ENDOGENOUS PROTEIN FLOW IN THE GUT OF THE SIMPLE-STOMACHED MAMMAL

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ABSTRACT

The set of studies was undertaken to examine different aspects of the measurement of endogenous ileal nitrogen and amino acid loss in simple-stomached mammals and specifically to investigate the effect of the concentration of protein and peptides in the diet on endogenous ileal amino acid flows. Seven separate studies were conducted using rats and pigs.

1. The aim of the first study was to determine whether endogenous nitrogen (N) and amino acid flows at the terminal ileum change over time in the growing pig fed a protein-free diet. Male pigs (n=7, mean bodyweight 82 kg) with surgically implanted post-valve T-caecum (PVTC) cannulas received a casein-based diet for 8 days after which food was withheld from the pigs for 24 hours. The pigs then received a protein-free diet for a further 8 days during which time ileal digesta were collected continuously via the cannulas from 1300h to 1800h on each day. Endogenous ileal N and amino acid flows were determined on the digesta. There were no significant ($P>0.05$) effects of the duration of feeding of the protein-free diet on endogenous ileal total N or amino acid flows except for the amino acids glycine and cysteine, the mean flows of which significantly decreased over the 8-day experimental period ($P<0.01$ and $P<0.05$ for glycine and cysteine, respectively), from 1639 to 892 μg/g dry matter intake (DMI) for glycine, and 173 to 127 μg/g DMI for cysteine.

2. The enzyme hydrolysed protein, isotope dilution and guanidination methods can be used to determine endogenous ileal protein flows. The aim of the second study was to determine whether the isotope dilution and guanidination methods give similar estimates of endogenous ileal N and lysine flows, respectively, as the enzyme hydrolysed protein method. A test diet was prepared that contained guanidinated and enzymatically hydrolysed (MW <5,000 Da) casein labelled with ^{15}N. Male rats (n=30, mean bodyweight 178 g) and male pigs (n=6, mean bodyweight 19.2 kg) received a preliminary EHC-based diet for 7 days. The test diet was given to the rats and pigs on the following day and digesta were sampled at the terminal ileum of the animals following euthanasia. Endogenous ileal lysine flows were determined using the enzyme hydrolysed protein and guanidination methods. Endogenous ileal N flows were determined using the enzyme hydrolysed protein and isotope dilution methods. The guanidination method led to significantly ($P<0.05$ and $P<0.01$ for the rat and pig, respectively) lower mean endogenous lysine flows compared with the enzyme hydrolysed protein method (298 vs 382 and 214 vs 287 μg/g DMI in the rat and pig, respectively). The isotope dilution method led to significantly ($P<0.001$ for the rat and $P<0.05$ for the pig) lower mean endogenous ileal N flows compared with those determined using the enzyme hydrolysed protein

method (means of 1034 vs 1942 and 1011 vs 1543 μg/g DMI for the rat and pig, respectively). Given that the enzyme hydrolysed protein method is known to somewhat underestimate actual endogenous N and amino acid flows, it appears that the guanidination and isotope dilution methods notably underestimate endogenous flows at the terminal ileum.

3. The third experiment involved an *in vitro* study to examine the effectiveness of Centriprep-10 Concentrator devices for the ultrafiltration of digesta with the enzyme hydrolysed protein method for the determination of endogenous ileal N and amino acid flows. Different amounts of enzyme hydrolysed casein (EHC) were added to test tubes containing digesta collected from pigs that had received a protein-free diet for 5 to 8 days. The samples were centrifuged and then ultrafiltered using Centriprep-10 concentrators. The amount of N and amino acids that was deemed to have originated from the EHC and remained in the precipitate plus retentate fraction of digesta after processing, expressed as a percentage of the total amount of N or amino acid added to the tubes as EHC, ranged from 1.0 to 5.0% for N and averaged 2.4 to 5.8% for the amino acids. With Centriprep-10 concentrators there is a less than complete separation of N and amino acids originating from EHC from endogenous material in the digesta, which could potentially lead to a small overestimation (up to 2%) of endogenous ileal N and amino acid flows.

4. In experiment 4, endogenous ileal N and amino acid flows were determined using the enzyme hydrolysed protein method using a molecular weight (MW) cut-off for ultrafiltration of 10,000 Da, and were compared with flows determined using a MW cut-off of 3,000 Da. Digesta were sampled from the terminal ileum of male rats (n=24, mean bodyweight 179 g) that had received a diet containing EHC for 8 days. The digesta were pooled to give 6 pooled samples, each containing the digesta from 4 rats. Endogenous ileal N and amino acid flows were determined using the enzyme hydrolysed protein method with ultrafiltration using MW cut-offs of 10,000 Da and 3,000 Da. The endogenous ileal N and amino acid flows determined using a MW cut-off of 3,000 Da were greater than those determined following ultrafiltration at 10,000 Da, by 17% for N and on average 12% for the amino acids, with a range from 1.7% for arginine and phenylalanine to 26% for serine.

5. In the fifth experiment, the diurnal pattern of endogenous N flow at the terminal ileum of the pig was examined using the enzyme hydrolysed protein method. Male pigs (n=7, mean bodyweight 33 kg) had PVTC cannulas surgically implanted. The pigs received an EHC-based diet for 8 days. Digesta were continuously collected for 24 hours (0800h - 0800h) on each of the fifth and eighth days. During each hour of digesta collection, 10% (by weight) of the digesta collected for that hour for each

pig was sampled. Flows of dry matter and chromium were determined in the digesta, and endogenous N was determined after centrifugation and ultrafiltration (10,000 Da MW cut-off) of the digesta. The concentration of chromium in the digesta expressed on a digesta dry matter basis was relatively constant over the 24-hour periods, with no statistically significant ($P>0.05$) differences from 1200h - 0800h. The ratio of endogenous N to chromium at the terminal ileum was also relatively constant with no statistically significant ($P>0.05$) differences from 1300h - 0800h. The net outcome of endogenous protein secretion and reabsorption in the small intestine appears to be relatively constant over time in the meal-fed animal.

6. The aim of experiment 6 was to determine whether dietary peptide concentration affects endogenous ileal N and amino acid flows in the growing pig. Entire male pigs (n=8, mean bodyweight 33 kg) had PVTC cannulas surgically implanted. The pigs received the diets (0, 5, 10 and 20% EHC) for 8-day periods in a Latin Square design with a basal casein-based diet given to the pigs for 6-day periods in between the experimental diets. Digesta were collected continuously for 24 hours on each of the fifth and eighth days. The endogenous ileal N and amino acid flows were determined directly for pigs receiving the protein-free diet or after centrifugation and ultrafiltration (10,000 Da MW cut-off) for pigs on the EHC-based diets. Mean endogenous ileal N flows were 1753, 1948, 2851 and 5743 μg/g DMI when the pigs received diets containing 0, 5, 10 and 20% EHC, respectively. There was a significant ($P<0.05$) effect of dietary peptide concentration on the endogenous ileal flow of N and for all of the amino acids, with an increase in endogenous ileal N and amino acid flow with increasing dietary EHC concentration.

7. The final experiment was conducted to corroborate the results described in the sixth study on the effect of dietary peptide/protein concentration on endogenous ileal lysine flow. Male rats (n=108, mean bodyweight 170 g) received diets containing 5, 10, 15, 20, 25 or 30% zein over a period of 8 days. Zein is nearly devoid of lysine and tryptophan, so the diets were supplemented with lysine and tryptophan for the first 6 days and lysine and tryptophan were given to the rats via intraperitoneal injections over the final 2 days of this period. Digesta were sampled (after euthanasia) from the terminal ileum of the rats on the eighth day and pooled to give 6 samples (3 rats per pooled sample) per diet. All lysine present in the digesta was assumed to be of endogenous origin. Increasing the amount of zein in the diet led to a significant ($P<0.0001$) increase in endogenous lysine flow through the terminal ileum.

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INTRODUCTION

Many nitrogen-containing compounds, including amino acids, peptides and proteins, diffuse into or are secreted into the gastrointestinal tract during the digestion of food. These compounds are termed endogenous, as opposed to dietary, or exogenous material. Endogenous nitrogen-containing material includes secretions containing digestive enzymes produced by the salivary glands, pancreas and the mucosal lining of the stomach and intestines, and bile acids from the liver. Mucus is secreted from cells along the entire gastrointestinal tract, and epithelial cells are sloughed off from the intestinal mucosa. Serum albumin is also present in the gastrointestinal tract. Most (70-80%) of this endogenous nitrogen is reabsorbed before the digesta leave the small intestine (Souffrant, 1991), but the remaining 20-30% enters the large intestine.

It is important for several reasons to have knowledge of the amount of endogenous protein that is not reabsorbed by the animal. Endogenous amino acid losses from the digestive tract are an important component contributing to amino acid requirements in humans and animals. Consequently, information on these losses is needed to allow a factorial estimation of amino acid requirements. Also, it is important to have knowledge of these losses for the determination of dietary protein and amino acid digestibility coefficients for application in practical diet formulation and for describing the protein quality of foods for both humans and farm animals. When comparing the protein quality of different feedstuffs, "*apparent*" ileal digestibility values, which are determined by subtracting the amount of amino acids leaving the terminal ileum from the amount of amino acids that were ingested by the animal, must be corrected for the endogenous material present in the digesta to give "*true*" digestibility coefficients.

The earliest attempt to determine endogenous nitrogen flow involved measuring faecal nitrogen excretion for rats given a protein-free diet (Mitchell, 1924). However, due to the profusion of micro-organisms in the hindgut, faecal values do not accurately reflect the excretion of unabsorbed dietary and endogenous amino acids from the animal. It has been estimated that 80% of the nitrogen present in the faeces of the pig is of bacterial origin (Mason, 1980). Both the level of fermentation and the susceptibility of exogenous and endogenous proteins to bacterial attack will determine the amount of endogenous ileal amino acids appearing in the faeces (Mason and Palmer, 1973). The ileal flow of amino acids is more representative of the unabsorbed dietary and endogenous amino acid flow than is the faecal flow. Accordingly, the currently accepted approach to determining the amount of endogenous amino acids that are not absorbed by the animal is to make measurements on digesta collected from the

terminal ileum. However, the ileal measure of digestibility poses certain technical difficulties. Firstly, ileal digesta must be collected, and secondly, the protein that is secreted into the animal's gut during digestion (the endogenous protein), needs to be distinguished from unabsorbed dietary protein.

This review commences with a description of the anatomy and physiology of the different parts of the gastrointestinal tract in the monogastric mammal including a description of the nitrogenous secretions into the digestive tract and the digestion and absorption of proteins of both endogenous and exogenous origin. The second part of the review discusses methods for the collection of ileal digesta for the measurement of endogenous ileal protein flows. Methods that have been developed to quantify endogenous ileal protein flows are then reviewed. Finally, dietary factors that influence endogenous ileal amino acid flow are discussed.

ANATOMY AND PHYSIOLOGY OF THE GASTROINTESTINAL TRACT IN SIMPLE-STOMACHED MAMMALS

The gastrointestinal system comprises the muscled digestive tract and its associated accessory organs. The digestive tract consists of a tube that is continuous with the skin at the mouth and anus and is divided according to its functional and anatomical characteristics.

The General Structure of Tubular Organs

The walls of the stomach and intestines are made up of four layers (Madara and Trier, 1994). The *tunica mucosa* is nearest the lumen and consists of three layers. The first layer is the *lamina epithelialis mucosae* or epithelium. The second layer, the *lamina propria mucosae* is formed of connective tissue and has a defensive role as well as being important in the nourishment of the epithelium. The *lamina muscularis mucosae, the third layer of the tunica mucosa, consists* of one or more layers of smooth muscle and is responsible for local motility.

The *tunica submucosa* lies underneath the *tunica mucosa* and is formed of connective tissue containing blood vessels and nerves.

The third layer of tubular organs is the *tunica muscularis.* This is usually well-developed and generally consists of two layers of smooth muscle. Typically, the inner

layer of muscle is circular and the outer longitudinal. Between the two layers there are normally vascular and neural plexus, including autonomic gangliona. Muscular movements of the *tunica muscularis* are responsible for the movement of digesta through the lumen of the organ.

The *tunica adventitia* or *tunica serosa* is the final outer layer of tissue surrounding the digestive tract.

The Oral Cavity

Food enters the gastrointestinal system through the mouth. It is tasted with the tongue, masticated with the teeth and mixed with saliva, a fluid produced by specialised glands in the oral cavity called the salivary glands. These are classified into major and minor glands. The major salivary glands are located away from the oral mucosa and secrete saliva into the oral cavity through one or more extra-glandular ducts (Young and van Lennop, 1978; Jamieson, 1988). There are three major pairs of salivary glands; the parotid, submandibular (also referred to as the mandibular and submaxillary gland) and the sublingual glands (Young and van Lennop, 1978; Jamieson, 1988; Cook *et al.*, 1994). The minor salivary glands consist mainly of small packages of glands in the labial, palatine, buccal, glossopalatine lingual and sublingual submucosae (Green and Embery, 1985) in close proximity to the *lamina epithialis mucosae* (Banks, 1986) and secrete saliva through numerous short excretory ducts (Young and van Lennop, 1978).

Saliva has several functions. It moistens food, presenting the food in solution to the taste buds on the tongue and lubricates the oesophagus, to assist movement of the food bolus to the stomach. The enzyme α-amylase is present in the saliva and initiates the breakdown of starch by hydrolysing α-1,4 glucosidic bonds. Lysozyme has been shown to be present in human submandibular and parotid saliva and lyses bacteria such as *Staphylococcus* and *Streptococcus* by hydrolysing a component of the bacterial cell wall (Sanford, 1982). Human saliva also contains histatins that can kill *Candida* (Oppenheim *et al.*, 1988).

The secretion of saliva has been reviewed by Burgen (1967), Ellison (1967), Schneyer and Schneyer (1967), Kidder and Manners (1978), Jacobson (1981), Young and Schneyer (1981), Davenport (1982), Sanford (1982), van Lennop *et al.* (1986), Young *et al.* (1987) and Cook *et al.* (1994). Most salivary glands do not have a spontaneous secretion (Young *et al.*, 1987). Some glands, however, such as the sublingual glands

in dogs and cats, the rabbit submandibular gland (Emmelin, 1953; Smaje, 1973; Young *et al.*, 1981) and the rat parotid gland (Castle, 1998) do secrete spontaneously. Salivary ducts have been shown to be absorptive (Yudilevich *et al.*, 1979; Bustamante *et al.*, 1981), therefore in other species small volumes of spontaneous secretion might be completely reabsorbed before they reach the mouth.

Stimulation of the sympathetic nerves supplying the salivary glands provokes the secretion of protein including α-amylase (Schneyer, 1974; Garrett and Thulin, 1975; Gjörstrup, 1979; Gjörstrup, 1980; Olsen *et al.*, 1988; Castle, 1998) but little fluid (Thulin, 1976; Gjörstrup, 1977; Case *et al.*, 1980). The amylase is washed out by the vigorous flow of fluid produced following parasympathetic stimulation (Emmelin *et al.*, 1973; Thulin, 1976; Gjörstrup, 1977; 1980; Case *et al.*, 1980; Davenport, 1982; Castle, 1998) due to mastication and taste stimuli (Babkin, 1950; Chauncey and Shannon, 1960; Kerr, 1961; Gjörstrup, 1981; Mackie and Pangborn, 1990).

No specific hormone is known to stimulate salivary secretion. However, the electrolyte composition of saliva can be modified by adrenal mineralocorticoids, which lower salivary sodium concentration and increase the concentration of potassium ions in the human (Sanford, 1982).

In humans, the saliva produced by the submandibular glands contains between 1.0 and 5.0 mg of protein per ml of saliva (Davenport, 1982). Kidder and Manners (1978) estimated that the volume of saliva secreted each day in the pig is 15-18 litres and the saliva stimulated by feeding cereal diets has a nitrogen content of 0.40-1.05 mg/ml of saliva (Kidder and Manners, 1978). This corresponds to a secretion of between 6.0 and 18.9 g of nitrogen in the saliva each day. Juste (1982) found the saliva produced by pigs receiving cereal diets to contain 0.63-0.95 mg/ml of protein, with a volume of 13.1-32.1 litres per day, corresponding to a secretion of 8.3-30.5 g of protein (approximately 1.3-4.9 g of nitrogen) per day.

Stomach

The stomach is located behind the left side of the diaphragm, attached to the distal end of the oesophagus and is divided into the cardia, fundus, body and pylorus. Food enters at the cardiac region through the oesophageal sphincter, moves through the fundus and body sections, and then exits to the small intestine through the pylorus.

The stomach is a storage organ for ingested food and controls the rate at which digesta enter the intestine (Davenport, 1982). Contractions of the muscular walls of the stomach mechanically break down the ingesta. Parietal cells in the mucosa lining the body region of the stomach secrete acid that helps sterilise digesta before they reach the more permeable absorptive surfaces of the small intestine. The acid also provides an acidic environment for the action of the enzyme pepsin. The parietal cells also secrete intrinsic factor, which is necessary for the absorption of vitamin B_{12}. Mucus is secreted from most of the stomach mucosa and protects the lining of the stomach from the acidic environment.

Proteolysis of ingested food (as well as salivary proteins) is initiated in the stomach by the enzyme pepsin. Pepsinogen, the inactive precursor, is released from chief (peptic) cells in the fundic and antral regions of the stomach (Hirschowitz, 1957; Hersey, 1994). Pepsinogen is activated to form pepsin by the hydrolytic removal of a peptide from the N-terminal end of the molecule (Kidder and Manners, 1978; Hersey, 1994). The activation of pepsin is then autocatalytic. This activation is usually initiated at pH 4 (Taylor, 1968), with no pepsin activity above pH 6 (Taylor, 1959a).

Once pepsin has been activated, there are two optimum pH ranges for its activity: 1.6-2.4 and 3.3-4.0 (Taylor, 1959b). Food entering the stomach normally has a pH of seven. The pH slowly decreases as the food is mixed with the acid. The digesta does not, however, always remain in the stomach for sufficient time to allow the pH to fall to the lower of pepsin's optimum pH ranges and optimise the action of pepsin. The time required to lower the pH of the stomach contents depends on the nature of the diet (Rogers and Harper, 1966), and the amount and type of protein. The full potential of pepsin in hydrolysing protein is probably rarely achieved. Gastric proteolysis exposes the peptide bonds which will be susceptible to hydrolysis by the more specific intestinal enzymes (Gitler, 1964).

Conflicting results have been obtained on the importance of the stomach for protein digestion. Taylor (1968) concluded that peptic digestion is non-essential for protein hydrolysis and absorption, as a deficiency in pepsin secretion due to gastrectomy or pernicious anaemia did not result in an increased nitrogen excretion. Everson (1952) found protein assimilation to be affected by gastrectomy in a third of human patients and over three-quarters of the dogs studied. In the pig, gastrectomy has been shown to significantly reduce the digestibility of fat, dry matter and protein (Cunningham, 1967).

