ca 9.7, P 5.0.

[2] Sigma Chemical Company, St Louis, U.S.A. Type I from bovine milk. M.W.<5,000 Da.

At the conclusion of the experiment the pigs were euthanased by a 10 ml intracardial injection of sodium pentobarbitone (300 mg/ml) following anaesthesia with halothane. A postmortem examination was carried out to determine the position of the cannula in the ileum and the extent of gastrointestinal adhesions, so as to ensure there had been unobstructed flow of ileal digesta from the cannulae.

Chemical Analysis

The ileal digesta were thawed, and the two 24 hour collections for each pig at each level of dry matter intake were combined and homogenised. Three digesta subsamples of approximately 200 g were taken for each pig at each feeding level. Two of these subsamples were freeze-dried and finely ground, while the other was subjected to centrifugation and ultrafiltration as described by in Chapter 5. The high molecular weight fraction (retentate: M.W.>10,000 Da) following ultrafiltration, was added to the precipitate and subsequently freeze-dried, and finely ground. The freeze dry matter content of the whole ileal digesta for each pig at each feeding level was determined. Oven dry matters were determined for feed refusals, diets, whole digesta and digesta precipitate plus retentates. Chromic oxide concentrations were determined in the relevant feed refusals, diets and whole digesta. Total nitrogen (N) and amino acids were determined in the diets and ileal digesta precipitate plus retentates.

Amino acids were determined after acid hydrolysis using a Pharmacia LKB

Alpha Plus amino acid analyser. Samples of 5-7 mg of freeze dry matter were hydrolysed in 500 µl of 6 M HCl plus 1% phenol for 24 hours at 110±2°C in glass tubes sealed under vacuum. Cystine, methionine and tryptophan are partly destroyed under acid hydrolysis and were therefore not determined. Nitrogen was determined by the Kjeldahl method using a Kjeltec Auto 1030 Analyser (Tecator, Sweden). Chromic oxide concentrations were determined by the method of Fenton and Fenton (1979). All chemical analyses were performed in duplicate.

Data Analysis

The recovery of chromic oxide in the ileal digesta for each pig at each feeding level was calculated based on the amount of chromic oxide fed to the animal in the first meal of the 24 hour collection period and that found in the ileal digesta collected over the entire 24 hour period. The total endogenous ileal nitrogen and amino acid flows for each pig at each feeding level were calculated on an oven dry matter basis according to the equation:

$$\text{Endogenous ileal nutrient flow (mg day}^{-1}\text{)} = \frac{\text{nutrient digesta concentration (mg g}^{-1}\text{) x dry matter flow (g day}^{-1}\text{)}}{\text{coefficient of Cr recovery}}$$

The endogenous ileal amino acid excretion data were analysed to determine the effect of food dry matter intake (GLM Procedure, SAS Institute Inc., U.S.A.). The data from the two trials (squares) were pooled for analysis. The model was:

$$y_{ijklm} = \mu + Seq_i + Pig_j + Col_k + \beta_1 Dmi_{ijklm} + \beta_2 Dmi^2_{ijklm} + Lvl_m + e_{ijklm}$$

where y_{ijklm} = endogenous ileal excretion (mg day^{-1})

μ = overall mean

Seq_i = effect of treatment sequence i (1-4)

Pig_j = pig (1-16)

Col_k = time; 8 day periods (1-4)

Dmi = actual food dry matter intake (g day^{-1})

β_1 = regression coefficient for the linear effect of actual Dmi

β_2 = regression coefficient for the quadratic effect of actual Dmi

Lvl = treatment level of food dry matter intake (lack of quadratic fit)

e_{ijklm} = residual error

RESULTS

The pigs remained healthy and readily consumed the experimental diets, except for 2 pigs in the second trial which developed ulceration of the pars

oesophagea. They were subsequently removed from the experiment. From the examination of the position of the cannula and the extent of gastrointestinal adhesions around the cannula, it appeared that there was unobstructed flow from all but one pig. The chromium recovery and endogenous amino acid excretion data for this pig, however, were no different from that of other pigs so the data were retained.

The coefficients of chromic oxide recovery ranged from 0.62 to 0.95 with means (±SE) of 0.76 (±0.018) and 0.78 (±0.018) for the two experimental periods. There was no effect of food dry matter intake on the chromic oxide recovery. The liveweights of the pigs during the experimental periods ranged from 40 to 60 kg liveweight. The overall mean pig liveweight during the experimental periods was 48 kg. There was a significant effect of treatment on endogenous ileal excretion for the combined data. The total ileal amino acid, nitrogen and dry matter excretions (mg day^{-1}) at the different levels of food dry matter intake are presented in Table 6.2. There was an overall increase in ileal excretion of amino acids and nitrogen with increasing level of food dry matter intake.