There is a small but continuous basal secretion of pepsinogen from chief cells in humans and rats (Hirschowitz *et al.*, 1957; Hersey, 1994), and an intermittent secretion in cats and dogs (Hirschowitz *et al.*, 1957). Vagal stimulation, which occurs during the process of eating, is a powerful stimulus for additional pepsinogen secretion (Davenport, 1982; Skak-Nielsen *et al.*, 1988). Pepsinogen secretion is also stimulated by the hormone secretin, released from the duodenal mucosa following acidification of the duodenum (Walsh, 1981). Cholecystokinin, secreted from cells in the mucosa of the upper small intestine, also stimulates pepsinogen release (Hersey *et al.*, 1983, Kasbekar *et al.*, 1983). The different stimulants of pepsinogen secretion are synergistic (Davenport, 1982).

The sources of nitrogen in gastric juice are enzymes, mucus and desquamated cells. The daily nitrogen secretion in the stomach of the pig has been reported to be 9.6 g (Tkacev, 1980) and 8 g (Zebrowska *et al.*, 1983). The concentration of protein in gastric juice has been shown to be 3.3 g/l in the human (Richmond *et al.*, 1955) and 10-11 g/l in the dog (Davenport, 1982).

Small Intestine

The small intestine is divided into three parts; the duodenum, jejunum and ileum (Dyce *et al.*, 1996). The duodenum is the most proximal part of the intestine, starting at the pylorus of the stomach. Ducts from the pancreas and liver enter this part of the intestine. When the small intestine enters the mesentery, it becomes the jejunum. The boundary between the jejunum and ileum is structurally indistinct.

Almost all protein digestion occurs in the small intestine (Borgström *et al.*, 1957) and there is a considerable absorption of the products of digestion as well as water and electrolytes from salivary, gastric, pancreatic and hepatic secretions. Digesta, along with salivary and gastric secretions, have a low pH when they enters the duodenum from the stomach. The digesta are mixed with pancreatic juice, bile and intestinal secretions, increasing the pH to between six and seven (Kidders and Manners, 1978).

Pancreas

The pancreas lies between the stomach and duodenum and produces both endocrine and exocrine secretions (Jamieson, 1988; Low and Zebrowska, 1989). The exocrine secretion contains digestive enzymes that are synthesised in, and secreted from,

pancreatic acinar cells (Johnson, 1981; Davenport, 1982; Jamieson, 1988; Solomon, 1994), and an aqueous bicarbonate secretion released by the centroacinar cells (Johnson, 1981; Jamieson, 1988). This bicarbonate secretion neutralises the duodenal contents, preventing damage to the duodenal mucosa by gastric acid and pepsin. It also elevates the pH of the digesta into the optimal range for the activity of the pancreatic enzymes (Johnson, 1981). The pancreas plays a very important role in digestion. Following a total pancreatectomy in the dog, nitrogen absorption an hour after feeding is decreased by 30% compared with the "intact" dog (Douglas *et al.*, 1953; Shingleton *et al.*, 1955). A decrease of up to 70% in the apparent digestibility of nitrogen after ligature of the pig pancreatic duct has also been reported (Pekas *et al.*, 1964).

Pancreatic secretion is stimulated by stomach distension via a vago-vagal reflex (Johnson, 1981; Davenport, 1982; Solomon, 1994), by the gastric hormone gastrin and the hormones secretin and cholecystokinin (CCK) (Konturek *et al.*, 1987; Camello *et al.*, 1994; Soloman, 1994). Secretin is secreted by endocrine cells in the duodenal mucosa in response to the presence of acid in the duodenal contents. Secretin stimulates the release of a mainly bicarbonate solution from the pancreas (Wang and Grossman, 1951; Hong *et al.*, 1967; Meyer *et al.*, 1970; Solomon, 1994). In general, the release of CCK from the duodenal mucosa is stimulated by the products of fat digestion (Cuber *et al.*, 1987; Douglas *et al.*, 1988; Low and Zebrowska, 1989) and peptones (Low and Zebrowska, 1989; Shimizu *et al.*, 1994) and provokes the release of a mainly enzymic secretion from the pancreas (Camello *et al.*, 1994; Solomon, 1994). There are conflicting reports in the literature regarding the exact stimuli for CCK release and the stimuli may be species specific. In the rat, intact dietary proteins have been shown to strongly stimulate CCK release (Liddle *et al.*, 1985; Liddle *et al.*, 1986), but amino acids and fat are reported to have little effect (Liddle *et al.*, 1985; Liddle *et al.*, 1986). In contrast, Shimizu *et al.* (1994) found that mixed amino acid solutions do stimulate CCK secretion in the rat and Guan and Green (1996) found peptic hydrolysates of bovine serum albumin (BSA) to be more effective at stimulating pancreatic secretion than intact BSA. In the dog, the presence in the intestines of intact proteins such as serum albumin, haemoglobin, casein and gelatin do not stimulate pancreatic secretion (Meyer and Kelly, 1976), whereas crude enzymic digests of protein, containing peptides and amino acids are effective stimulants of CCK release (Wang and Grossman, 1951) and pancreatic enzyme secretion (Thomas and Crider, 1941; Wang and Grossman, 1951; Meyer and Kelly, 1976). Meyer *et al.* (1976) found only phenylalanine to be an effective stimulator of CCK release in the dog,

whereas Konturek *et al.* (1973) found CCK secretion in the dog to be provoked by almost all L-isomers of amino acids, with both tryptophan and phenylalanine the most effective. In the human, mixtures of amino acids stimulate CCK secretion (Watanabe *et al.*, 1986) with phenylalanine, valine, methionine and tryptophan being the most effective stimulators of CCK secretion (Go *et al.*, 1970).

Acetylcholine (ACh) is another strong stimulator of pancreatic enzyme secretion. Vagal stimulation during feeding results in the release of ACh from parasympathetic nerves (Low and Zebrowska, 1989). Truncal vagotomy reduces secretion from the pancreas in response to a meal by approximately 60% (Johnson, 1981), indicating the importance of this mechanism. The stimulatory effects of CCK on pancreatic release are augmented by ACh and *vice versa.*

Most pancreatic enzymes are secreted into the duodenum as inactive precursors. The enzymes found in pancreatic juice include trypsin (secreted as the inactive zymogen trypsinogen), chymotrypsin (secreted as chymotrypsinogen), carboxypeptidase, pancreatic amylase, lipolytic enzymes (pancreatic lipase and phospholipase A and B), the nuclease ribonuclease I and elastase.

Trypsinogen is initially converted to trypsin, its active form, by the enzymic action of enterokinase which is located in the brush border of the duodenal mucosal cells (Holmes and Lobley, 1970; Lobley and Holmes, 1970; Nordström and Dahlqvist, 1970; Nordström and Dahlqvist, 1971; Hadom *et al.*, 1971; Lowe, 1994). The activation of trypsinogen is then autocatalytic at pH 7.0-9.0. Trypsin then activates the other pancreatic enzymes (Yamashina, 1956; Gray and Cooper, 1971).

The proteolytic pancreatic enzymes may be classified as either endopeptidases or carboxypeptidases (exopeptidases) depending on where they act on the protein chain. Trypsin, chymotrypsin, elastase (pancreatopeptidase E) and elastase II are endopeptidases, hydrolysing susceptible peptide bonds within the protein chain, provided the bond is accessible to the enzyme. The carboxypeptidases (A and B) remove an amino acid residue from the carboxyl end of the protein chain (Holmes and Lobley, 1970; Nordström and Dahlqvist, 1970; Nordström and Dahlqvist, 1971; Hadom *et al.*, 1971, Lowe, 1994) and are particularly effective on trypsin and chymotrypsin hydrolysates, thus continuing the proteolysis carried out by pancreatic endopeptidases (Gray and Cooper, 1971).

Pancreatic amylase is secreted in its active form. It hydrolyses α-1,4-glucosidic bonds in starch, breaking unbranched starch into maltose and maltotriose. The products from branched starch after the action of amylase are glucose, maltose, maltotriose and a mixture of dextrins.

Pancreatic lipase acts on water-soluble triglycerides, breaking them down to fatty acids and 2-monoglycerides. Colipase, which is also secreted from the pancreas, and bile salts are required for the action of pancreatic lipase. Phospholipase A and B (activated by trypsin) break down the phosphate-containing lipids lecithin and lysolecithin, releasing fatty acids.

The quantity of pancreatic enzymes secreted is thought to be approximately ten times the amount required to digest the amounts of food normally ingested (Corring, 1980a) thus ensuring maximum digestion. Secretion of proteolytic enzymes from the pancreas is approximately proportional to the quantity of nitrogen orally ingested in the rat (Schick *et al.*, 1984) or intestinally infused in humans (Vidon *et al.* 1978). The volume of pancreatic juice also increases as meal size increases. However, the total amount of protein produced daily by the pancreas has been shown to be the same in rats fed a starch-rich diet as those fed a protein-rich diet. The enzymic profile of the secretion changes, but not the total protein concentration (Abdeljlil and Desnuelle, 1964).

Approximately 40% of the total nitrogen in pig pancreatic secretion is of a non-protein origin (Corring and Jung, 1972). In the 45 kg pig, daily pancreatic juice flows of 2500 ml (Corring, 1980b), 1850 ml (Corring *et al.*, 1990) and 1270-4960 ml (Partridge *et al.*, 1982) have been reported, containing 18 g, 12 g and 6.7-9.8 g of protein, respectively. In the 34 kg pig, 13-19 g of protein has been reported to be secreted in pancreatic juice daily (Zebrowska, 1985), and 11-12 g of protein has been reported to be secreted in the pancreatic juice of the 40 kg pig (Zebrowska *et al.*, 1983).

The Gall Bladder and Bile Secretion

Bile contains bile salts, which are powerful emulsifying agents that assist in the emulsification, hydrolysis and absorption of fat. Bile salts are also the major breakdown products of cholesterol (Wiseman, 1964; Haslewood, 1967; Weisbrodt, 1981; Davenport, 1982; Corring *et al.*, 1989; Hofmann, 1994).

Bile is secreted by the polygonal cells in the liver into ducts that converge to eventually form the cystic duct that enters the gall bladder. In the gall bladder, bile is concentrated due to the absorption of water and electrolytes, namely Na^+, Cl^- and HCO_3^-. The cystic duct from the gall bladder converges with the hepatic duct and then passes to the duodenum. Bile enters through the sphincter of Oddi into the duodenum following contraction of the gall bladder. The hormone CCK, released as described above, causes the gall bladder muscle to contract (Sanford, 1982). The secretion of CCK is inhibited by the presence in the intestine of mixtures of bile acids and amino acids or fat, but not bile acids alone. With no CCK present, the gall bladder ceases to contract and the flow of bile into the small intestine ceases (Sanford, 1982). Stimulation of the sympathetic nervous system causes the gall bladder to relax, while parasympathetic stimulation via the vagus nerve, which occurs during feeding, provokes gall bladder contraction.

The major organic solutes present in bile are bile acids, phospholipids, cholesterol and bilirubin. Mucus is also present in bile (Haslewood, 1978). Bile acids are water-soluble derivatives of cholesterol. The two primary bile acids are choleic acid and chenodeoxycholic (chenic) acid, which are synthesised in the liver and conjugated with glycine or taurine. Conjugated bile acids are less likely to be precipitated by acid or calcium and are absorbed more slowly in the proximal small intestine than unconjugated acids.

As the conjugated primary bile acids pass along the small intestine, approximately 75% are unaltered. They are reabsorbed in the ileum by active transport and returned to the liver via the portal circulation. The remaining 25% are deconjugated by bacteria in the ileum (Sanford, 1982) and most are reabsorbed and reconjugated in the liver. Each day, 25-33% of the primary bile acids are excreted in the faeces or converted by small intestinal bacteria into secondary bile acids. A proportion of the secondary bile acids is absorbed in the same way as the primary bile acids and the remainder is excreted.

In humans, between 700 and 1200 ml of bile can be secreted each day (Sanford, 1982). Human bile contains 1.0-4.0 g/100 ml of proteinaceous material such as mucins, protein and enzymes (Haslewood, 1967). In the pig, the amount of bile secreted each day has been reported to be 1.7 l/24 hrs (Sambrook, 1981) 2.06 l/24 hrs (Juste *et al.*, 1979), 2.07-2.45 l/24 hrs (Laplace and Ouaissi, 1977) and 1.82 l/24 hrs (Corring *et al.*, 1990). The total nitrogen that is secreted in the bile of the pig has been

reported to be 1.8-1.9 g/24 hrs (Sambrook, 1978; Sambrook, 1981) and 1.7 g/24 hrs (Corring *et al.*, 1990). Most of this nitrogen secreted within bile will be reabsorbed.

The Brush Border

The brush border of the intestine contains many peptidases, including an endopeptidase (Fulcher and Kenny, 1983; Yoshioka *et al.*, 1988), aminopeptidases (Maroux *et al.*, 1973; Svensson *et al.*, 1978; Benajiba and Maroux, 1980), carboxypeptidases (Skovbjerg, 1981; Yoshioka *et al.*, 1988; Erikson *et al.*, 1989), an aminotripeptidase (Adibi and Kim, 1981), and dipeptidases (Norén *et al.*, 1973; Sjostrom *et al.*, 1978). Dipeptidases are also present in the cytosol of enterocytes (Peters, 1970).

Larger peptides are split by aminopeptidases in the brush border while small peptides are absorbed intact and hydrolysed by aminopeptidases within mucosal cells (Peters, 1970; Kim *et al.*, 1974). *In vitro* studies have shown that cytosolic peptidases in the rat and human are capable of hydrolysing di- and tri-peptidases, but not larger peptides. In contrast, the brush border enzymes have been shown to hydrolyse large protein substrates including a heterohexapeptide (Peters *et al.*, 1972; Kim *et al.*, 1974).

Intestinal enzyme activities are distributed regionally. For example, alkaline phosphatases are predominant in the duodenum, disaccharides such as lactosephlorizin hydrolase and sucrose isomaltase are predominant in the jejunum (Skovberg, 1981) and aminopeptidases such as dipeptidyl peptidase IV and aspartate aminopeptidase are predominant in the ileum (Skovberg, 1981).

Other Proteinaceous Secretions into the Small Intestine

In addition to digestive enzymes and bile, protein enters the small intestine in the form of mucus, desquamated cells and leaked plasma protein.

The mucosa of the stomach and intestine contains cells that synthesise and secrete mucus into the lumen of the gastrointestinal tract. This mucus provides a protective buffer zone between the mucosa and the luminal contents, as a stable unstirred layer. Low molecular weight solutes (<1000 daltons) can move through the mucus layer, but larger macromolecules can not. In the stomach and duodenum, mucus combines with bicarbonate, and protects the epithelium from acid and digestive enzymes.

Other gastrointestinal secretions such as enzymes and IgA, lactoferrin as well as microorganisms, sloughed cells and ingested matter at various stages of digestion may also be present in the mucus layer (Mantle and Allen, 1989).

The proteolytic enzymes pepsin, chymotrypsin and trypsin reduce the viscosity of mucin solutions by breaking the side chains of the protein moiety (Hashimoto *et al.*, 1963). The protein core cannot be further fragmented unless the carbohydrate chains are removed. This occurs primarily in the colon by bacterial flora (Mantle and Allen, 1989). It has been suggested that there are two regions in the glycoprotein molecule. One is glycosylated, therefore resistant to proteolysis, while the other is not glycosylated, and is partially susceptible to proteolysis (Scawen and Allen, 1977).

In the mouse, the turnover time for the whole gastrointestinal epithelial cell population averages approximately 60 hours in the duodenum, jejunum, ileum and colon (Cheng and Bjerknes, 1982). This turnover time is the time taken to replace the total number of epithelial cells in the small intestine. Different cell types vary in their turnover time. Every minute, 20-30 million cells are lost from the small intestine in the human (Croft *et al.*, 1968), which is equal to 287g of cells per 24 hours (Croft and Cotton, 1973).

Plasma proteins are present in low concentrations in saliva (Young and Schneyer, 1981; Young *et al.*, 1987) and secretions entering the stomach and intestines (Jeffries and Sleisenger, 1968). This protein is usually rapidly hydrolysed, and reabsorbed in the intestine.

The total quantity of protein that is secreted through the intestinal wall in humans has been studied using a washing technique which prevented contamination of the segment of intestine being studied from digestive secretions from other sources (da Costa, 1971). The average secretion of protein from a 5 cm segment of duodenum was 3 g/24 hrs and da Costa (1971) estimated that up to 180 g of protein could be secreted into the small intestine in 24 hours.

The amount of protein secreted in intestinal juice per 24 hours in pigs following collection of intestinal fluid from an isolated intestinal loop has been reported to be 50-62 g after feeding of a 16% protein diet (Horszczaruk *et al.*, 1974). Buraczewska (1979) reported that the daily secretion of protein through the small intestinal wall in 50-70 kg pigs averages 90 g when the intestine is perfused with a solution that is isosmotic with blood.

Amino Acid Absorption from the Intestines

After the preceding hydrolytic reactions, the proteinaceous material presented to the absorptive surfaces of the small intestine consists of a mixture of undigested proteins, small peptides and free amino acids from both endogenous and dietary sources. Approximately one third of the nitrogenous material presented for absorption is in the form of free amino acids and there are also many small peptides. The predominant site of amino acid absorption is dependent on the species. The proximal jejunum is the predominant site in the rat (Borgström *et al.*, 1957; Schlüssel, 1959) and human (Nixon and Mawer, 1970), whereas in the pig, the jejunum and ileum are the predominant sites of amino acid absorption (Leibholz, 1982).

Most amino acids are absorbed actively against a concentration gradient (Wiseman, 1953; Wiseman, 1956), with co-transport of a Na^+ ion (Moe and Jackson, 1987; Ganapathy *et al.*, 1994). The appropriate Na^+ concentration for this form of transport (high Na^+ concentration intracellularly and low Na^+ concentration extracelluarly) is maintained by Na^+/K^+ ATPase in the basolateral membrane (Quigley and Gotterer, 1969). Other amino acids can be absorbed by simple diffusion and facilitated diffusion (Na^+-independent) (Stevens *et al.*, 1984). The significance of each method of amino acid absorption is dependent on the initial amino acid concentrations. The amount of amino acids taken up by diffusion makes a progressively greater contribution to the amino acid uptake as the substrate concentration increases (Stevens *et al.*, 1984).

Each amino acid has a characteristic absorption rate (Wiseman, 1956). This absorption rate varies with the presence of other amino acids, suggesting competition for the same carrier mechanisms (Wiseman, 1954; Spencer and Samiy, 1961; Cohen and Haung, 1964; Matthews and Laster, 1965).

Small peptides including di-peptides (Newey and Smyth, 1959; Matthews *et al.*, 1968) and tri-peptides (Matthews *et al.*, 1968) can move intact into mucosal cells. Larger peptides (up to 6 amino acids long) are hydrolysed by brush border peptidases. Once peptides have been absorbed from the intestine into enterocytes, they are hydrolysed by di- and tri-peptidases present in the cytosol of enterocytes and then released into the portal blood in the form of amino acids (Newey and Smyth, 1959; Newey and Smyth, 1960). It appears that some peptides are not hydrolysed to their constituent amino acids before entering the circulation (Prockop *et al.*, 1962; Perry *et al.*, 1967; Adibi, 1971; Warshaw *et al.*, 1971; Worthington *et al.*, 1974; Gardner, 1984; Gardner,

1994). For example, it has been shown that when tritium-labelled bovine serum albumen (^{3}H-BSA), with a molecular weight of over 50,000, is infused into the duodenum of the adult rat, approximately 2% of the ^{3}H-BSA is absorbed intact into the lymph and blood (Warshaw *et al.*, 1971). In another study in the adult rat (Worthington, 1974), intact BSA was also found in the blood following feeding of BSA. Conversely, in a study using enzyme hydrolysates of milk proteins in pigs, the rate of appearance of amino acids in the portal vein reached 100% with no peptides found (Rérat *et al.*, 1988). The fate of any peptides that may be present in the mesenteric circulation is unclear. They may be utilised by tissues, hydrolysed in the plasma or transported into tissue cells and then hydrolysed (Webb, 1990). It has also been proposed that these peptides may modulate health and behaviour (Gardner, 1994).

In enterocytes, amino acids may be incorporated into proteins, catabolised through transamination or deamination to provide a source of organic acids and energy for the enterocytes, or transaminated to synthesise non-essential amino acids and regulatory peptides. Most of the amino acids are released into the portal blood (Rérat and Corring, 1991).

Microbial Activity in the Small Intestine

In the duodenum of the pig, there appears to be little microbial activity (Dierick *et al.*, 1986; Knudsen and Jensen, 1991). In the ileum, however, there is significant microbial activity (Dierick *et al.*, 1986; Knudsen and Jensen, 1991; Jørgensen and Jensen, 1994) and it has been reported (Dierick *et al.*, 1983; Poppe *et al.*, 1983) that 25-30% of the total protein in ileal digesta is bound in bacterial protein. Although microbial protein is not strictly endogenous, it can not be easily separated from the endogenous material, and thus is analysed with endogenous protein. Caine *et al.* (1999) estimated that 30-46% of 'endogenous' ileal nitrogen in the pig may be of bacterial origin.

The presence of microbes in ileal digesta has implications for the storage of digesta post-collection. If not treated, the microbes will continue to be active, degrading some amino acids in the digesta and incorporating others into microbial protein. This will change the amino acid profile of the endogenous (and exogenous) protein. Therefore, following collection, digesta should be acidified to prevent further microbial activity, or immediately frozen.

The Hindgut

The main function of the hindgut is the digestion of complex carbohydrates and the absorption of water and electrolytes from the digesta. Undigested protein, along with the peptides and free amino acids that have not been absorbed in the small intestine, enter the hindgut where most are metabolised by micro-organisms (Just, 1980; Parsons *et al.*, 1982; McNeil, 1988). In most monogastric animals, few or no amino acids are absorbed intact from the large intestine (Zebrowska, 1973; Zebrowska, 1975; Zebrowska, 1978; Just *et al.*, 1981; Schmitz *et al.*, 1991a; Darragh *et al.*, 1994). Most nitrogen is absorbed in the form of ammonia, with some as amines or amides. Normally, this nitrogen is of no nutritional value to the animal and is ultimately excreted in the urine as urea (Zebrowska, 1973; Zebrowska, 1982; Just, 1983; McNeil, 1988). The microbes also synthesise amino acids, particularly lysine and methionine (Just, 1980; Low, 1980). These amino acids are excreted in the faeces, along with those from the digesta that remain unabsorbed.

COLLECTION OF ILEAL DIGESTA

Several methods have been devised for collecting digesta from the terminal ileum of mammals, such as the rat and pig. These include the slaughter technique, anastomosis and cannulation techniques. Many of these methods rely on the use of an indigestible dietary marker, such as chromic oxide, which is included in the diet so that the total endogenous ileal flows can be determined from a sample of digesta. The following section commences with a review of dietary indigestible markers by way of introduction to a description of the methods that can be used for the collection of ileal digesta.

Indigestible markers

In many situations, it is not practical or possible to collect all of the digesta that pass through the terminal ileum during a 24-hour period. To determine endogenous ileal flows, an indigestible marker may be used to allow the results obtained for a sample of digesta to be extrapolated to a more quantitative value.

The main criterion for an indigestible marker is that it is not digested or absorbed by the animal and can be quantitatively collected in the faeces. The suitability of a marker can be assessed by determining the fraction of the marker fed to the animal that is recovered in the faeces. Ideally the recovery of the marker should be 100%, or a constant value, which would allow the use of a correction factor. The marker must also

have no effects on the digestion and/or absorption of any dietary constituents and must be able to be accurately determined chemically. The marker must be able to be completely and uniformly mixed into the diet, and move through the intestines with the substances that are being measured. Markers that have been used when measuring endogenous ileal protein flows include "internal" markers such as acid insoluble ash, which are already present in the diet and "external" markers such as chromic oxide and titanium dioxide, which are added to the diet.

Chromic oxide is not toxic to the animal (Kotb and Luckey, 1972) and has been completely (98-102%) recovered following quantitative faecal collection in many studies (Lloyd *et al.*, 1955; Ehle *et al.*, 1982; Saha and Gilbreath, 1991; Saha and Gilbreath, 1993; Bakker and Jongbloed, 1994; Mroz *et al.*, 1996). Other studies, however, have found lower recoveries, in the order of 85% (Moughan *et al.*, 1991), 78-95% (Petry and Enders, 1974), 78-86% (Moore, 1957), 75-80% (Jagger *et al.*, 1992) or 75% (McCarthy *et al.*, 1974).

Possible explanations for the discrepancy in chromium recovery include under-estimation of feed refusals and thus over-estimation of the amount of feed consumed by the animal, or incomplete collections of faeces or losses during the preparation of faeces for chromium analysis (Moore, 1957). Saha and Gilbreath (1991) and Fuller *et al.* (1994) have suggested that some chromium may be retained in the digestive tract. Moore (1957) reported the observation that chromium with a small particle size gave lower recoveries than that with a larger particle size when fed to pigs. When chromium is used as a digestibility marker, the retention of a fraction of the chromium in the digestive tract would be the only one of these possibilities that may affect the results, assuming that there is no absorption of chromium from the digestive tract. Even if there is a small amount of chromium retention in the digestive tract, as long as the animal is fed chromium for several days before sampling commences, the chromium exchange should be in "equilibrium" and the amount of chromium in the faeces (or total digesta) should equal that ingested in the diet. Overall, chromic oxide appears to be suitable for use as a marker when complete collections of digesta are not possible.

Slaughter Technique

The slaughter method was first suggested by Payne *et al.* (1968) as a simple method for the collection of ileal digesta in birds. It has also been applied to other simple-stomached animals such as the rat and pig (Butts *et al.*, 1991; Donkoh *et al.*, 1994a; Donkoh *et al.*, 1994b; van Barneveld *et al.*, 1994; van Wijk *et al.*, 1998). The

technique involves the removal of a distal section of the small intestine from the anaesthetised or recently euthanased animal and collection of the contents of the removed segment of intestine. However, mucosal cells are rapidly shed into the gut lumen following death and may be present in the digesta (Badawy *et al.*, 1957; Fell 1961), which will increase the nitrogen content of the digesta. This problem can be minimised if barbiturates are used to euthanase the animal, and the digesta are collected immediately after death (Badawy *et al.*, 1957; Thorpe and Thomlinson, 1967).

When digesta are collected from the rat or pig using the slaughter method, the animal is often fed at hourly intervals for several hours before sample collection. This induces a relatively constant flow of digesta at the terminal ileum, ensuring that the sample is as representative as possible of "normal" digesta. Alternatively, digesta can be collected at a set time after the commencement of feeding. Four and nine hours after feeding are suitable sampling times in the rat (Donkoh *et al.*, 1994a; van Wijk et al, 1998) and pig (Donkoh *et al.*, 1994b), respectively.

The major advantage of the slaughter method is its simplicity and ethical acceptability compared to cannulation and anastomosis techniques. Moreover, there is minimal interference with the animal's digestive tract prior to sampling and there are no limitations on the type of diet fed to the animal as there may be with cannulated animals, because of potential blockages of the cannulas. This method has the further advantage that digesta can be sampled from several parts of the digestive tract.

However, there exists the possibility of a bias in results due to a non-representative sample because of the limited amount of digesta collected. Furthermore, measurements can only be made at one time for each animal and non-digestible dietary markers must be used.

In the growing pig, controlled studies comparing protein digestibility obtained using the slaughter method and simple T-cannulated animals have produced similar mean results and similar variances about the mean (Moughan and Smith, 1987; Donkoh *et al.* 1994b). The slaughter technique appears to be a valuable method for the collection of digesta samples from small animals such as the rat, and this method is also useful for application to larger animals such as the pig.

Anastomosis

Anastomosis has been used to collect ileal digesta both in the pig (Fuller and Livingstone, 1982; Hennig *et al.*, 1986) and rat (Fuller *et al.*, 1994). There are several forms of this technique, as shown in Figure 1. All of these variations involve transecting the ileum anterior to the ileo-caecal sphincter and joining this to the side of the descending colon. In variation 1, the large intestine is not isolated and will remain partially functional. The disadvantage of this variation is the possible contamination of ileal digesta with material from the large intestine. In the second variation, the large intestine is isolated and the ileum attached to the rectum (Green *et al.*, 1987; Köhler *et al.*, 1992a). The large intestine may, however, remain partially functional resulting in a build up of gas from microbial fermentation, which can lead to the animal's death due to constriction of the gastrointestinal tract in the abdomen. Variation 3 is similar to variation 2, but a simple cannula is inserted into the isolated colon (Green *et al.*, 1988) to allow the digesta initially present and products of residue fermentation to escape (Green *et al.*, 1987). Anastomised pigs need supplements of sodium and other minerals to compensate for the lack of a fully functional large intestine (Hennig *et al.*, 1989; Laplace *et al.*, 1989; Fuller, 1991).

The major advantage with anastomosis techniques is that digesta can be collected quantitatively via the anus, which eliminates the need for a dietary marker. Also, there are no problems with blockage and digesta leakage that may occur with some cannulation methods.

Several studies have been carried out to examine the metabolic and physiological effects of anastomosis in the pig. Köhler *et al.* (1992a) demonstrated that pigs with an anastomosis have a significantly ($P<0.01$) lower liveweight gain compared with intact animals, although a decreased liveweight gain compared to intact animals was not found by Yin *et al.* (1993). Köhler *et al.* (1992b) reported that blood concentrations of urea, creatinine, Na, K, base excess and bicarbonate, and the pH of the blood were all significantly different in anastomised pigs compared with intact pigs, as were the animals' overall Na and K balances. Nitrogen retention has been reported to be lower in pigs with an anastomosis than in intact pigs (Hennig *et al.*, 1989, Köhler *et al.* 1992a). Volatile fatty acid levels in the ileal digesta of anastomised pigs (Köhler *et al.*, 1992a) have been reported to be higher than comparable reported values for intact (Argenzio and Southworth, 1974; Clemens *et al.*, 1975) or T-cannulated pigs (Fuller, 1991), indirectly indicating increased microbial activity. This is supported by results obtained by Köhler *et al.* (1992a) showing an increase in diaminopimelic acid (DAPA),

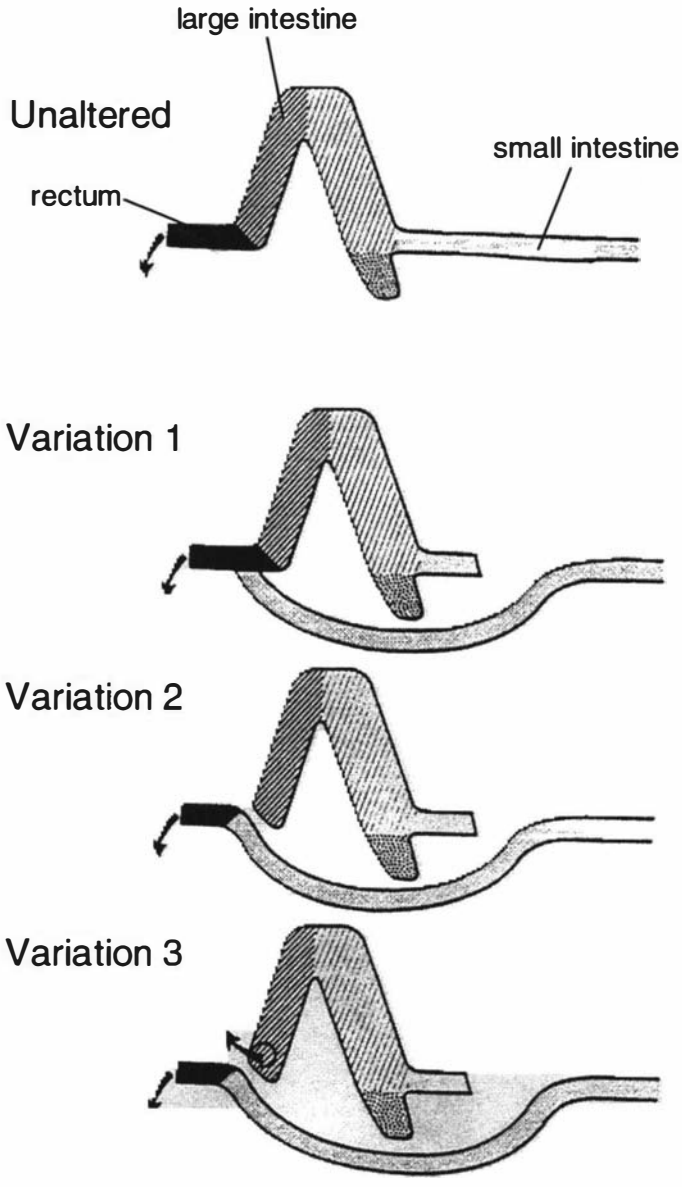

Figure 1 Schematic diagram of the different methods for ileo-rectal anastomosis (modified from Sauer *et al.*, 1989).

which is found only in bacteria, in the digesta from anastomised pigs above that found in intact pigs. Anastomised pigs are reported to have a 12% higher maintenance requirement for energy than intact animals (Herrman *et al.*, 1989) and Hennig *et al.* (1989) recommended that diets for anastomised pigs should be supplemented with easily soluble carbohydrates at a rate of 100 g/kg dry matter intake for the normal growth of these animals. Histological changes in the terminal ileum of anastomised pigs have also been reported (Fuller, 1991; Redlich *et al.*, 1993; Redlich *et al.*, 1997).

Comparisons of anastomised pigs with cannulated pigs have shown generally similar values for the digestibility of crude protein and amino acids (Hennig *et al.*, 1990; Leterme *et al.*, 1990; Fuller, 1991; Hennig *et al.*, 1991; Hennig *et al.*, 1992; Yin *et al.*, 1993; Torrallardona *et al.*, 1997), but not in all cases (Darcy-Vrillon and Laplace, 1985; Darcy-Vrillon and Laplace, 1990; Köhler *et al.*, 1991). Statistically significant differences between anastomised and cannulated pigs have, however, been reported for the digestibility of amino acids (Leterme *et al.*, 1990; Köhler *et al.*, 1991; Hennig *et al.*, 1992; Yin *et al.*, 1993). Dry matter (Fuller, 1991; Köhler *et al.*, 1991; Yin *et al.*, 1993) and organic matter digestibilities (Hennig *et al.*, 1991; Yin *et al.*, 1993) have been found to differ between anastomised and cannulated pigs, although this may depend on the diet given to the pigs (Torrallardona *et al.*, 1997). The transit time of digesta in anastomised pigs has been reported to be shorter than that for T-cannulated pigs (Leterme *et al.*, 1991). Nitrogen balance tests with anastomised pigs have demonstrated that they excrete significantly less nitrogen in the urine and more in the excreta (ileal digesta) than do T-cannulated pigs, although the two methods have been reported to give statistically equivalent results for the biological value or net protein utilisation of pea proteins (Leterme *et al.*, 1990).

Overall, the evidence suggests that digestion and metabolism in anastomised pigs is altered compared to intact pigs, and this must call into question the validity of this technique.

T-Cannula

The most straightforward cannulation technique is the surgical implantation of a simple T-shaped cannula in the terminal ileum as described by Livingstone *et al.* (1977) and Gargallo and Zimmerman (1980). The intestinal flow is divided, with a portion continuing along the intestine and the remainder being diverted through the cannula. It is assumed that the portion collected from the cannula is representative of the total flow, with no fractionation of the components due to the division of flow. This may not

always be so, especially with high fibre diets. The stem of the cannula may become blocked with fibrous material, particularly in the case of high fibre diets.

T-cannulas do not allow complete collection of digesta, so the technique requires a marker in the diet, although if digesta are continuously collected through the T-cannula, high marker recoveries (62-95%) are possible (Butts *et al.*, 1993a).

Comparison of T-cannulated pigs with intact pigs has shown no effect of the presence of the cannula on appetite and growth rate (Livingstone *et al.*, 1977). As noted above, comparisons between T-cannulated pigs and the slaughter method have yielded similar results for ileal amino acid digestibility for pigs fed meat and bone meal-based (Donkoh *et al.*, 1994b) or barley-based (Moughan and Smith, 1987) diets.

Re-entrant Cannula

Another technique used in the pig for the collection of ileal digesta is the re-entrant cannula as described by Cunningham *et al.* (1962). To surgically implant a re-entrant cannula, the terminal ileum is transected 15-20 cm from the ileocaecal junction and the two ends are sealed. A T-cannula is then inserted into the intact ileum near the sealed stump (the proximal cannula) and a second cannula is inserted into the caecum near the ileocaecal junction (the distal cannula). The cannulas are exteriorised and connected with a length of plastic tubing. The tubing is removed for sampling, and a quantitative collection of digesta can be made (Easter and Tanksley, 1973), thus eliminating the need for a marker in the diet. Leakage of digesta around the stem of the reentrant cannula in pigs has, however, been reported (Köhler *et al.*, 1990; Köhler *et al.*, 1991), in which case a marker may be required to correct for any losses of digesta. Beynen *et al.* (1986) demonstrated that faecal digestibility of organic matter, crude protein, fibre and amino acids does not differ between intact pigs and the same pigs after surgical preparation with a re-entrant cannula. This suggests that the presence of the re-entrant cannula does not significantly affect digestion in the animal.