The relationships between the daily ileal excretion of endogenous amino acids and nitrogen (mg day^{-1}) and food dry matter intake (g day^{-1}) are presented in Table 6.3. These relationships were all linear ($P<0.05$) with positive slopes, with the exceptions of lysine, glutamic acid and phenylalanine which increased in a positive curvilinear manner with increasing food dry matter intake (Table 6.4). There was a significant lack of quadratic fit for phenylalanine so the cubic effect of food dry matter intake was fitted to the data. The relationship for endogenous ileal phenylalanine excretion was sigmoidal.

DISCUSSION

The present study used T-cannulation for the collection of ileal digesta. This method was chosen because it minimises surgical interference on the functioning of the gastrointestinal tract. The techniques of re-entrant cannulation and ileo-rectal anastomosis require complete transsection of the intestine which disrupts the myoelectric complex (Laplace and Borgida 1976; Wenham and Wyburn 1980; Sauer *et al.* 1989), causes hypertrophy of the intestine near the cannulas (Laplace and Borgida 1976), disrupts the function of the ileo-caecal sphincter (Laplace and Borgida 1976, Sauer *et al.* 1989) and the large intestine (Just 1983). Studies with pigs fitted with re-entrant cannulas are also hampered by problems that result from blockage of the cannula by digesta, particularly at higher food dry matter intakes

Table 6.2 Least square means[1] for daily ileal amino acid, nitrogen and dry matter excretions for the 50 kg liveweight pig at different levels of dietary intake

	Level of Feeding[2]								
	0.05	**0.06**	**0.07**	**0.08**	**0.09**	**0.10**	**0.11**	**0.12**	**Overall SE**
Lysine	594	721	786	918	952	1069	1133	1154	30.6
Histidine	347	443	453	559	568	619	642	733	18.7
Arginine	548	602	659	727	791	811	820	949	21.0
Aspartic acid	1384	1844	2008	2218	2385	2690	2700	3132	85.6
Threonine	816	976	1029	1311	1355	1462	1501	1703	42.7
Serine	894	1051	1107	1438	1534	1673	1766	2067	55.9
Glutamic acid	2613	3997	4126	5298	5498	6283	6072	7406	68.4
Proline	2226	2720	2845	3101	3026	3516	3606	4083	90.6
Glycine	1258	1898	1879	2164	2329	2619	2806	3239	87.8
Alanine	673	887	899	1082	1092	1207	1245	1551	39.8
Valine	766	946	1000	1261	1133	1322	1369	1673	40.6
Isoleucine	599	811	782	963	1038	1109	1249	1427	36.4
Leucine	723	927	982	1098	1210	1262	1314	1572	38.0
Tyrosine	489	592	635	699	724	782	803	916	19.7
Phenylalanine	594	670	699	764	799	837	859	985	18.3
Nitrogen[3]	2.77	3.67	3.89	4.24	4.54	4.97	5.33	5.90	0.154
Dry Matter[3]	264	414	488	619	667	787	948	1203	42.3
Food Dry Matter Intake[4]	0.90	1.08	1.27	1.45	1.63	1.81	1.99	2.17	

1 mg day^{-1}.
2 $liveweight^{0.75}$ day^{-1}.
3 g day^{-1}
4 kg day^{-1}

Table 6.3 Regression relationships between daily ileal excretions of endogenous amino acids and nitrogen and food dry matter intake for the 50 kg liveweight pig

	Regression Equation[1]	Residual Standard Deviation
Histidine	$y = 0.5x - 56$	48.5
Arginine	$y = 0.9x - 274$	51.4
Aspartic Acid	$y = 1.6x + 40$	242.3
Threonine	$y = 0.9x - 11$	74.9
Serine	$y = 0.7x + 614$	112.8
Proline	$y = 1.8x + 370$	193.8
Glycine	$y = 1.6x + 104$	147.1
Alanine	$y = 0.7x + 69$	71.5
Valine	$y = 0.6x + 298$	56.1
Isoleucine	$y = 0.8x - 53$	58.8
Leucine	$y = 0.6x + 268$	59.6
Tyrosine	$y = 0.2x + 435$	49.8
Nitrogen	$y = 2.7x + 122$	584.2

[1] All slopes and intercepts were significant ($P<0.001$). y = endogenous ileal excretion (mg day^{-1}); x = actual food dry matter intake (g day^{-1}).

Table 6.4 Regression relationships between daily ileal excretions of lysine, glutamic acid and phenylalanine and food dry matter intake for the 50 kg liveweight pig

	Regression Equation[1]	Residual Standard Deviation
Lysine	$y = 0.8x - (1.3 \times 10^{-4})x^2 + 142$	76.7
Glutamic acid	$y=6.2x-(9.1\times10^{-4})x^2-1784$	516.4
Phenylalanine	$y=1.0x-(4.1\times10^{-4})x^2+(1.0\times10^{-7})x^3-29$	30.5

[1] All slopes and intercepts were significant ($P<0.001$). y = endogenous ileal excretion (mg day^{-1}); x = actual food dry matter intake (g day^{-1}).

(Van Leeuwen *et al.* 1987; Sauer *et al.* 1989). Wenham and Wyburn (1980) have shown that the implantation of single T-cannulae into the small intestine of sheep resulted in minimal disturbance of intestinal motility. Moreover, Moughan and Smith (1987) found no difference in amino acid digestibility after digesta were collected from pigs prepared with simple T-cannulas or direct samples from the terminal ileum after euthanasia with a barbiturate. The post-valve T-caecum cannulation technique would appear to allow almost complete collection of ileal digesta with minimal interference of the gastrointestinal function (Köhler *et al.* 1990). This technique, however, has only been recently reported in the literature and consequently was not used in the present study.

The apparatus for collecting digesta from the cannulas, in the present study was altered during the overall trial because of concerns regarding the extent of digesta recovery. Analysis of the chromium recovery, however, indicated that the two collection approaches were equally satisfactory. Close to 80% of the administered marker was recovered during collection of digesta.

The results of the present study indicate that there was a significant effect of food dry matter intake on endogenous ileal amino acid and nitrogen excretion for growing pigs under peptide alimentation. The present study, however, did not distinguish between the effects of increased levels of the different dietary components with increasing food dry matter intake. Increased dietary protein has been reported to increase the levels of protein secreted by the pancreas in the pig (Corring and Saucier 1972, Ozimek *et al.* 1984). Higher levels of dietary fat cause an increase in pancreatic lipase secretion (Ozimek *et al.* 1983, 1985, Corring and Chayvialle 1987), and increased dietary starch has been reported to cause an increase in pancreatic amylase secretion (Corring and Chayvialle 1987) and increased secretion of the brush border sugar hydrolases (Puigserver *et al.* 1986). Increased levels and types of dietary fibre have been shown to stimulate gastric, pancreatic and intestinal secretions (Low and Rainbird 1983, 1984, Vahouny *et al.* 1985, Zebrowska 1985, Langlois *et al.* 1987, Low 1989, Fuller and Cadenhead 1991), and increase endogenous ileal loss of amino acids from the pig fed a protein-free diet (Sauer *et al.* 1977, Van Weerden *et al.* 1980, Taverner *et al.* 1981, Green *et al.* 1987, De Lange *et al.* 1989). In contrast, Furuya and Kaji (1991) found no effect of dry matter intake or dietary fibre on endogenous ileal amino acid and nitrogen output of the growing pig. Further work needs to be conducted to define the effects of specific dietary components on endogenous loss.

The total daily ileal excretion of amino acids and nitrogen found in the present study were higher than those found by Holmes *et al.* (1974), Zebrowska *et al.* (1975), Van Weerden *et al.* (1980) and De Lange *et al.* (1989) following 24 hour

collection of digesta from protein-free fed pigs. The present results, however, were similar to those found by De Lange *et al.* (1990) using the ^{15}N-isotope dilution technique, and by Krawieltzki *et al.* (1990) who exchanged ^{15}N labelled and non-labelled digesta between pigs. The previous study with pigs (Chapter 5) relied upon sampling digesta at death, whereas the present trial employed total collection of digesta. Both studies indicate higher endogenous loss under peptide feeding.