In addition to the relatively complex surgical intervention, this method has several disadvantages. Firstly, the small intestine is completely transected, which interrupts the transmission of migrating myoelectric complexes (Laplace and Borgida, 1976; Wenham and Wybum, 1980) that provoke intestinal movement responsible for normal digesta flow. While the cannula is in "re-entrant mode" digesta are propelled through the cannula solely due to the pressure generated by the entrance of more digesta into the cannula. Consequently, blockages are common with all diets except those that

have been finely ground. This problem is exacerbated by the reduced motility of the small intestine and it is not possible to obtain adequate samples for many diet ingredients without additional grinding of the diet. There may also be an hypertrophy of the small intestine close to the cannula (Laplace and Borgida, 1976).

A modification of the original re-entrant cannulation procedure has been developed (Darcy *et al.*, 1980), called the post-ileocolic valve fistulation technique, whereby the proximal cannula is located immediately distal to the ileocaecal valve. This maintains the integrity of the small intestine and the movement of digesta through the ileo-caecal valve. However, the surgery required to insert this cannula is extensive and the two parts of the cannula are not joined. Digesta are collected continuously from the proximal cannula and subsequently returned intermittently through the distal cannula. Although fewer, there are still many instances of cannula blockages with the modified technique (Fuller, 1991) and additional grinding of some dietary ingredients is still required to obtain adequate digesta samples (Fuller *et al.*, 1994).

Post-valve T-Caecum Cannula

The insertion of the post-valve T-caecum (PVTC) cannula in the pig (van Leeuwen *et al.*, 1991) involves the removal of approximately two-thirds of the caecum and the insertion of a cannula into the remaining segment, opposite the ileo-caecal valve. When the bung is removed from the cannula, the difference in the intra-abdominal and atmospheric pressures causes the ileocaecal valve to collapse into the mouth of the cannula. This causes the digesta leaving the ileum to drain via the cannula.

The major advantage of this approach is that the small intestine is not transected, so there are minimal effects on ileal muscle movement. Also, the bore of the cannula is sufficiently large to prevent blockages, hence there is no restriction on the type of diet that can be fed to the animal. However, the removal of a substantial part of the caecum may alter the metabolism of the animal, and this remains to be thoroughly investigated. Mean marker recoveries of ileal digesta of 71.4% (Köhler *et al.*, 1991), 71.6, 71.9, 90.8, and 106.4% (den Hartog *et al.*, 1988; Köhler *et al.*, 1990) have been reported with PVTC cannulated pigs.

The effect on the animal of long-term (12 weeks) PVTC cannulation has been examined by Köhler *et al.* (1992a, b). Cannulated pigs were compared with intact, non-cannulated pigs for growth performance, N retention, the weights of the liver, spleen and adrenal glands, Na and K balances, and blood variables including the pH

and concentrations of urea, creatinine, Na, K, Cl, Mg, bicarbonate and base excess. For all of the parameters tested, except for the concentrations of urea and potassium in the blood, there were no significant differences between the two groups of pigs. It was concluded that the PVTC cannula does not significantly alter the metabolism of the pig and, therefore, is suitable for the collection of digesta in metabolic studies.

MEASUREMENT OF ENDOGENOUS PROTEIN FLOW

Traditionally, endogenous ileal amino acid flow is determined after feeding animals a protein-free diet. This approach, however, has major disadvantages and over the last decade, a considerable amount of research has been undertaken to develop alternative approaches.

Protein-free Diets

This approach involves feeding the animal a protein-free diet, collecting digesta from the terminal ileum, and then determining the nitrogen and amino acid flows in the digesta. All of the nitrogen and amino acids appearing in the digesta must, by definition, be of endogenous origin.

The method has been criticised, however, due to the "unphysiological" nature of the treatment, as the animals are in a negative nitrogen balance. Protein-free feeding may result in a reduction in the amount of protein secreted into the gut (Snook and Meyer, 1964; Fauconneau and Michel, 1970; Corring and Saucier, 1972; Horszczaruk *et al.*, 1974; Schneeman *et al.*, 1977; Buraczewska, 1979; Buraczewski, 1980; Rodriguez *et al.*, 1982; Schneeman, 1982; Rodriguez *et al.*, 1983) and a general decrease in the rate of protein synthesis in the body and gut (Munro and Goldberg, 1964; Millward *et al.*, 1976; Simon, 1989; Muramatsu, 1990). There may also be an increase in the breakdown and reutilisation of enzymes secreted into the gut (Snook and Meyer, 1964; Fauconneau and Michel, 1970). All of these effects potentially lead to a decrease in endogenous protein loss under protein-free feeding. Conversely, however, it is possible that the reduced enzyme synthesis and activity in the protein-free state may result in a lowered digestibility of endogenous protein. This could lead to an accumulation of endogenous nitrogen at the terminal ileum. It should be noted, however, that the protein-free diet is generally fed to the animals for a period of 7-days or more before digesta samples are taken, to allow the animal's metabolism to adjust to the diet. The protein-free diet could, theoretically, be given to the test animals for a shorter period of time before samples are taken, thus avoiding or

minimising the effects on metabolism. Applying the method for a shorter period of time may result in estimates of endogenous ileal amino acid flows that are more physiologically relevant than those determined when the protein-free diet is fed to the animals for a longer time. This possibility needs investigating.

High excretions of proline in association with protein-free feeding have been documented (Sauer *et al.*, 1977; Taverner *et al.*, 1981; Sauer, 1982; Skilton *et al.*, 1988). When animals are fed a protein-free diet and enter a negative nitrogen balance, body proteins and especially muscle protein are broken down releasing large amounts of alanine or glutamine (Rodwell, 1985). Glutamine is metabolised by the intestinal tissue to form proline, amongst other compounds (Rodwell, 1985; Rogers and Phang, 1985), thereby causing the high excretion of proline in the digesta of animals fed a protein-free diet (Sauer and de Lange, 1992). There is also evidence (Nagchaudhuri and Sharma, 1972) that the efficient absorption of proline is dependent on an adequate supply of protein in the diet. Endogenous ileal proline flows may or may not be over-estimated following protein-free alimentation, however, there is clearly a substantial alteration of whole body metabolism which will also affect gut metabolism when a protein-free diet is fed to an animal. Therefore, estimates of endogenous amino acid flow determined using the protein-free method are unlikely to be accurate.

Linear Regression

The regression approach for determining endogenous ileal amino acid flows involves feeding the animal increasing amounts of dietary protein (at a constant dry matter intake) and then determining the endogenous amino acid flows by extrapolating the ileal amino acid flows to zero dietary protein intake. This allows endogenous losses to be determined under more normal conditions of protein alimentation.

The main assumption made with this method is that the increase in the protein content of the ileal digesta with increasing dietary protein is due to an increased amount of undigested dietary protein, with no change in the amount of endogenous protein. This assumption may be unsound. For example, the rate of enzyme secretion from the stomach and pancreas may vary with protein intake (Fauconneau and Michel, 1970; Schneeman, 1982). Further work on the relationship between endogenous protein flows and dietary protein concentration is needed to confirm or negate this assumption.

The regression method relies upon mathematical extrapolation, with its associated errors. Consequently, the estimates of endogenous loss often have high standard errors, although the estimates reported by Fan *et al.* (1995) had relatively small standard errors when a wide range of dietary protein concentrations was used (4-24% dietary crude protein). Endogenous ileal amino acid flows determined using the regression approach appear to depend upon the range of dietary protein concentrations. Fan *et al.* (1995) reported large differences in the estimated endogenous ileal amino acid losses determined using the regression method due to differences in the ranges of graded dietary protein levels used.

It should also be noted that at low levels of protein intake, which are generally used with the regression method, enhanced losses of proline can be expected (Taverner, 1979), as is the case for protein-free feeding.

Comparisons of the regression method with the protein-free method have shown that the two methods give similar results (Taverner *et al.*, 1981; Leibholz, 1982; Leibholz and Mollah, 1988; Furuya and Kaji, 1989; Donkoh *et al.*, 1995; Mariscal-Landín *et al.*, 1995), which, given the above discussion, also calls into question the accuracy of the regression approach.

Synthetic Amino Acid Based Diets

This approach was developed to determine endogenous loss without the test animal going into negative body nitrogen balance with the accompanying metabolic adaptation, as occurs with the protein-free method. Semi-synthetic diets are formulated containing mixtures of synthetic amino acids as the sole source of nitrogen. In some of the diets selected dietary non-essential or essential amino acids are excluded, in the latter case, feeding of the diet is accompanied by intravenous infusion of the deleted essential amino acid. The amounts of the excluded amino acids in ileal digesta allow a direct determination of endogenous amino acid flows.

Experiments using this approach with rats and pigs (Skilton *et al.*, 1988; Darragh *et al.*, 1990; Butts *et al.*, 1993b) have shown that the endogenous ileal amino acid flows of lysine, aspartic acid, serine, glutamic acid and alanine are not significantly different from those determined using the protein-free method. The endogenous ileal flows of proline and glycine determined using the protein-free method were significantly greater than those determined with the synthetic amino acid based diet.

This is consistent with the hypothesis that increased flows of endogenous proline and glycine occur in animals fed a protein-free diet.

Protein-free Diets with Intravenous Amino Acid Infusion

De Lange *et al.* (1989a) intravenously infused pigs, on a protein-free diet, with either saline (control group) or a balanced mixture of free amino acids. There were no statistically significant differences in the endogenous ileal amino acid flows between the pigs with a negative nitrogen balance (saline infusion) compared with the pigs having a positive nitrogen balance (amino acid infusion), except that significantly more proline was excreted by the pigs infused with saline. This result has been confirmed more recently by Leterme *et al.* (1996a). These results, together with those obtained using synthetic amino acid based diets, suggest that a negative body nitrogen balance *per se,* does not result in lowered endogenous ileal amino acid flows.

Natural Proteins Devoid of Specific Amino Acids

The use of natural proteins devoid of specific amino acids with intravenous infusion of any essential amino acids not present in the protein, is another direct approach to determining endogenous ileal amino acid flow. With this approach, the test animal is in positive body nitrogen balance, the gut tissues have a direct supply of amino acids and digestive breakdown products (peptides) are present. However, widespread use of this technique is restricted by the limited commercial availability of such proteins.

Zein, a purified protein from maize, is almost completely lacking in lysine and tryptophan and, therefore, can be used to directly determine endogenous lysine and tryptophan flows. Butts *et al.* (1993b) fed zein-based diets to young pigs. Lysine and tryptophan were intravenously infused to supply the animals with these essential amino acids. Animals receiving the zein-based diet had statistically significantly higher ileal lysine flows than those fed protein-free diets. This suggests that proteins or protein breakdown products (peptides) may affect endogenous ileal amino acid flows, at least for lysine.

Guanidination

The guanidination method, also known as the homoarginine method, involves the guanidination of dietary protein using the agent *O*-methylisourea. Lysine residues in the protein are transformed to homoarginine, a lysine derivative that does not occur naturally in body tissue. By determining both the homoarginine and lysine contents of ileal digesta collected after feeding an animal the guanidinated diet, endogenous lysine flow can be calculated (Hagemeister and Erbersdobler, 1985; Rutherfurd and Moughan, 1990). The guanidination method may be applied in two ways. Firstly, the protein source may be partially guanidinated (the indirect method, e.g. Hagemeister and Erbersdobler, 1985). Secondly, the protein source may be completely guanidinated (the direct method, e.g. Rutherfurd and Moughan, 1990). With the direct approach, since no dietary lysine is given, all ileal lysine must be endogenous by definition.

The guanidination reaction has been applied to a diverse range of feedstuffs, including casein (Rutherfurd and Moughan, 1990; Erbersdobler and Lee, 1991; Drescher *et al.*, 1994; Roos *et al.*, 1994: Frik *et al.*, 1997; Nyachoti *et al.*, 1997), fish protein (Ravindran *et al.*, 1996), meat and bone meal (Ravindran *et al.*, 1996), gelatin (Moughan and Rutherfurd, 1990; Moughan and Rutherfurd, 1991) and vegetable proteins such as wheat protein (Frik *et al.*, 1997) soya bean protein (Rutherfurd and Moughan, 1990; Moughan and Rutherfurd, 1991; Marty *et al.*, 1994), barley and canola meal (Nyachoti *et al.*, 1997).

There are a number of assumptions that must be made with the guanidination method, all of which apply to the indirect method for the homoarginine method and some to the direct method, as detailed below. Firstly, for both the direct and indirect methods, it must be assumed that homoarginine does not cause altered protein metabolism in the animal, thus affecting endogenous nitrogen loss. Drescher *et al.* (1994) determined the digestibility of the milk proteins casein, β-lactoglobulin and lactoferrin in the miniature pig. All of the milk proteins were either singly-labelled with ^{15}N or double labelled with ^{15}N and homoarginine. The nitrogen digestibility of all of the proteins was lower for the guanidinated diets than the non-guanidinated diets, which may indicate that there is an altered digestion in animals fed guanidinated diets. It should be noted, however, that the β-lactoglobulin and lactoferrin diets contained low amounts of these proteins (13 and 8%, respectively) with the remainder of the protein component being un-labelled casein. Therefore the level of homoarginine labelling would have been

rather low in the β-lactoglobulin- and lactoferrin-containing diets. The effects of guanidination of proteins on protein digestion and metabolism need further investigation.

Another assumption that applies to both the direct and indirect methods is that guanidinated protein is digested and the amino acids are absorbed at similar rates to the original protein. Schmitz *et al.* (1991b) demonstrated that the chymotryptic hydrolysis (*in vitro)* of guanidinated and non-guanidinated casein proceed at nearly the same rate. However, tryptic hydrolysis of guanidinated casein was shown to proceed at a slower rate than that for non-guanidinated casein (Schmitz *et al.*, 1991b; Hara *et al.*, 1995). Although this difference may not be of physiological relevance, as tryptic capacity probably does not limit the rate of proteolysis (Corring, 1980a), it does indicate a difference in the proteolysis of guanidinated proteins compared to that for non-guanidinated proteins.

It must also be assumed that there is no significant arginase activity within the gastrointestinal tract (including that due to the action of microbes) which could break down homoarginine to lysine resulting in an overestimation of endogenous lysine. That arginase activity is very low has been confirmed for the rat (Schuttert *et al.*, 1991) and chicken (Siriwan *et al.*, 1994). When the indirect method is used, it is assumed that homoarginine is not recycled into the small intestine following absorption. This has been confirmed in the pig (Schmitz *et al.*, 1991b) and chicken (Angkanapom *et al.*, 1997). Also for the indirect method, the transformation of lysine to homoarginine in the guanidinated protein must occur randomly, so that the protein is homogenously labelled with homoarginine. Siriwan *et al.* (1994) have confirmed this assumption by sequential *in vitro* proteolysis of guanidinated proteins followed by amino acid analysis of the digested fractions. Guanidinated casein and soyabean proteins were shown to contain constant ratios of homoarginine to other amino acids in all of the fractions. There may, however, be difficulties with homogeneously guanidinating heat-treated proteins (Erbersdobler and Lee, 1991).

A further assumption that must be made when using the indirect approach is that homoarginine is released from the protein and absorbed to the same extent as lysine. The direct method does not rely on this assumption. Moughan and Rutherfurd (1990) found similar endogenous losses of lysine in the rat using the direct and indirect methods which lends support to this assumption.

Finally, with the guanidination method it must be assumed that guanidination does not alter the activity of antinutritional factors present in the feedstuff, thus resulting in a difference in the digestion and absorption of the guanidinated protein compared to non-guanidinated protein. This assumption is yet to be fully tested.

The main advantage with the guanidination method is that it allows the endogenous ileal excretion of lysine to be determined with the animal being fed a near-normal diet. A disadvantage is that it provides direct information about the endogenous flow of lysine only, and the flows of other amino acids are estimated by assuming a constant ratio of lysine to the other amino acids in the endogenous protein. A further disadvantage is that homoarginine, although partially transformed to lysine, accumulates in the liver over time and has toxic effects on the animal, thus restricting the duration of experiments, especially when the direct method is used. Whereas the direct method does not rely on all of the assumptions that are inherent with the indirect method, there are often difficulties in achieving 100% guanidination of feedstuffs as required for the direct method.

Overall, the guanidination method appears to offer considerable promise for the determination of endogenous ileal amino acid flows. Endogenous flows can be determined in animals that are in a physiologically normal state, after ingestion of a wide range of dietary protein sources. Further studies are required to fully establish the validity of some of the assumptions inherent in this method. Comparisons of endogenous ileal lysine flows determined using the guanidination and protein-free diet approaches have shown a lower lysine flow with the protein-free approach (Moughan and Rutherfurd, 1990).

Isotope Dilution

The use of isotopes allows the determination of endogenous protein flows at the terminal ileum while the animal is fed a diet that contains protein. The animal's body nitrogen pool (de Lange *et al.*, 1990; Huisman *et al.*, 1992; Schulze *et al.*, 1994) or the food protein (Leterme *et al.*, 1994; Roos *et al.*, 1994) is labelled with either a stable or radioactive tracer (Souffrant *et al.*, 1982; Souffrant, 1991).

The stable isotope ^{15}N is the most commonly used tracer, allowing undigested dietary nitrogen to be distinguished from endogenous nitrogen and the proportion of endogenous nitrogen in the digesta to be calculated from dilution of the isotope.

Labelling the animal's body nitrogen pool (the ^{15}N-perfusion technique), usually involves continuous intravenous infusion of labelled amino acids. The ratio of labelled nitrogen in the digesta to that in the precursor pool for gut protein synthesis is used to calculate endogenous nitrogen flows in ileal digesta. Usually a single ^{15}N-labelled amino acid is infused into the animal. ^{15}N-labelled leucine is the most commonly used amino acid as it is presumed to be readily metabolised resulting in labelling of other amino acids by transamination. However, the ^{15}N label of leucine is not transferred to lysine or threonine, and the isotopic enrichment of other amino acids is less than that of the labelled leucine (de Lange *et al.*, 1992; Lien *et al.*, 1997a; Lien *et al.*, 1997b). Alternatively, the use of mixtures of ^{15}N-labelled amino acids has been suggested (Reeds *et al.*, 1997; Leterme *et al.*, 1997) and may avoid this problem.