In the present study there were small food refusals at the higher feeding levels, so the absolute food dry matter intakes were used in deriving the regression relationships between endogenous ileal excretion and food dry matter intake. Most of the relationships between endogenous ileal amino acid excretion and food dry matter intake derived here were linear, however, for lysine, glutamic acid and phenylalanine curvilinear relationships provide a better description of the data. The present relationships quantify the effect of food dry matter intake on endogenous ileal amino acid excretions in the pig, and given that they have been determined under more normal conditions than those determined with the traditional protein-free approach, provide more accurate values for the determination of amino acid requirements by the factorial method, and in the determination of true amino acid digestibilities of feedstuffs. The data given here pertain to the 50 kg liveweight pig and caution should be exercised in extrapolating to other liveweight pigs.

In conclusion, food dry matter intake strongly influences endogenous excretion from the ileum of the growing pig. The amount of endogenous amino acids excreted from the ileum of the pig at a given level of dietary dry matter intake is likely to vary for diets with different ingredient compositions, but the present relationships, which were derived under more physiologically normal conditions than feeding a protein-free diet, provide interim data on the magnitude of small intestinal amino acid losses. Further research is required to determine the effects of the level of dietary protein, fibre and dry matter *per se* on endogenous ileal amino acid excretion.

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SUMMARY AND CONCLUSIONS

The studies reported here aimed to refine a methodology for the determination of endogenous ileal amino acid excretion under conditions of peptide alimentation and to further elucidate the effect of dietary peptides and protein on net endogenous amino acid excretion from the terminal ileum of monogastric mammals. The peptide alimentation method as used by Darragh *et al.* (1990) and Moughan and Rutherfurd (1990) in which endogenous ileal amino acid excretion was determined after feeding rats a hydrolysed casein-based diet, relied on the assumption that the dietary amino acids were completely absorbed. Moughan *et al.* (1990) proposed a modification to this method which removes the need to make assumptions concerning the completeness of absorption of the dietary amino acids. In the modified method, the animal is fed a semi-synthetic diet containing enzymically hydrolysed casein (EHC) as the sole nitrogen source. Ileal digesta are collected and the protein separated by ultrafiltration (molecular weight exclusion limit 10,000 Da) using disposable plastic ultrafilters. The high molecular weight (MW>10,000 Da) fraction resulting from the ultrafiltration provides a measure of endogenous amino acid flow. Dietary amino acids and peptides that have not been absorbed are removed in the low molecular weight fraction. The low molecular weight fraction will also contain non-protein nitrogen and endogenous free amino acids and peptides. The levels of endogenous free amino acids and peptides are expected to be low, but their removal in the low molecular weight fraction will lead to a degree of underestimation of the actual endogenous loss of amino acids.

The initial studies in the research programme (Chapters 2, 3 and 4) were undertaken to develop and evaluate the above described novel method for determining endogenous ileal amino acid excretion. Three prefiltration treatments (perchloric acid protein precipitation, trichloroacetic acid protein precipitation and centrifugation) of rat ileal digesta were examined, and centrifugation was established as the most satisfactory method for removing insoluble proteins and other solid material from the raw digesta. The accuracy (>90% effective separation) of the ultrafiltration devices for filtering molecules on the basis of molecular weight and shape was confirmed, and it was established that distilled water was as satisfactory a medium as physiological saline for flushing digesta from the terminal ileum of animals. Finally, the molecular composition of the endogenous digesta nitrogen-containing material was determined to give an indication of the extent of underestimation of endogenous amino acid loss following application of the peptide

alimentation/digesta ultrafiltration method.

The first experiment used the growing rat to determine endogenous ileal amino acid flows under peptide alimentation using the ultrafiltration technique with centrifugation of the digesta prior to filtration, and compared these flows with those obtained under peptide alimentation but without treatment of digesta, or under protein-free feeding. The endogenous amino acid flows for the rats fed EHC were generally higher for the total digesta than for the digesta following centrifugation plus ultrafiltration. The protein-free fed rats had significantly lower ileal amino acid flows than those for rats fed the EHC-based diet with ultrafiltration plus centrifugation of digesta. The present findings support those of earlier studies (Darragh *et al.* 1990, Moughan and Rutherfurd 1990) that endogenous ileal amino acid loss in the laboratory rat is enhanced under peptide feeding. On average 21% of the total digesta nitrogen was present as free amino acid plus peptide nitrogen. The latter value was somewhat higher than that reported (11% of total digesta N) in a similar study with the pig (Moughan and Schuttert 1991). Overall, it was concluded that the underestimation of endogenous ileal amino acid excretion following ultrafiltration of the digesta is relatively low. The findings from the initial studies supported the application of the proposed new method for the determination of total endogenous ileal amino acid excretion.