It is vital to choose an appropriate precursor pool when the animal's body nitrogen pool is labelled with ^{15}N (Moughan *et al.*, 1992a). The TCA-soluble blood plasma fraction is commonly used to indicate ^{15}N enrichment of the gut endogenous material. It is assumed that the free amino acid pool in the TCA-soluble fraction is used for the synthesis of body protein and thus for most of the endogenous secretions into the digestive tract. This is not true, however, for small intestinal mucosa secretions at least, in which case absorbed dietary amino acids are used directly for protein synthesis (Alpers, 1972). The ^{15}N-enrichment of the TCA-soluble plasma fraction is lower than that of pancreatic secretions and of the urinary urea and ammonia (Souffrant *et al.*, 1993). Potentially, plasma enrichment underestimates the enrichment of endogenous nitrogen secreted into the intestinal lumen which leads to an overestimation of endogenous protein excretion (Lien *et al.*, 1997b). More work is required to isolate the actual precursor pool or pools for endogenous secretion.

When ^{15}N-labelled diets are required to be fed to animals, most commonly the food proteins are labelled by applying ^{15}N-labelled fertilisers or salts to growing plants, with the expectation that most of the amino acids in the plant protein will be labelled with ^{15}N. ^{15}N-labelled milk can be produced by infusing ^{15}N-labelled salts into the rumen of the lactating cow. With the use of ^{15}N-labelled diets, the assumption must be made that labelled and unlabelled food amino acids are absorbed equally, and that the endogenous nitrogen secreted into the gut does not become labelled to a significant extent during the course of the experiment. Leterme *et al.* (1996b), however, have observed that a proportion of the absorbed dietary amino acids are rapidly

synthesised into body protein and resecreted as endogenous protein, which will lead to an underestimation of endogenous nitrogen.

A limitation with the ^{15}N method is that only endogenous nitrogen is directly measured, not the amino acids. Thus to calculate endogenous amino acid flows it must be assumed that there is a constant ratio in the endogenous material between nitrogen and the amino acids.

Endogenous ileal nitrogen flow determined using the isotope dilution method with ^{15}N-labelled pigs is greater than that determined when a protein-free diet is fed to the animals (de Lange *et al.*, 1990) and similar to that determined using the enzyme hydrolysed protein method described below (Schulze *et al.*, 1995a).

The ^{15}N-isotope dilution technique is a valuable method for studying factors that influence the recovery of endogenous protein in digesta, such as antinutritional factors and plant fibre, but requires modification to improve accuracy. It appears that when the diet is labelled with ^{15}N, the endogenous ileal flows of the animal will be underestimated, whereas when ^{15}N-perfusion is used, the determined endogenous nitrogen flows will be overestimated. Comparison of endogenous ileal nitrogen flows determined using the two forms of administration of ^{15}N in the pig has confirmed that the endogenous flows determined with the ^{15}N-perfusion technique are significantly greater than those determined using ^{15}N-labelled diets (Leterme *et al.*, 1994)

The stable carbon isotope ^{13}C has also been investigated as a potential label for the determination of endogenous protein flows through the terminal ileum. When infused intravenously or given orally to the test animal ^{13}C is incorporated into body protein (Minson *et al.*, 1975). Feedstuffs that are normally used to formulate diets differ in the degree of labelling with ^{13}C, so diets can be prepared with different abundances of ^{13}C. Arentson and Zimmerman (1995) have determined the endogenous ileal protein flows in pigs by feeding ^{13}C-labelled diets and obtained endogenous flows similar to those previously obtained using ^{15}N. Leterme *et al.* (1997) directly compared endogenous protein flows determined following intravenous infusion of ^{13}C-leucine with those determined following infusion of ^{15}N-leucine in the pig and found lower values for endogenous ileal nitrogen flows with the ^{13}C method (1.4 vs 5.7 mg/g dry matter intake for the ^{13}C and ^{15}N labels, respectively). This difference in endogenous flows between the two labels was suggested to be due to an overestimation of endogenous N with ^{15}N

due to underestimation of the enrichment of the endogenous N which occurs when the TCA-soluble blood plasma fraction is used as the precursor pool, as described above. The use of ^{13}C for the determination of endogenous protein flows has the same disadvantages as ^{15}N in terms of recycling of the label and the determination of an appropriate precursor pool, but the advantage of using ^{13}C over ^{15}N is the lower cost of labelled diets. When ^{13}C-labelled leucine is intravenously administered to the animal, however, the ^{13}C can not be transferred to other amino acids, so only the endogenous flow of leucine can be determined, which limits the usefulness of this method.

Enzyme Hydrolysed Protein Method

The enzyme hydrolysed protein method, also referred to as the peptide alimentation method, was proposed by Moughan *et al.* (1990). It allows endogenous ileal nitrogen and amino acid flows to be determined in animals fed a diet containing dietary peptides and free amino acids as the sole source of nitrogen. Usually, the animal is fed an enzymatically hydrolysed casein (EHC) based diet, containing a mixture of free amino acids and oligopeptides with no peptides being larger than 5,000 Daltons. Digesta are collected, centrifuged and then ultrafiltered, to remove any material smaller than the filtration cut-off of 10,000 Daltons. The >10,000 Dalton fraction is then used to estimate the flow of endogenous material.

A major advantage of the peptide alimentation method is that it allows the endogenous flows of total nitrogen and all amino acids to be determined directly, as opposed to the homoarginine and ^{15}N methods in which cases only lysine or nitrogen, respectively, are measured directly.

With the peptide alimentation method, however, there may be some loss of small peptides and free amino acids of endogenous origin when the ultrafiltrate is discarded, leading to an underestimation of the total endogenous amino acid flow. The extent of this underestimation has been studied by ultrafiltering digesta collected after feeding animals a protein-free diet so that all of the nitrogenous material present in the digesta is endogenous. Moughan and Schuttert (1991) found that only 11% of the amino N in the digesta of the growing pig, was present as molecules <10,000 Da. Butts *et al.* (1992) performed a similar experiment in the growing rat and found 33% of the total N in the digesta to comprise molecules <10,000 Da. Leterme *et al.* (1996a) ultrafiltered the soluble fraction of digesta obtained after feeding pigs a protein-free diet and found that some 22% of the total digesta N was present in the <10,000 Da fraction. In the latter two studies, the pH of the digesta was not decreased to prevent autolysis in the

digesta post-collection, thus the values may be overestimated. Marty *et al.* (1994) found that free amino acids comprised 7.2-9.2% of the total nitrogen flow in pigs fed a protein-free diet.

It should be recognised that protein or peptide alimentation may affect the composition as well as the amount of endogenous excretion, and that higher or lower than normal amounts of free amino acids and small peptides may occur with protein-free feeding. It is difficult to accurately determine the amounts of endogenous small (<10,000 Da) amino molecules in the ileal digesta of protein-fed animals, though based on first principles it would be expected that the concentration of such molecules at the terminal ileum would be low.

The removal of endogenous material in the ultrafiltrate will lead to some degree of underestimation of endogenous amino acid loss with the peptide alimentation method. The accuracy of the enzyme hydrolysed protein method could be improved by the use of ultrafiltration devices with a molecular weight (MW) cut-off of 5,000 Da or even 3,000 Da. The extent of the effect that this would have on the endogenous flows determined using this method remains to be evaluated.

The effect of peptide size on endogenous ileal amino acid flow is unknown. If peptide size does have an effect on endogenous ileal amino acid flow, then the extent of hydrolysis of the protein may influence the results obtained using the enzyme hydrolysed protein method. An assumption with this method is that the protein source, which has been hydrolysed artificially, simulates the products of natural gastric digestion. When the hydrolysed protein is fed to the animal, it is assumed that the peptides entering the small intestine are of similar size to those that would enter the small intestine if non-hydrolysed protein had been fed to the animal. If this is not so, and the size of the peptides affects endogenous amino acid flow, then the results obtained using the enzyme hydrolysed protein method may be inaccurate.

Further, it is possible that small undigested dietary peptides may bind to the endogenous protein fraction and thus inflate the endogenous estimates. Butts *et al.* (1991) have evaluated the ultrafiltration devices used with the enzyme hydrolysed protein method and found them to be effective for the separation of amino acids and purified peptides and proteins. However, the effectiveness of the ultrafiltration devices for the separation of dietary peptides from digesta has not been examined.

The enzyme hydrolysed protein method allows the determination of basal endogenous nitrogen and amino acid excretions under seemingly physiological conditions. However, there will be some degree of underestimation of the endogenous flows with this method due to the presence of some endogenous free amino acids and peptides in the discarded ultrafiltrate. Further studies are required to test the validity of some of the assumptions that must be made with this method. Endogenous ileal nitrogen and amino acid flows in mammals, determined using the enzyme hydrolysed protein method, are greater than those determined using the protein-free method (Darragh *et al.,* 1990; Butts *et al.,* 1991; Moughan *et al.,* 1992b; Butts *et al.,* 1993*a;* Donkoh, *et al.* 1995; Hendriks *et al.,* 1996; Leterme *et al.,* 1996a; Rutherfurd and Moughan, 1997) and synthetic amino acid based diets (Butts *et al.*, 1993b), and do not differ significantly from those determined feeding zein-based diets (Butts *et al.*, 1993b) or using the isotope dilution method applied by labelling the animal's nitrogen pool with ^{15}N (Schulze *et al.*, 1995a)

DIETARY FACTORS INFLUENCING ENDOGENOUS ILEAL AMINO ACID FLOW

The diet consumed by an animal affects the flow of endogenous ileal nitrogen and amino acids. Factors such as the dry matter intake of the animal, the amount of fibre in the diet, antinutritional factors and the presence of peptides in the digesta have all been reported to influence endogenous ileal protein flows.

Dry Matter Intake

Mitchell (1924) was the first to suggest that dry matter intake ("roughage") affects endogenous nitrogen flows. Rats consuming roughage (in the form of filter paper) were found to have a higher concentration of nitrogen in the faeces per gram of the original diet than rats that did not have access to the roughage.

Several studies have focused on the effect of dietary dry matter intake on endogenous nitrogen and amino acid flows at the terminal ileum. Buraczewska and Horaczynski (1983) fed casein based diets at three levels of dry matter intakes to pigs, and reported a significant increase in the amount of nitrogen and most amino acids at the terminal ileum as dry matter intake (DMI) increased.

Mariscal-Landín *et al.* (1995) suggested that at low feed intakes (<70 g/kg liveweight$^{0.75}$) endogenous protein flows are related to pig body size as opposed to DMI. This hypothesis would explain the lack of effect of dry matter intake on endogenous nitrogen flows through the terminal ileum found by Furuya and Kaji (1992) who fed pigs at levels of 46, 69 and 92 in one experiment and 27, 40 and 54 g diet/kg bodyweight$^{0.75}$ in a second experiment.

Butts *et al.* (1993a) reported a linear increase in endogenous ileal nitrogen and amino acid flows in pigs when the dietary DMI was increased, with the flow of endogenous nitrogen increasing from 2.77 to 5.90 g/day when DMI was increased from 0.91 to 2.19 kg/day. Except for the lowest feed intake level, the feed intakes of the pigs in this trial were all greater than 70 g/kg$^{0.75}$. Thus it appears that DMI influences endogenous ileal nitrogen and amino acid flows through the terminal ileum and it is now standard to report endogenous flows on a dry matter intake basis.

Fibre

Endogenous ileal nitrogen and amino acid flows, when expressed on a DMI basis, increase with increased dietary fibre intake (Sauer *et al.*, 1977; Taverner *et al.*, 1981; Bergner, 1982; Fuller and Kadenhead, 1991; Sève *et al.*, 1994; Mariscal-Landín *et al.*, 1995; Schulze *et al.*, 1995b; Leterme *et al.*, 1996c). This increase in endogenous protein flow is reported to reach a maximum level and then plateau (Taverner *et al.*, 1981; Sève *et al.*, 1994; Mariscal-Landín *et al.*, 1995). The source of fibre may influence the extent of the increase in ileal flow of endogenous nitrogen and amino acids. For example, the addition of purified wood cellulose to pig diets has been reported to have little effect on endogenous ileal protein flows (De Lange *et al.*, 1989b; Furuya and Kaji, 1992; Leterme *et al.*, 1992), whereas the addition of pectin (de Lange *et al.*, 1989b; Mosenthin *et al.*, 1994) and purified neutral detergent fibre (Schulze *et al.*, 1995b) to diets cause a marked increase in endogenous flows in the pig.

The cause of the increased endogenous protein flow that occurs with increased dietary fibre is not clear. It could be due to increased loss of endogenous protein into the intestines, decreased reabsorption of the endogenous proteins or a combination of the two effects. Taverner *et al.* (1981) suggested that an increased mucin secretion is involved. Indirect evidence for this has been provided by Satchithanandam *et al.* (1990), who reported an increase in the amount of luminal mucin in rats fed diets containing guar gum, compared with those fed a fibre-free diet.

There is conflicting evidence in the literature on the effect of dietary fibre on exocrine pancreatic secretions. Mosenthin and Sauer (1991) reported that the addition of either Alphafloc or barley straw to a diet had no effect on exocrine pancreatic secretion. The same result was reported by Mosenthin *et al.* (1990) regarding the addition of pectin to pig diets. In contrast, Partridge *et al.* (1982) speculated that the difference in pancreatic secretion found in the pig between two iso-nitrogenous diets may have been due to the greater fibre content of the diet which provoked the greater pancreatic secretion. Ikegami *et al.* (1990) reported that the effect of increased fibre content on pancreatic secretion was dependent on the type of fibre in the diet. In their study, the diets that led to increases in exocrine pancreatic secretion were those containing fibre with higher viscosity, whereas the diets with low viscosity fibre did not provoke increases in pancreatic secretion. In a study by Larsen *et al.* (1993) in rats, increased fibre viscosity *per se* resulted in an increased level of ileal endogenous nitrogen and all amino acids except for tyrosine, phenylalanine and arginine. Generally, the amino acid composition did not change, suggesting either the secretion of larger amounts of amino acids from a similar origin, or a decrease in endogenous amino acid absorption. In this study, there was also an increase in the concentration of sialic acids in the digesta as the fibre level was increased, indicating an increased concentration of mucoproteins.

Schulze *et al.* (1994) found no change in the levels of diaminopimelic acid (DAPA) in ileal digesta with greater endogenous flows following the feeding to pigs of diets containing fibre, compared to pigs fed diets with very little fibre. DAPA is present exclusively in some bacteria. Jørgensen and Jensen (1994), however, reported an increase in the production of CH_4 in the intestine of pigs fed a high fibre diet, which was suggested to be due to greater microbial activity. Therefore, there is still doubt as to whether the increased endogenous ileal protein flows that occur with increased dietary fibre intake are at least partially due to an increase in bacteria numbers in the small intestine.

It has been suggested that fibre may adsorb amino acids and peptides, which would prevent their absorption from the gastrointestinal tract, and result in a greater flow of endogenous amino acids and peptides with high fibre diets (Bergner *et al.*, 1975; Sauer *et al.*, 1991).

Antinutritional Factors

Antinutritional factors (ANFs) are substances that may be present in feedstuffs and produce detrimental effects on animals such as reduced growth, feed conversion

and/or health (Burns, 1987; Birk, 1989; Huisman, 1989). The presence of some antinutritional factors, such as protease inhibitors, tannins and lectins, in the diet has been shown to have an effect on endogenous nitrogen and amino acid flows through the terminal ileum, as discussed below.

Protease inhibitors are proteins that bind to proteases and form stable complexes, inhibiting the actions of the enzymes (Burns, 1987; Birk, 1989). They are present in many legume seeds and oilseeds (Huisman *et al.*, 1990). Protease inhibitors can bind to many proteases, such as the pancreatic enzymes trypsin (trypsin inhibitors) and chymotrypsin (chymotrypsin inhibitors). The inclusion of trypsin inhibitor (TI) proteins in the diet has been shown to increase the endogenous nitrogen flow at the terminal ileum (Schulze, 1994). This increased endogenous nitrogen flow could be due to an increase in the secretion of enzymes from the pancreas, a reduction in the proteolysis and subsequent absorption of endogenous protein or a combination of the two possibilities. Increased secretion of pancreatic enzymes occurs in mice (Roy and Schneeman, 1981), rats (Temler *et al.*, 1984) and chickens (Gertler and Nitsan, 1970) given diets containing protease inhibitors. This has been suggested to be due to the protease inhibitor-enzyme complex inactivating the trypsin, thus causing the release of more cholecystokinin, which stimulates pancreatic secretion (Birk, 1989; Liener and Kakade, 1980). The amount of undigested dietary nitrogen at the terminal ileum of the pig has been shown to increase when TI proteins are present in the diet (Schulze, 1994). Therefore, it seems likely that the increased endogenous ileal nitrogen flow that occurs when protease inhibitors are present in the diet is due to a combination of an increase in the secretion from the pancreas and a decrease in the digestion and reabsorption of endogenous protein.

Tannins are another ANF present in many legumes and oilseeds (Huisman *et al.*, 1990). When fed to animals, they are thought to form indigestible complexes with digestive enzymes and feed proteins (Mangan, 1988; Longstaff and McNab, 1991). This inhibits the actions of the enzymes and prevents the feed proteins from being absorbed from the gut. Yu *et al.* (1995) reported that while the addition of cottonseed hulls, which contain high levels of tannins, to rat diets resulted in an increased flow of endogenous nitrogen and some amino acids in the rat, this did not appear to be due to an effect of tannins. Jansman *et al.* (1995), however, found an increase in both endogenous and exogenous nitrogen flow through the terminal ileum of the pig when they were fed diets which contained tannins. This increased endogenous excretion of nitrogen from the terminal ileum may be due to the decreased activity of proteolytic

enzymes that has been reported to occur in the pig following feeding of a diet rich in tannins (Jansman *et al.*, 1994). This may result in a decreased digestion and subsequent absorption of endogenous protein, and an increased secretion of proteolytic enzymes, in the same manner as for protease inhibitors.

Lectins, also known as haemagglutinins, are also present in many legume seeds (Huisman *et al.*, 1990) that are used in animal diets. Lectins have been reported to reduce the production of endocrine cells and gut hormones in the walls of the digestive tract (King *et al.*, 1983) and can bind to the gut mucosa (Egberts *et al.*, 1984) resulting in damage to the intestinal wall (Donatucci *et al.*, 1987; Liener, 1986; Pusztai *et al.*, 1990). These effects on the gastrointestinal tract may result in impaired digestion and absorption of nutrients and the inclusion of lectins in the diet has been shown to increase endogenous nitrogen flows through the terminal ileum of the pig (Schulze *et al.*, 1995c). This may be due to extra shedding of the damaged cells lining the intestines, increased mucus secretion and the loss of more plasma proteins into the intestines. The absorption of the breakdown productions of both endogenous and exogenous proteins may also be decreased due to damage to the walls of the small intestine by lectins.