Having been used successfully with the laboratory rat, the refined peptide method was then applied in a study with growing pigs to confirm the effect of dietary peptides on endogenous loss in this species. In the same study, the effect of state of body nitrogen balance (protein-free vs synthetic amino acid diet) on the endogenous ileal amino acid loss was also determined. The traditional method for determining endogenous loss by feeding a protein-free diet has been criticised for creating a physiologically abnormal state in the pig (Low 1980), which may lead to a decreased rate of whole body protein synthesis (Millward *et al.* 1976, Muramatsu 1990) and thus affect the amount of protein entering the gut. Recent studies (Skilton *et al.* 1988, Darragh *et al.* 1990) determined the endogenous excretions of non-essential amino acids at the terminal ileum of rats fed a diet containing a mixture of synthetic amino acids as the sole nitrogen source, but devoid of the amino acid under consideration. The animals were supplied with a balanced array of dietary amino acids for protein metabolism, thus overcoming the problem of a possible decreased rate of whole body protein synthesis, consequent upon feeding a protein-free diet. In both studies, the endogenous amino acid flows were not different to those for rats fed a protein-free diet. Further, De Lange *et al.* (1989) reported lower endogenous ileal protein excretion for pigs given a protein-free diet but with

parenteral administration of amino acids compared with pigs given a protein-free diet and infused with saline. Another approach to the estimation of endogenous amino acid flow which has not yet been used experimentally, is to feed an animal a natural protein that is devoid of specific amino acids, and to measure the flows of those amino acids at the terminal ileum. Zein is naturally deficient in both lysine and tryptophan.

The mean endogenous ileal lysine flows in the present work for the zein and EHC fed pigs were not significantly different from each other but were higher than those for the protein-free and synthetic amino acid fed pigs, whose mean flows were not significantly different from each other. The mean endogenous ileal flows for amino acids other than lysine were higher for the EHC fed pigs compared to the animals on the protein-free diet, except for proline, glycine and arginine. The similar endogenous ileal lysine excretion for pigs receiving a synthetic amino acid-based diet and thus in positive nitrogen balance, and protein-free fed pigs in negative nitrogen balance, supports recent findings by other workers (Skilton *et al.* 1988, De Lange *et al.* 1989, Darragh *et al.* 1990) that negative body nitrogen balance *per se* does not lead to a lowered endogenous ileal excretion.

The similar endogenous ileal lysine excretion for pigs receiving the EHC based diet with centrifugation plus ultrafiltration of digesta and the zein fed pigs is consistent with recent findings (Moughan *et al.* 1990, Moughan and Schuttert 1991) that there are relatively low proportions (11-21% of total digesta nitrogen) of endogenous peptides and free amino acids in digesta which leads to only a small underestimation of endogenous loss when determined by the peptide alimentation method. The presence in the gut of dietary peptides or protein appears to enhance amino acid excretion from the terminal ileum of the pig above that found with protein-free or synthetic amino acid alimentation. This supports the earlier findings with the laboratory rat.

Lower proteolytic enzyme activities in the pancreas and intestine have been reported (Kimura *et al.* 1977, Schneeman 1982) for animals fed a protein-free diet and the presence of dietary peptides in the gut has been reported to be more effective than free amino acids in stimulating the rates of trypsin and chymotrypsin synthesis (Temler *et al.* 1983, Puigserver *et al.* 1986). The traditional method of determining endogenous loss by feeding a protein-free diet appears to underestimate endogenous ileal amino acid excretion in the rat and pig, and probably simple-stomached animals generally.

The true digestibility coefficients for zein calculated using either the endogenous ileal amino acid flows determined under peptide or protein-free

alimentation were markedly higher than the apparent digestibility coefficients. The method for determining ileal endogenous amino acid flow significantly affected the estimation of true digestibility. The protein-free endogenous ileal amino acid flows gave lower true amino acid digestibility coefficients than did the flows from the EHC fed pigs. It appears that the choice of method for determining endogenous amino acid flow may strongly influence the resultant true amino acid digestibility coefficients for feedstuffs given to the growing pig.

In the final experiment of this study (Chapter 6), the effect of food dry matter intake on endogenous ileal amino acid loss under a defined feeding regime was determined. The EHC ultrafiltration method was used with growing pigs. Statistical relationships were derived and provide interim data on the effect of dry matter intake on endogenous ileal amino acid excretion determined under physiologically normal conditions. These data should not be extrapolated beyond the levels of feed intake examined or to other pig liveweights. The results provide information on the overall effect of dietary dry matter intake on endogenous ileal amino acid excretion. The effects of the levels of the different dietary ingredients on endogenous loss, however, were not examined in the present study.

A summary of endogenous ileal nitrogen excretion for the growing pig is shown in Table 7.1. The direct estimate and that determined under peptide alimentation are generally higher than those determined by the protein-free and regression methods. As established earlier the protein-free and regression estimates of endogenous ileal amino acid loss for the pig are very similar. The direct estimate for endogenous excretion obtained from the secretion data, however, should be interpreted with some caution. This estimate may be unreliable because of the unavailability of amino acid data for salivary and gastric secretions. Also, an overall mean digestibility coefficient for endogenous protein was used to estimate the total amount of the secretions remaining unabsorbed at the end of the small intestine. This may not be appropriate due to the individual amino acids of a protein being digested and absorbed at different rates. Also, the mean absorption values for endogenous protein found in the literature are highly variable (54-85% of total nitrogen secreted is reabsorbed). Given these considerations, the data in Table 7.1 should only be interpreted broadly to indicate agreement between protein-free alimentation and the regression method, with the higher peptide alimentation excretion being closer to the direct estimate.

Overall it is concluded that the peptide alimentation method is better than the protein-free and regression techniques for the routine determination of endogenous ileal amino acid excretion, but this method does have some limitations. The main

Table 7.1 Endogenous ileal amino acid excretion for the growing pig (g day^{-1}) determined directly, under protein-free alimentation, by the regression method and under peptide alimentation

Nitrogen	Direct[1]	Protein-Free Alimentation[2]	Regression[3]	Peptide Alimentation[4]
Nitrogen	8	2.5	2.7	4.5

1 from Table 1.3 and based on assumed absorption of endogenous digesta nitrogen of 70% (Rérat *et al.* 1976, Low 1982, Rérat 1990)
2 from Table 1.4 (assuming pigs were fed at 0.09 metabolic bodyweight)
3 from Table 1.5 (assuming pigs were fed at 0.09 metabolic bodyweight)
4 from Table 6.2 (pigs fed at 0.09 metabolic bodyweight)

limitation of the method is that it underestimates endogenous ileal amino acid excretion, because endogenous free amino acids and peptides are discarded in the low molecular weight ultrafiltration fraction. A further limitation is that the method does not allow the effect of protein *per se* on endogenous loss or the effect of different amino acid compositions of dietary proteins to be investigated. Also, it is possible that estimates of endogenous loss determined with the peptide alimentation method may be influenced in some way by the enzymic hydrolysate of casein itself and thus be an artefact of this particular dietary treatment.

There is a possibility that there is binding of the undigested dietary free amino acids and peptides to the endogenous proteins in ileal digesta, which could result in an overestimation of endogenous loss. The binding of free amino acids and peptides to plasma proteins has been reported to occur in blood (Williams *et al.* 1972, Ohara and Ariyosha 1979). There is evidence from the present study, however, that shows indirectly that this does not occur in digesta. The endogenous ileal amino acid flows for the pigs fed the synthetic amino acid diet were not higher than those for the protein-free fed pigs indicating that the dietary free amino acids were not trapped in endogenous proteins. It is also noteworthy that the studies of Moughan and Rutherfurd (1990) and De Lange *et al.* (1990) using the homoarginine and ^{15}N-tracer methods, respectively, corroborate the present findings that that the presence

gut of dietary protein or peptides enhances endogenous ileal nitrogen excretion. It is also possible that the ultrafiltration devices did not adequately separate digesta amino acids and proteins, but from the results of the preliminary studies this would appear unlikely. This possibility could be investigated by quantitatively adding free amino acids (e.g. homoarginine) and/or peptides to ileal digesta and measuring the recovery of the added material following centrifugation and ultrafiltration.

Further research is required on the effect of levels of dietary protein, fibre, fat and starch, and age of animal on endogenous ileal amino acid loss. Also clarification of the physiological mechanisms involved in the changes in endogenous amino acid secretion and excretion with changes in diet is required. The effects of other dietary protein hydrolysates and the degree of hydrolysis of the dietary nitrogen on the quantity and amino acid composition of endogenous ileal amino acid excretion need to be determined to ensure the present results are not an artefact of the enzymically hydrolysed casein diet.

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