The Presence of Peptides in the Diet

When animals are fed a protein-free diet, endogenous ileal amino acid flows appear to be lower than those determined when a peptide or protein containing diet is fed to the animal (Darragh *et al.*, 1990; de Lange *et al.*, 1990; Moughan and Rutherfurd, 1990; Butts *et al.*, 1991; Moughan *et al.*, 1992b; Butts *et al.*, 1993b; Donkoh *et al.*, 1995; Hendriks *et al.*, 1996; Leterme *et al.*, 1996a; Rutherfurd and Moughan, 1997). However, there is no difference in the endogenous ileal amino acid flows determined when a diet containing synthetic amino acids as the sole source of dietary nitrogen is fed to animals compared to the flows determined under protein-free alimentation (Skilton *et al.*, 1988; Darragh *et al.*, 1990; Butts *et al.*, 1993b). When the feeding of a protein-free diet to an animal is accompanied by intravenous infusion of amino acids in the pig, the endogenous ileal amino acid flows also do not differ from those determined when saline is infused intravenously (de Lange *et al.*, 1989a; Leterme *et al.* 1996a). These findings suggest that the difference in endogenous ileal protein flow found when a peptide- or protein-containing diet is fed to an animal is not due to the negative body nitrogen balance of the animals *per se.*

There is little difference between the endogenous lysine flows at the terminal ileum of rats fed guanidinated gelatin, soyabean or casein compared with those fed an EHC based diet (Moughan and Rutherfurd, 1990; Moughan and Rutherfurd, 1991). This is also true for pigs fed a zein-based diet compared with those fed an EHC-based diet (Butts *et al.*, 1993b) and suggests that the source of the protein *per se* does not affect endogenous ileal protein flow.

There seems to be little doubt that the presence of protein or protein-breakdown products (peptides) in the digestive tract supports higher endogenous ileal protein flow. It is not known, however, whether the concentration of peptides in the diet influences endogenous ileal protein flows. The relationship may be a gradually changing or an "all or nothing" relationship and this needs to be determined.

SUMMARY AND CONCLUSIONS

Nitrogen enters the digestive tract from several sources during digestion, including secretions from salivary glands, the stomach, the pancreas and the liver, as well as mucus and desquamated cells. A proportion of this nitrogen is not reabsorbed by the animal and is present in the digesta that flows through the terminal ileum.

Several methods have been developed to determine the amount of endogenous protein flowing through the terminal ileum, all of which require collection of the ileal digesta. The slaughter method appears to be the most appropriate method of collection for small animals such as the rat and chicken. This method can also be used for larger monogastric animals such as the pig. There is strong evidence that ileo-rectal anastomosis causes major alterations to digestive and metabolic processing in the anastomised animal, and thus does not appear to be a suitable method for the collection of ileal digesta. With T-cannulation, there are still questions regarding possible fractionation of digesta, particularly when the animals are fed diets with a high fibre content. An additional point of concern with T-cannulas is the effect of the cannula on intestinal motility. Re-entrant cannulation allows a complete collection of digesta, but due to the transection of the intestine, motility is reduced and blockages of the cannula are common. Many diet ingredients require grinding to allow a digesta sample to be collected via a re-entrant cannula. With PVTC cannulation, there is no transection of the intestine and the digesta are collected after passing through the ileo-caecal junction. Also, due to the large bore of the cannula, there are no problems with blockages of the cannula and thus no restrictions on dietary ingredients. Digesta collections from PVTC cannulated pigs may not be quantitative, so an indigestible marker should be included in the diet. The PVTC cannulation procedure does not appear to cause a significant alteration in metabolism in the pig, and thus appears to be the current method of choice for the collection of ileal digesta from the pig.

Various methods have been used to determine the flows of endogenous nitrogen and amino acids through the terminal ileum. The traditional approach, involving the feeding of a protein-free diet to test animals for several days before digesta collection, results in an abnormal physiological state within the animal. The endogenous amino acid flows, therefore, are unlikely to be accurate. However, it is possible that the protein-free diet could be given to the test animal for a shorter period of time than the standard 7-10 days before digesta are collected, and this may give rise to more physiologically relevant estimates of endogenous ileal protein

flows. If, however, the metabolic adaptations within the animal to the protein-free diet occur rapidly (within 1-2 days), then the protein-free method will not give accurate estimates of endogenous ileal amino acid flows even if the diet is fed for a shorter length of time.

The validity of the regression method for determining endogenous ileal nitrogen and amino acid flows is questionable for two main reasons. Firstly, differences in the range of dietary protein levels used can result in large differences in the estimated endogenous ileal nitrogen and amino acid flows and, secondly, it relies on the assumption that there is no change in endogenous protein flow with increasing levels of dietary protein. This assumption still requires testing.

The use of natural proteins devoid of specific amino acids is limited, as there are few such proteins available. Zein, which is almost completely devoid of lysine and tryptophan, has been used to study the increased endogenous protein flow which occurs when proteins or peptides are present in the diet (Butts *et al.*, 1993b) and the results confirm those found using other methods.

The guanidination, isotope dilution and enzyme hydrolysed protein methods are techniques whereby the test animal receives a diet containing proteins or peptides and the animal is in a physiologically normal nitrogen balance when digesta are collected. However, there are assumptions that must be made with each of these three methods and that remain to be fully tested. The main disadvantage with labelling the diet with ^{15}N is the rapid recycling of the label resulting in underestimation of the endogenous protein flows determined. Labelling the animal with ^{15}N (isotope perfusion) should be done by using mixtures of ^{15}N labelled amino acids as opposed to the most common method, where a single ^{15}N-labelled amino acid is infused. Doubt still remains as to an appropriate precursor pool for use with the isotope perfusion technique. The use of the TCA-soluble blood plasma as the precursor pool, as is currently the standard method, may result in overestimation of the determined endogenous nitrogen flows (Lien *et al.*, 1997b). It is possible that a single precursor pool for endogenous protein does not exist, and a weighted set of pools will have to be used.

The enzyme hydrolysed protein method for the determination of endogenous ileal protein flows still requires validation. The endogenous flows determined with this method are underestimated due to the presence of endogenous material in the

ultrafiltrate that is discarded. The extent of this underestimation is not clear and requires more investigation. Conversely, it is also possible that dietary enzyme hydrolysed protein may covalently bind to the endogenous fraction of digesta, and thus result in an overestimation of endogenous ileal nitrogen and amino acid flows.

The guanidination, isotope dilution and enzyme hydrolysed protein methods are in widespread use for the determination of endogenous ileal protein flows. No controlled experimental comparisons have been made, however, to determine whether the three methods give the same or different values for endogenous ileal nitrogen and amino acid flow and this would appear to be important information.

Factors such as dietary dry matter intake, fibre and the presence of antinutritional factors in the diet increase endogenous ileal amino acid flow. Whereas it is known that the presence of protein or peptides in the diet also increases endogenous ileal amino acid flow, little is known about how this occurs, or the type of relationship that may exist between dietary peptide concentration and endogenous ileal amino acid flow.

Based on this background, the aims of the work described here were as follows:

- To determine whether the protein-free method could be applied for a shorter period of time to give "physiologically" relevant values for endogenous ileal nitrogen and amino acid flows.
- To perform a direct comparison of the enzyme hydrolysed protein, guanidination and isotope dilution methods to determine whether the three methods give similar estimates of endogenous nitrogen and lysine flows.
- More validation is required for the enzyme hydrolysed protein method. Firstly, the extent of the underestimation that occurs with the enzyme hydrolysed protein due to discarding endogenous amino acids in the filtrate fraction of the digesta needs to be examined further. Secondly, it still remains to be determined whether dietary peptides bind to the endogenous fraction and inflate endogenous ileal nitrogen and amino acid flows. Another aim of the work reported here was to examine these points so that the enzyme hydrolysed protein method can be used with greater confidence.
- To use the enzyme hydrolysed protein method to examine the time course of endogenous ileal nitrogen flow over 24-hour periods in the peptide-fed pig, as a step towards elucidation of the mechanisms involved in the peptide-stimulated increase in endogenous nitrogen flows.

- To determine whether a relationship exists between dietary peptide concentration and endogenous amino acid flow, using the enzyme hydrolysed protein method and a method adopting the use of zein protein.

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Chapter 2

Effect of the Duration of Feeding of a Protein-Free Diet on Endogenous Ileal Nitrogen and Amino Acid Loss in the Growing Pig

The protein-free method for the determination of endogenous ileal nitrogen and amino acid flows in simple stomached mammals involves feeding a protein devoid diet to the animals for a period of 7-10 days before the collection of ileal digesta. All of the nitrogen and amino acids in the collected digesta must be of endogenous origin. The aim of this study was to determine whether endogenous ileal nitrogen and amino acid flows change over time as a protein-free diet is given to the animals.

Effect of the Duration of Feeding of a Protein-Free Diet on Endogenous Ileal Nitrogen and Amino Acid Loss in the Growing Pig

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ABSTRACT

The aim of the study was to determine whether endogenous nitrogen and amino acid excretions at the terminal ileum change over time in the growing pig fed a protein-free diet for 8 days. Seven entire male pigs with surgically implanted post-valve T caecum cannulas were fed a semi-synthetic casein based diet for 8 days. Food was withheld from the pigs for 24 hours, after which they were fed a protein-free diet for a further 8 days at a rate of 10% of metabolic body weight ($W^{0.75}$) per day. Chromic oxide was included in the protein-free diet as an indigestible marker. Ileal digesta were collected continuously from 1300h to 1800h on each day of the experimental period. Endogenous ileal nitrogen flows were determined for each pig each day the protein-free diet was given and endogenous ileal amino acid flows for the first and eighth days. There were no significant ($P>0.05$) effects of the duration of feeding of the protein-free diet on endogenous ileal total nitrogen or amino acid flows except for the amino acids glycine and cysteine, the flows of which significantly decreased over the 8-day period ($P<0.01$ and $P<0.05$ for glycine and cysteine, respectively), from (mean $\pm$ SEM) 1639 $\pm$ 217 to 892 $\pm$ 212 µg/g dry matter intake (DMI) for glycine, and 173 $\pm$ 13 to 127 $\pm$ 19 µg/g DMI for cysteine. The relative contributions (moles of each amino acid as a proportion of total moles of amino acids) of threonine, glycine and cysteine decreased significantly ($P<0.05$) and that of proline increased significantly ($P<0.05$) during the 8 days that the protein-free diet was fed to the pigs.

INTRODUCTION

It is important to be able to determine with accuracy the amount of endogenous protein in ileal digesta (Tamminga *et al.*, 1995; Boisen and Moughan, 1996a; Nyochoti *et al.*, 1997). The traditional method of determination involves feeding the animal a diet that is virtually devoid of protein. With this approach, a strength of which is its simplicity, all protein present in the digesta must be of endogenous origin. The protein-free method, however, has been criticised as being unphysiological as the animal is in a negative body nitrogen balance. Protein-free feeding may result in a reduction in the amount of gastric and pancreatic enzymes secreted (Fauconneau & Michel 1970, Schneeman 1982) and an increased rate of breakdown and reabsorption of secreted enzymes (Snook & Meyer 1964, Fauconneau & Michel 1970). There may be a general decrease in the rate of protein synthesis in the body and gut with the feeding of a protein-free diet (Millward *et al.* 1976, McNurlan & Garlick, 1981).

The standard procedure used to determine endogenous ileal amino acid flows using the protein-free method is to feed a protein-free diet to the animal for a period of 7 days or more, allowing the animal's metabolism to adjust to the protein-devoid diet before collecting ileal digesta. It is possible, however, that the protein-free diet could be fed to the animal for a shorter period of time to avoid or minimise the effects of metabolic adjustment. This may give rise to more physiologically relevant endogenous amino acid flows compared to those determined using the traditional approach.

The present study was conducted to determine endogenous ileal nitrogen and amino acid flows over time (daily) in the growing pig fed a protein-free diet. Of particular interest, was whether endogenous ileal nitrogen flow was higher at the beginning of an 8-day protein-free feeding period.

MATERIALS AND METHODS

Seven Large White x (Large White x Landrace) entire male pigs with a common sire, and with an overall mean liveweight of (mean $\pm$ SEM) 81.6 $\pm$ 3.3 kg at the commencement of the trial were housed individually in smooth-walled steel metabolism crates in a room maintained at 22 ± 1°C. Ethics approval for the study was granted by the Massey University Animal Ethics Committee.

Surgery

A post-valve T caecum (PVTC) cannula was inserted into the caecum of each pig for the collection of ileal digesta (van Leeuwen *et al.,* 1991), with anaesthesia and post-operative care as described by Hodgkinson *et al.* (1999) (refer Chapter 7 of this thesis). The cannulas were made of medical grade silastic tubing with an internal diameter of 25 mm and an external diameter of 29 mm.

Diets and Feeding

A casein-based basal diet and a protein-free experimental diet were prepared (Table 1). The determined total nitrogen content and amino acid composition of the "protein-free" diet are given in Table 2. The "protein-free" diet contained 0.63 g N/kg diet, which corresponds to 0.36% crude protein.

Table 1 Ingredient compositions of the basal and experimental diets (g/kg, air-dry basis).

Ingredient	Diet	
	Basal	Protein-free
Casein	120	-
Soya bean oil	35	35
Cellulose	50	50
Sucrose	70	70
Wheaten cornflour	719	833
Vitamin/mineral mix[1]	2.5	2.5
Dicalcium phosphate	2.5	2.5
Sodium chloride	0.3	0.3
Potassium carbonate	0.5	0.5
Magnesium sulphate	0.2	0.2
Chromic oxide	-	6

[1] Pig grower/finisher vitamin, mineral premix, Danmix, Nutritech, Auckland, New Zealand, containing (per kg diet) 4,000,000 IU vitamin A, 800,000 IU vitamin D, 12,000 IU vitamin E, 800 mg vitamin K, 400 mg vitamin B1, 1 g vitamin B2, 800 mg vitamin B6, 4 mg vitamin B12, 84 mg folic acid, 4 g pantothenic acid, 6 mg biotin, 6 g niacin, 20 g choline, 120 mg selenium, 200 mg cobalt, 400 mg iodine, 50 g copper, 40 g iron, 18 g manganese, 48 g zinc and 4 g zinc bacitracin.

Throughout the trial, the pigs were fed twice daily (08:00 and 17:00h) at a level of 0.10 of metabolic body weight ($W^{0.75}$) per day. The diets were mixed with water (1:1 w/v) immediately prior to feeding and water was freely available between meals. Chromic oxide was included in the protein-free diet as an indigestible marker.

The pigs were fed the basal diet for 8 days. Each pig was weighed and its food intake adjusted accordingly at the end of the 8 day preliminary period. Following the preliminary period, the pigs had food withheld for 24 hours and were then given the protein-free diet for a further 8 days. Ileal digesta were collected continuously (using plastic bags attached to the cannulas) from 1300h to 1800h on each day that the pigs received the protein-free diet. The bung was removed from the cannula an hour before the collection commenced as suggested by van Leeuwen *et al.* (1991), to allow the ileo-caecal valve to move so that it was protruding into the lumen of the cannula instead of the intestinal lumen. The plastic bags were emptied at least hourly, and the digesta were frozen (-20 °C) hourly after adjustment to pH 3.5 by the addition of 6 M H_2SO_4. This procedure was adopted to reduce enzyme and bacterial activity in the digesta post collection.

Table 2 Nitrogen and amino acid contents of the "protein-free" diet (mg/kg dry matter).

Amino acid	Concentration
LYS	0.13
HIS	0.03
ARG	0.08
ASP	0.19
THR	0.08
SER	0.10
GLU	0.31
PRO	0.07
GLY	0.12
ALA	0.12
CYS	0.10
VAL	0.10
MET	0.08
ILE	0.08
LEU	0.14
TYR	0.07
PHE	0.08
Nitrogen	0.63

Chemical Analysis

Digesta were freeze-dried and finely ground. The diet (protein-free) and digesta were then analysed for total nitrogen, chromium and dry matter. The protein-free diet and digesta collected on the first and eighth days that the protein-free diet was fed to the pigs were also analysed for amino acids.

Total nitrogen was determined in duplicate. The samples were combusted at 1050 °C in oxygen gas. The nitrogen was then reduced to N_2 by a catalyst and this was measured by a thermal conductivity cell using a Leco FP2000 (Leco Corporation, St Joseph, Michigan, USA).

Amino acids were determined following hydrolysis of duplicate samples (5-7 mg) in 1 ml of 6 mol/L glass-distilled HCl containing 0.1% phenol in glass tubes sealed under vacuum, for 24 hours at 110 $\pm$ 2 °C. Amino acid concentrations were measured using a Waters ion exchange HPLC system calibrated against a reference amino acid mixture of known concentration. The peaks of the chromatograms were integrated using the dedicated software Maxima 820 (Waters, Millipore, Milford, MA), which identifies the amino acids by retention time against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively, and the weight of each amino acid was calculated using free amino acid molecular weights. No corrections were made for losses of amino acids during hydrolysis. Cysteine and methionine are destroyed during acid hydrolysis, so were determined by oxidation of duplicate samples (3-4 mg) with 1 mL of performic acid (1 part 30% H_2O_2 to 9 parts 88% formic acid) for 16 hours at 0 °C. The samples were then neutralised with 0.15 mL of 50% (w/w) HBr prior to acid hydrolysis. Tryptophan, which is also destroyed during acid hydrolysis, was not determined.

The chromium contents of the diet and ileal digesta were determined using an Instrumentation Laboratory Atomic Absorption Spectrophotometer according to the method described by Costigan & Ellis (1987).

Data Analysis

The total digesta dry matter (DM) collected each day was determined, and the concentrations of nitrogen (N) and chromium (Cr) per gram of digesta dry matter were also determined. The ratio of nitrogen to chromium in the digesta collected each day was calculated.

Endogenous nitrogen and amino acid flows (related to the ingestion of 1 g of dry matter) were determined using the following equation:

Endogenous flow =

$$\frac{\text{Concentration of compound in digesta} \times \text{Diet chromium concentration}}{\text{Digesta chromium concentration}}$$

The units were μg/g dry matter intake.

A single-factor repeated measures analysis was undertaken using the statistical programme SAS (version 6.12, 1997) to determine whether time (day of collection) had a significant effect on the total flow of DM during the collection period and on the concentrations of nitrogen and chromium in the digesta, as well as the nitrogen to chromium ratios and endogenous nitrogen and amino acid flows.

RESULTS

The pigs recovered rapidly from surgery and grew (mean $\pm$ SEM) 509 $\pm$ 54 g/day during the 8-day period that the casein-based basal diet was fed to the animals. Throughout the experiment, each pig consumed its full quota of feed at each meal. There was minimal leakage of digesta around the cannulas in the pigs during the experiment, and at *post mortem* following the study, tissue near the cannulation site appeared healthy. A mean of 28.6% (SEM 1.8%) of the chromium that was fed to the animals in the preceding meal was recovered in the digesta over the five-hour digesta collection period. The digesta sampled on the first day were green in colour and the chromium content (mean $\pm$ SEM, 27.6 $\pm$ 3.2 mg Cr/g DM) was not significantly different from that found on subsequent days, indicating that the samples resulted solely from the meal given on that day.

Total flows of digesta DM over the five hours of digesta collection, the concentrations of N and Cr and the N to Cr ratios in the digesta samples are given in Table 3. There was no statistically significant ($P>0.05$) effect of day of collection on the concentrations of N or Cr, or the N to Cr ratio. There was a significant ($P<0.05$) effect of day of collection on the amount of digesta dry matter collected.

Endogenous ileal total nitrogen flows over the eight days are shown in Figure 1. There was no statistically significant effect ($P>0.05$) of pig or day on endogenous ileal nitrogen excretion.

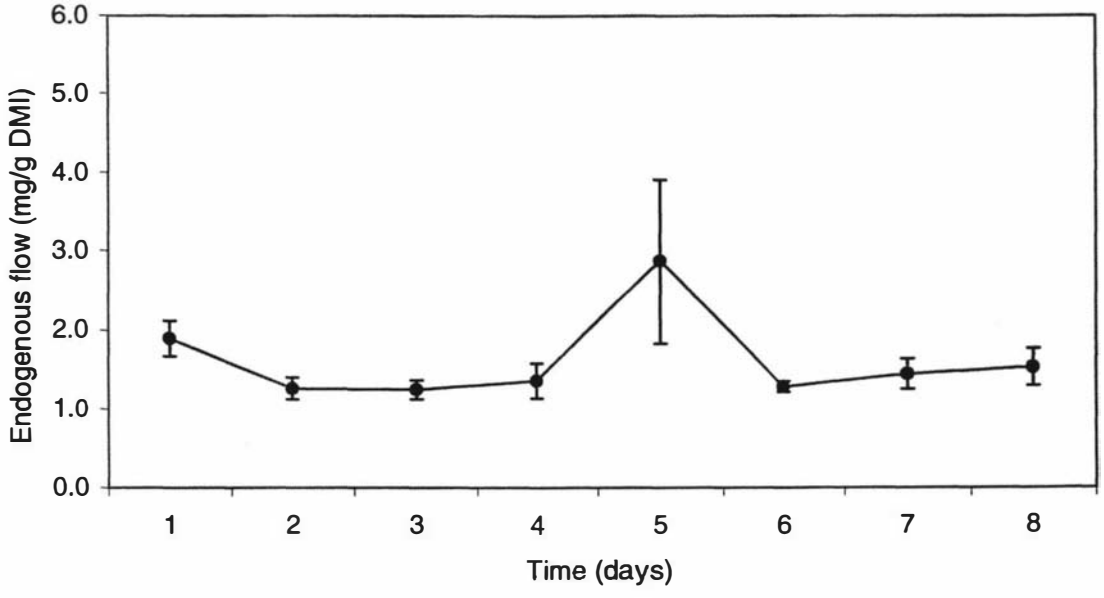
Table 3 Mean (n=7) amounts of dry matter (DM, g) collected over the 5 hour digesta collection period each day and digesta concentrations of chromium (Cr, mg/g DM) and nitrogen (N, mg/g DM) and the ratio of N to Cr (N/Cr, g N/g Cr) over eight days of feeding a protein-free diet to the growing pig.

Day	Total DM	Cr[1]	N[1]	N/Cr ratio[1]
1	4.7 (0.5)[2]	27.6 (3.2)	12.2 (1.2)	0.46 (0.05)
2	4.1 (0.3)	36.4 (2.6)	11.3 (1.4)	0.31 (0.03)
3	4.2 (0.2)	38.7 (1.5)	11.8 (1.0)	0.31 (0.03)
4	4.9 (0.4)	36.5 (2.2)	12.1 (1.9)	0.33 (0.05)
5	3.5 (0.4)	32.9 (3.1)	18.1 (2.8)	0.68 (0.23)
6	5.6 (0.7)	35.6 (5.2)	10.8 (1.3)	0.31 (0.02)
7	4.2 (0.5)	33.4 (4.2)	12.0 (2.5)	0.35 (0.05)
8	6.1 (0.9)	27.2 (2.6)	10.9 (2.5)	0.38 (0.06)

[1]Effect of time non-significant (P>0.05).

[2]The number in brackets following the estimate is the SEM.

Figure 1 Mean ± SEM (n=7) endogenous ileal nitrogen flow over eight days of feeding a protein-free diet to the growing pig.



There were no statistically significant differences in daily endogenous amino acid flow between days 1 and 8 for all of the amino acids determined, except for glycine and cysteine (Table 4), where flows were lower on day 8. For glycine, this difference was of a considerable order.

Table 4 Mean (n=7) endogenous ileal flows (μg/g dry matter intake) of amino acids on the first and eighth days of the feeding of a protein-free diet to the growing pig.

Amino acid	Endogenous flow		Statistical significance[1]
	Day 1	Day 8	
LYS	318 (64)[2]	350 (74)	NS
HIS	174 (26)	166 (42)	NS
ARG	237 (38)	277 (75)	NS
ASP	743 (106)	520 (113)	NS
THR	602 (40)	458 (84)	NS
SER	510 (91)	403 (87)	NS
GLU	843 (172)	680 (100)	NS
PRO	1058 (289)	2058 (750)	NS
GLY	1639 (217)	892 (212)	**
ALA	454 (64)	662 (294)	NS
CYS	173 (13)	127 (19)	*
VAL	424 (57)	277 (52)	NS
MET	123 (23)	108 (15)	NS
ILE	300 (56)	218 (32)	NS
LEU	497 (64)	353 (65)	NS
TYR	252 (39)	268 (71)	NS
PHE	278 (38)	236 (37)	NS

[1] NS, not significant; * P<0.05; ** P<0.01.

[2] The number in brackets following the estimate is the SEM.

DISCUSSION

The endogenous ileal nitrogen and amino acid flows on the eighth day that the protein-free diet was fed to the pigs were generally similar in magnitude to results from previous studies where pigs have been given a protein-free diet for a period of 8-12 days (Taverner et al, 1981; Mariscal-Landin *et al.*, 1990; Moughan and Schuttert, 1991; Moughan *et al.*, 1992; Butts *et al.*, 1993). The endogenous nitrogen flow on day four was similar to that found by Leterme *et al.* (1992) in pigs that had received a protein-free diet for a period of four days.

Chemical analysis of the experimental diet which was formulated to be devoid of protein, demonstrated that some nitrogenous material was present (0.63 g total

nitrogen/kg diet). If all this material was protein, the diet would contain 0.36% crude protein. It was expected that the animal would absorb much of this, but even if dietary amino acids remained unabsorbed, they would comprise only a low proportion of the amino acids present in the digesta at the terminal ileum. The overall result would not be materially affected.

The daily amounts of digesta dry matter collected varied over the eight days of the study with large fluctuations from one day to the next. This could have been due to a more complete collection of the digesta passing through the ileo-caecal valve on some days than others, or it may indicate differences in the rate of passage of digesta through the ileum over time as the protein-free diet was fed to the pigs. Examining the data from individual pigs revealed no common patterns in the total amount of DM collected each day from one pig to another. Darcy *et al.* (1980) collected digesta continuously for 24 hours after feeding growing pigs a protein-free diet. Large inter-animal variances in digesta dry matter flows were reported, especially during the period 7-10 hours after the meal, when the dry matter flows were maximal. In the present study, digesta were collected from 5 to 10 hours following the meal. Laplace *et al.* (1983) examined the relative importance of different sources of variance for the rate of passage of digesta through the ileo-caeco-colic junction in the pig. The two main sources of variation were found to be inter-animal variation and the reaction of individual pigs to particular diets. The influence of inter-animal variation on rate of digesta flow was greatest when the digesta contained a high relative flow of endogenous materials as would be the case with the semi-synthetic diets fed in the present study. If the differences in dry matter collection over the study period were due to incomplete digesta collection on some days, this would not affect the results of the study, assuming that there was no fractionation of the digesta that moved through the cannula from that which by-passed the cannula, as endogenous nitrogen and amino acid flows were calculated based on chromium flows.

When expressed on a dry matter basis, the concentrations of total chromium and nitrogen and the nitrogen to chromium ratio in the ileal digesta did not differ significantly ($P>0.05$) over time as the protein-free diet was fed to the pigs. There were, however, noticeable differences in the mean concentration of nitrogen in the digesta between days, with a relatively greater mean concentration of nitrogen in the digesta collected on day 5, and a correspondingly greater mean N/Cr ratio. The endogenous ileal nitrogen flows in two of the pigs were markedly higher on day 5

than on any other collection day for these animals, resulting in an overall greater mean flow and a high standard error for the data from day 5. There were high inter-animal variations in these data and the observed differences did not reach statistical significance.

The endogenous ileal nitrogen and amino acid flows were relatively constant over time as the protein-free diet was fed to the pigs although the tendency was for the endogenous flows of nitrogen and amino acids to be lower on the eighth day than the first day of digesta collection. The endogenous flows of cysteine and glycine decreased between the first and eighth days of sampling, suggesting a decrease in the secretion of a protein or proteins rich in these two amino acids. A decreased secretion of such proteins may also have resulted in a slight decrease in the endogenous flows of other amino acids, but not of sufficient magnitude to reach statistical significance. Alternatively, the decreased flow of cysteine and glycine may have been due to an increased reabsoption of these amino acids, or a combination of decreased secretion and increased reabsorption.

When animals are fed a protein-free diet and enter a negative body nitrogen balance, proline and, to a lesser extent, glycine are usually found at very high levels in the ileal digesta (Skilton *et al.*, 1988; Moughan and Schuttert, 1991; Moughan *et al.*, 1992; Butts *et al.*, 1993). The high level of proline is thought to be due to the breakdown of muscle protein to supply the amino acids required for metabolism (Sauer and de Lange, 1992). There is also evidence that the efficient absorption of proline is dependent on an adequate supply of protein in the diet (Nagchaudhuri and Sharma, 1972). Glycine shares a common absorption pathway with proline. In the present study, there was no statistically significant difference in the endogenous proline flow between days 1 and 8 due to the high variation between animals on day 8, although the mean flow for day 8 was nearly double that found on day one. A difference may have been masked due to the high variances in the data from day 8. On day 1, except for glycine, proline was the most abundant amino acid present in the ileal digesta. On day 8, proline was the most abundant amino acid, with a mean concentration of over double that for the second most abundant amino acid, glycine.

The number of moles of each amino acid per gram dry matter intake on days 1 and 8 were calculated and the amount of each amino acid was expressed as a percentage of the total moles of amino acids. There were statistically significant ($P<0.05$) decreases observed for threonine, glycine and cysteine, and an increase in

proline concentration, indicating differences in the relative amino acid composition of the endogenous protein from day 1 to day 8.

Overall, there was little effect of the duration of feeding of a protein-free diet on the absolute amino acid flows which infers that adjustment in the animal's metabolism due to the protein-free state either mostly occurred within the first 30 hours that the pigs were without dietary protein or that the protein-free regimen did not elicit changes in endogenous flow. In rats, there is evidence to suggest that hepatic and urea cycle enzyme activities reach a new steady state within 20 hours of changes in dietary protein concentration (Das and Waterlow, 1974) and it may be that the animals had almost reached a new steady metabolic state by the time digesta sampling commenced. Corring *et al.* (1984) examined the effect on exocrine pancreatic secretion of feeding pigs a protein-free diet for 8 days. Pancreatic secretions were collected and analysed daily. They reported that neither the protein concentration nor the total amount of protein secreted in pancreatic juice significantly changed over time in pigs fed the protein-free diet compared with these factors when the pigs were fed a diet containing 14% protein. The proportions of the amino acids in the secreted pancreatic juice were also reported by Corring *et al.* (1984) not to change, except for methionine, which decreased compared to the proportions in the same pigs fed a 14% protein diet.

Comparison between endogenous nitrogen excretion in pigs fed a protein-free diet and that of pigs fed diets containing peptides, has shown a considerably greater endogenous flow in pigs fed peptide containing diets (Moughan *et al.* 1992, Butts *et al.* 1993, Leterme *et al.* 1996; Souffrant *et al.*, 1997). It has been suggested that the greater endogenous nitrogen and amino acid flows in peptide-fed animals are due to a stimulatory effect of dietary peptides on the endogenous secretions (Darragh *et al.*, 1995; Boisen and Moughan, 1996b; Moughan *et al.*, 1998). The present results suggest that if there is any adjustment to the absence of dietary peptides, this is almost complete within 30 hours. It is not practical for the protein-free method to be applied over a time period of less than 30 hours, as there would not be certainty that the digesta sampled did not contain dietary nitrogen from the previous meal.

It is concluded that when the growing pig is fed a protein-free diet, there is no effect of the duration of time that the animal has received the protein-free diet on endogenous ileal nitrogen or amino acid flows from between 30 hours and 9 days, with the exception of the amino acids glycine and cysteine. The relative contributions of

threonine, proline, glycine and cysteine on a molar basis do alter during this period of time.

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Chapter 3

Direct Comparison of the Enzyme Hydrolysed Protein, Guanidination and Isotope Dilution Methods for Determining Endogenous Ileal Protein Flow in the Growing Rat and Pig

The results of the study reported in the previous Chapter of this thesis demonstrated that endogenous ileal nitrogen and amino acid flows determined in digesta collected from pigs 30 hours after the pigs had received the last protein-containing meal did not significantly differ from those determined after the pigs had received a protein-free diet for eight days. Given that endogenous ileal nitrogen and amino acid flows determined in digesta collected from animals that have received a protein-free diet for seven to ten days are thought to be underestimated, this suggests that the protein-free method is not a suitable method for the determination of endogenous ileal nitrogen and amino acid flows. Other methods that may be used to determine endogenous ileal protein flow include the enzyme hydrolysed protein, isotope dilution and guanidination methods. These latter methods allow determination of endogenous ileal protein flows in animals that are receiving a diet that contains protein or peptides. No direct comparison has been made previously among the latter three methods to determine whether they give similar estimates of endogenous flows. Therefore, the aim of this study was to compare endogenous ileal nitrogen and lysine flows determined using the isotope dilution and guanidination methods, respectively, with those determined using the enzyme hydrolysed protein method in the growing pig and rat.

Direct Comparison of the Enzyme Hydrolysed Protein, Guanidination and Isotope Dilution Methods for Determining Endogenous Ileal Protein Flow in the Growing Rat and Pig

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ABSTRACT

The aim of this study was to determine whether the isotope dilution and guanidination methods give similar estimates of endogenous ileal nitrogen and lysine flows, respectively, as the enzyme hydrolysed protein method in the growing rat and pig. The test diet contained casein labelled with ^{15}N, guanidinated and enzymatically hydrolysed (MW < 5,000 Da). Male rats (n=30; mean bodyweight 178 g) and entire male pigs (n=6; mean bodyweight 19.2 kg) received a preliminary enzyme hydrolysed casein based diet for 7 days. The rats had free access to the diet for 10 minutes each hour for 8 hours per day; the pigs were given the diet at a rate of 10% of their metabolic body weight per day divided into 8 hourly feeds. The test diet was given to the rats and pigs on the following day in the same manner as the preliminary diet. Digesta were sampled at the terminal ileum after the animals had been anaesthetised. The guanidination method gave significantly ($P<0.05$ and $P<0.01$ for the rat and pig, respectively) lower endogenous lysine flows than the enzyme hydrolysed protein method (means of 298 vs 382 and 214 vs 287 μg/g dry matter intake, DMI, in the rat and pig, respectively). Endogenous nitrogen flows determined using the enzyme hydrolysed protein method were significantly ($P<0.001$ for the rat and $P<0.05$ for the pig) greater than those determined using the isotope dilution method (means of 1942 vs 1034 and 1543 vs 1011 μg/g dry matter intake, DMI, in the rat and pig, respectively). The guanidination method may underestimate endogenous ileal lysine flow in both the rat and pig. The isotope dilution method (labelled diet) may also underestimate endogenous ileal nitrogen flow in the rat and pig.

INTRODUCTION

When calculating amino acid requirements for growing animals or true ileal digestibility coefficients it is important to be able to determine with accuracy the amount of protein of endogenous origin that is present in ileal digesta (Tamminga *et al.*, 1995; Boisen and Moughan, 1996a; Nyochoti *et al.*, 1997a). Several methods have been developed to this end. Three of these approaches, the enzyme hydrolysed protein, isotope dilution and guanidination methods, allow the determination of endogenous protein flow through the ileum when dietary peptides are present in the gut lumen.

The enzyme hydrolysed protein method, proposed by Moughan *et al.* (1990), involves feeding the test animal a semi-synthetic diet containing enzymatically hydrolysed casein (molecular weight <5,000 Da) as the sole source of nitrogen. After the digesta are collected, the high molecular weight fraction (>10,000 Da), which contains the endogenous protein, is separated by centrifugation and ultrafiltration and is analysed for total nitrogen and amino acids.

The isotope dilution method involves the use of the stable isotope ^{15}N and can be applied by feeding diets labelled with ^{15}N to test animals (Leterme *et al.*, 1993; Leterme *et al.*, 1994; Roos *et al.*, 1994). Endogenous nitrogen in the digesta can then be distinguished from ^{15}N-labelled exogenous (dietary) nitrogen.

The guanidination method involves partially transforming dietary lysine to homoarginine. The latter amino acid (an analogue of lysine) is absorbed but not used for body protein synthesis. It is not present in endogenous gut protein and thus acts as a marker for lysine uptake. By determining both the homoarginine and lysine contents of ileal digesta collected after feeding an animal a guanidinated diet, the endogenous lysine flow can be calculated (Hagemeister and Erbersdobler, 1985; Rutherfurd and Moughan, 1990).

The enzyme hydrolysed protein method allows valid measurement of endogenous ileal nitrogen and amino acid flow, by definition, in the specific case pertaining to the administration of the hydrolysed protein itself. The determined endogenous ileal amino acid flow will be a valid estimate for the particular hydrolysate being fed to the animals. It should be noted, however, that there may be a slight underestimation of flow due to the loss of endogenous amino acids discarded with the ultrafiltrate but this is a defined systematic error. In the present study the endogenous total nitrogen and lysine flows determined using the enzyme hydrolysed protein method

were accepted as a suitable baseline and compared to those determined by the more commonly applied isotope dilution and guanidination methods. Casein, which was previously labelled with ^{15}N, was treated with O-methylisourea, to transform lysine to homoarginine. The guanidinated ^{15}N-labelled casein was then enzymatically hydrolysed. A diet was prepared with the enzymatically hydrolysed, guanidinated, ^{15}N-labelled casein as the sole nitrogen source. This diet was fed to growing rats and pigs, and the enzyme hydrolysed protein, isotope dilution and guanidination methods were used to determine endogenous ileal flows of total nitrogen and lysine. A unique aspect of the study was that comparisons among the methods were made within the same animal and the same diet.

MATERIALS AND METHODS

Preparation of Diets

Milk labelled with ^{15}N was collected from a lactating dairy cow following a 10-day period during which 10 g of ^{15}N-labelled $(^{15}NH_4)_2SO_4$ (96-atom-% enriched) was infused twice daily into the cow's rumen via a permanent rumen fistula. The milk was defatted and ^{15}N-labelled casein was isolated from the milk by precipitation with rennin. The ^{15}N-labelled casein was then washed and lyophilised. The nitrogen in the casein was shown to be enriched with 0.1125 atom-%-excess. Half of the ^{15}N-labelled casein was guanidinated as described by Schmitz *et al.* (1991). This part was mixed 1:1 (w/w) with the remaining non-guanidinated ^{15}N-labelled casein. The lysine in the final casein was shown to be 40-45% guanidinated based on determinations of homoarginine.

The resulting ^{15}N-labelled partially guanidinated casein was enzymatically hydrolysed (pancreatin with an enzyme-to-substrate ratio of 0.0089, at a pH of 8.0 for 3 hours) so that all material was less than 5,000 Da. The enzyme was then inactivated at 90 °C for 25 minutes, and the product freeze-dried. The molecular weight distribution of the peptides in the ^{15}N-labelled guanidinated hydrolysed casein (^{15}NGH-casein) was determined using an HPLC gel filtration column (Waters Millipore 625 HPLC system and PSK2000SW 60cm column). The eluting solvent contained 0.1% trifluoroacetic acid and 36% acetylnitrile. The sample was detected using a wavelength of 205 nm. In the ^{15}NGH-casein, 72% of the peptides were found to be between 250 and 5,000 Da, 15% were between 185 and 250 Da and the remaining 13% were less than 185 Da in size.

A diet was prepared (test diet) with the ^{15}NGH-casein as the sole source of protein. Two further diets were also prepared: an intact casein-based diet (basal diet) and an enzyme hydrolysed casein-based diet (preliminary diet). The enzyme hydrolysed casein used in the preliminary diet was chosen to have a similar molecular weight and amino acid profile as the ^{15}NGH-casein. Chromic oxide was included in the preliminary and test diets as an indigestible marker. The ingredient compositions of the diets are given in Table 1 and the determined total nitrogen and amino acid composition of the test diet in Table 2.

Table 1 Ingredient composition (g/kg air dry weight) of the experimental diets given to growing rats and pigs.

Ingredient	Basal Diet	Preliminary Diet	Test Diet
Casein	100	-	-
Enzyme hydrolysed casein (EHC)[1]	-	100	-
^{15}N-labelled guanidinated EHC[2]	-	-	100
Soya bean oil	35	35	35
Cellulose	50	50	50
Sucrose	70	70	70
Wheaten cornflour	739	733	733
Vitamin/mineral mix[3]	2.5	2.5	2.5
Dicalcium phosphate	2.5	2.5	2.5
Sodium chloride	0.3	0.3	0.3
Potassium carbonate	0.5	0.5	0.5
Magnesium sulphate	0.2	0.2	0.2
Chromic oxide	-	6	6

[1] New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand.

[2] Prepared as described in the text.

[3] Vitamin and Mineral Powder, Danmix, Nutritech, Auckland, New Zealand, containing (per kg diet) 4,000,000 IU vitamin A, 800,000 IU vitamin D, 12,000 IU vitamin E, 800 mg vitamin K, 400 mg vitamin B1, 1 g vitamin B2, 800 mg vitamin B6, 4 mg vitamin B12, 84 mg folic acid, 4 g pantothenic acid, 6 mg biotin, 6 g niacin, 20 g choline, 120 mg selenium, 200 mg cobalt, 400 mg iodine, 50 g copper, 40 g iron, 18 g manganese, 48 g zinc and 4 g zinc bacitracin.

Table 2 The determined total nitrogen and amino acid composition of the test diet (mg/g dry matter).

Amino acid	
LYS	4.46
HARG[1]	3.83
HIS	3.40
ARG	3.87
ASP	7.46
THR	4.34
SER	4.78
GLU	23.24
PRO	12.17
GLY	2.01
ALA	3.05
CYS	0.27
VAL	6.95
MET	3.16
ILE	5.51
LEU	9.96
TYR	5.43
PHE	5.63
Nitrogen	16.50

[1]Homoarginine

Rat Study

All protocols for experiments described in this study were approved by the Massey University Animal Ethics Committee. Thirty Sprague Dawley male rats with a bodyweight of (mean ± SEM) 179.0 ± 3.0 g were obtained from the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. The animals were housed singly in wire-bottomed cages which were designed to prevent coprophagy, at 22 ± 2 °C with a 12-hour light/dark cycle. Fresh water was available at all times. Throughout the trial the rats were given free access to food for ten minutes every hour over eight hours each day, starting at 0800h; the final meal of the day was at 1500h. The feeders were weighed before and after each meal.

The first seven days of the trial were a training period, during which the rats were given access to the basal diet and trained to eat in eight ten-minute periods per day as

described above. The rats then received the preliminary diet for a further seven days in the same manner.

On the next day (day 15), the rats were fed the test diet using the same procedure (ie. free access to the diet for 10 minutes every hour). Starting five hours after the first meal of the day, the rats were euthanased by asphyxiation with CO_2 gas and then decapitated. The body cavity was opened and the last 20 cm of the terminal ileum immediately dissected free, rinsed with distilled water to remove any traces of blood and hair and gently dried with absorbent paper. Care was taken not to apply pressure to the intestine. The digesta were slowly flushed out with deionised water from a syringe. The digesta samples were immediately frozen and stored at –20 °C until analysis. The maximum time from euthanasia until the digesta were collected was four minutes.

Pig Study

Six Landrace x Large White entire male pigs with a bodyweight of (mean ± SEM) 19.2 ± 0.5 kg were sourced from the Pig Research Unit, Massey University, Palmerston North, New Zealand. The pigs were housed individually in smooth-walled steel metabolism crates at the Animal Physiology Unit, Massey University, Palmerston North, New Zealand. The room was maintained at 22 ± 1 °C. Water was freely available at all times.

Throughout the trial, the pigs were fed the diets mixed with water (1:1) at 0.10 x metabolic body weight ($kg^{0.75}$) daily. For the first seven days of the study, the pigs were fed the basal diet with half of the daily allowance given at 0800h and the other half at 1700h. At the end of this period, the pigs were weighed and the food intake adjusted accordingly for the remainder of the trial. For the next seven days, the pigs were fed the preliminary diet and for the first two of these seven days, the daily allowance was fed in two equal portions, at 0800h and 1700h. For the following five days, the daily allowance was divided into eight equal portions with one portion fed to the pigs every hour commencing at 0800h daily during the first two days. The feeding times for each pig were staggered by 30 minutes during the final three days of this five-day period.

On the 15th day, the pigs were fed the test diet in the same manner (i.e. staggered hourly meals from 0800h). Exactly nine hours after the start of its first meal of the day,

each pig was given an intramuscular injection of azaperone (Stresnil, Janssen Pharmaceutica, Beerse, Belgium), anaesthetised by inhalation of halothane (Fluothane, ICI Pharmaceuticals, Lower Hutt, New Zealand) and euthanased by intracardial injection of sodium pentobarbitone (Pentobarb 500, Chemstock Animal Health Ltd, Christchurch, New Zealand). The final 20 cm of the terminal ileum was immediately dissected from the body and digesta were collected as described above for the rat study. The digesta were immediately frozen and stored at -20 °C until analysis. The maximum time from anaesthetising a pig until the digesta were collected was six minutes.

Chemical Analyses

The digesta from each rat were freeze-dried. The rat digesta samples were randomly divided into six groups each containing the digesta from five animals. The digesta from each group of five rats were pooled so that a sufficient amount of digesta was available for all chemical analyses, giving a total of six pooled samples. Each digesta pool was carefully mixed and divided into three portions. The digesta sample from each pig was freeze-dried, mixed and then divided into 3 portions.

The test diet was analysed for chromium, dry matter, total nitrogen, ^{15}N and amino acids as described below.

One portion of digesta from each sample from the rats and pigs was analysed for ^{15}N, ^{14}N and dry matter, for calculations with the isotope dilution method. The second portion from each sample was analysed for dry matter, chromium, and the amino acids lysine and homoarginine to allow calculations using the guanidination method.

The third portion of digesta from the rat and pig samples was resuspended in distilled water and acidified to pH 3.5 with 6 M sulphuric acid. The samples were stored overnight at 4°C, and then centrifuged at 7,000 G for 10 minutes. The supernatants were then ultrafiltered separately using Centriprep-10 ultrafiltering devices (Amicon Inc., Beverly, MA, USA) according to the manufacturer's instructions. The precipitate from the centrifugation step was added to the retentate (>10,000 Da) from the ultrafiltration step, and the material was freeze-dried. This portion was analysed for amino acids, nitrogen, chromium and dry matter for calculations with the enzyme hydrolysed protein method.

The chromium contents of the diet and ileal digesta were determined using an Instrumentation Laboratory Atomic Absorption Spectrophometer according to the method described by Costigan & Ellis (1987).

Total nitrogen for the enzyme hydrolysed protein method was determined in duplicate. The samples were combusted at 1050 °C in oxygen gas. The nitrogen was then reduced to N_2 by a catalyst and this was measured by a thermal conductivity cell using a Leco FP2000 (Leco Corporation, St Joseph, Michigan, USA).

The amino acid composition of the samples was determined as follows: Duplicate samples (5-7 mg) were hydrolysed in 1 ml of 6 mol/L glass-distilled HCl containing 0.1% phenol in glass tubes sealed under vacuum, for 24 hours at 110 $\pm$ 2 °C. Amino acid concentrations were then measured using a Waters ion exchange HPLC system calibrated against a reference amino acid mixture. The peaks of the chromatograms were integrated using the dedicated software Maxima 820 (Waters, Millipore, Milford, MA) which identifies the amino acids by retention time against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively, and the weight of each amino acid was calculated using free amino acid molecular weights. No corrections were made for losses of amino acids during hydrolysis.

Cysteine and methionine are partially destroyed during this hydrolysis procedure, so were determined following oxidation of duplicate samples (3-4 mg) with 1 mL of performic acid (1 part 30% H_2O_2 to 9 parts 88% formic acid) for 16 hours at 0 °C. The samples were then neutralised with 0.15 mL of 50% (w/w) HBr prior to acid hydrolysis. Tryptophan, which is also destroyed during acid hydrolysis, was not determined.

To determine the ^{15}N enrichment of the diet and digesta for the isotope dilution method, ground homogenised samples were analysed using an on-line combined CHN Elemental Analyser (EA 1108, Carlo Erba, Italy) and isotope mass spectrometer (IRMS Delta S, Finnigan, Germany) for ^{14}N and ^{15}N. This system is accurate to 0.00005 atom %. The measured ^{15}N enrichment of the diet and digesta were corrected for the natural ^{15}N-enrichment of the preliminary diet and digesta from animals that had not received a ^{15}N-labelled diet.

Data Analysis

For the enzyme hydrolysed protein method, endogenous ileal nitrogen and amino acid flows (related to the ingestion of 1 g of dry matter) were determined using the equation reported by Hodgkinson *et al.* (1999, refer Chapter 2 of this thesis). The units were μg/g dry matter intake (DMI).

For calculation of the lysine flows for the guanidination method, firstly the total lysine flow was calculated as follows:

Total lysine flow =

$$\frac{\text{Concentration of lysine in the digesta} \times \text{Diet chromium concentration}}{\text{Digesta chromium concentration}}$$

The units were μg/g DMI. The total homoarginine (HA) flow was calculated in the same manner. The endogenous lysine flow was then calculated using the equation below:

Endogenous lysine flow =

$$\text{Total lysine flow} - \frac{(\text{Total HA flow} \times \text{Concentration of lysine in the diet})}{\text{Concentration of HA in the diet}}$$

For the isotope dilution method, the endogenous nitrogen flows (related to the ingestion of 1 g of dry matter) were calculated using the following equations:

Exogenous nitrogen flow (μg/g DMI) =

$$\frac{^{15}\text{N enrichment of digesta (atom \%-excess)}}{^{15}\text{N enrichment of diet (atom \%-excess)}} \times \text{total nitrogen content of digesta } (\mu\text{g/g DM}).$$

Endogenous nitrogen flow (μg/g DMI) =

Total nitrogen flow (μg/g DMI) – exogenous nitrogen flow (μg/g DMI).

The data were first tested for homogeneity of variance using Bartlett's Test (Snedecor and Cochran, 1980) and all variances were found to be homogeneous ($P>0.05$). Paired t-test comparisons were carried out using the statistical programme SAS (version 6.12, 1997) to test for differences in the endogenous lysine flows determined using the enzyme hydrolysed protein and guanidination methods, and to test for differences in the endogenous nitrogen flows determined using the enzyme hydrolysed protein and isotope dilution methods.

An estimate of the nitrogen and amino acid composition of endogenous ileal excretions reported by Boisen and Moughan (1996b) was used to calculate the

endogenous nitrogen flow corresponding to the determined endogenous lysine flow based on the guanidination method determined in the pig. For the rat, endogenous ileal flows of nitrogen and lysine reported in the literature (Butts *et al.*, 1992; Donkoh *et al.*, 1995; Yu *et al.*, 1995) were averaged (average N:lysine ratio was 5.51 μg nitrogen per μg lysine at a set dry matter intake) and the mean value was used to calculate the endogenous ileal nitrogen flow corresponding to the endogenous lysine flow determined with the guanidination method applied in the rat. The endogenous nitrogen flows calculated for the rat and pig were compared with those determined using the isotope dilution and enzyme hydrolysed protein methods, using a paired t-test.

RESULTS

All of the rats and pigs appeared healthy throughout the study. The pigs ate all of their allocated dietary allowance throughout the experiment and the rats had a daily intake of (mean ± SEM) 14.3 ± 0.2 g of diet. During the 8-day period that they were given access to the preliminary and test diets, the growth rates were (mean ± SEM) 4.1 ± 0.2 g/day and 344 ± 31 g/day for the rats and pigs, respectively.

The endogenous lysine flows determined using the guanidination method in both the rat and pig were significantly ($P<0.05$ and $P<0.01$ for the rat and pig, respectively) lower than those determined using the enzyme hydrolysed protein method (Table 3). Endogenous nitrogen flows determined using the isotope dilution method in the rat and pig were significantly ($P<0.001$ and $P<0.05$ for the rat and pig, respectively) lower than those determined using the enzyme hydrolysed protein method (Table 3). The endogenous ileal amino acid flows determined using the enzyme hydrolysed protein method in the rat and pig are presented in Table 4.

The endogenous ileal nitrogen flows, calculated based on the endogenous lysine flows determined using the guanidination method and based on an assumption of the ratio of nitrogen to lysine in endogenous protein flows, were (mean ± SEM) 1641 ± 73 and 1179 ± 389 μg/g DMI in the rat and pig, respectively. In the pig, the endogenous nitrogen flow for the guanidination method was not significantly different from those determined using either the enzyme hydrolysed protein or isotope dilution methods. In the rat, the endogenous ileal nitrogen flow for the guanidination method was significantly ($P<0.05$) lower than that determined using the enzyme hydrolysed protein method but greater ($P<0.01$) than that determined using the isotope dilution method.

Table 3 Mean (± SEM, n=6) endogenous ileal nitrogen and lysine flows (μg/g DMI) determined using the enzyme hydrolysed protein[1], guanidination[2] and isotope dilution[3] methods in the growing rat and pig.

	Rat		Pig	
Method	Lysine	Nitrogen	Lysine	Nitrogen
Enzyme hydrolysed protein	382 ± 24	1942 ± 113	287 ± 70	1543± 262
Guanidination	298 ± 13	-	214 ± 73	-
Isotope dilution	-	1034 ± 51	-	1011 ± 164
Significance[4]	*	***	**	*

[1] Lysine and nitrogen flows through terminal ileum determined in >10,000 Da fraction of digesta following ultrafiltration.

[2] Lysine and homoarginine contents of diet and ileal digesta determined and endogenous lysine flow calculated.

[3] ^{15}N enrichment of diet and ileal digesta determined and endogenous N flows calculated from dilution of ^{15}N label in ileal digesta.

[4] NS not significant, * P<0.05, ** P<0.01, *** P<0.001, determined using paired t-test comparisons.

Table 4 Mean (± SEM, n=6) endogenous ileal amino acid flows (μg/g DMI) determined using the enzyme hydrolysed protein method[1] in the growing rat and pig.

Amino acid	Rat	Pig
HIS	307 ± 17	277 ± 47
ARG	282 ± 17	243 ± 75
ASP	1222 ± 76	733 ± 127
THR	782 ± 45	521 ± 69
SER	1552 ± 92	816 ± 169
GLU	2990 ± 171	1996 ± 352
PRO	706 ± 41	660 ± 110
GLY	786 ± 65	651 ± 98
ALA	484 ± 33	368 ± 86
CYS	134 ± 7	112 ± 24
VAL	798 ± 46	473 ± 86
MET	148 ± 7	117 ± 29
ILE	803 ± 48	412 ± 90
LEU	584 ± 36	448 ± 103
TYR	327 ± 21	155 ± 45
PHE	246 ± 16	166 ± 51

[1] Amino acid flows through terminal ileum determined in >10,000 Da fraction of digesta following ultrafiltration.

DISCUSSION

The aim of the present study was to evaluate the accuracy of the isotope dilution and guanidination methods for the determination of endogenous nitrogen and lysine flows at the terminal ileum of simple-stomached mammals. This was done by comparing endogenous ileal flows determined using the isotope dilution and guanidination methods with those determined using the enzyme hydrolysed protein method. The approach taken, with application of the three methods to a group of animals given the same diet, allowed an original comparison of the three methods in the same animals at the same time.

The enzyme hydrolysed protein (casein) method was used as a baseline for comparison as it gives valid estimates of endogenous nitrogen and amino acid flows in the specific case where hydrolysed casein is being fed to animals, as was the situation in the present study. When the enzyme hydrolysed protein method is applied more generally to determine endogenous ileal protein flows, the assumption is made that the molecular weight profile of the dietary peptides (ie. peptide size) does not effect the net endogenous protein flow, but this has not been fully tested. In the present study, however, hydrolysed casein was fed to all of the animals for all of the experimental treatments thus any effect of peptide size on endogenous ileal protein flow would be inherent to the one diet and would not affect the comparison between the methods. The enzyme hydrolysed protein (casein) method is expected to give valid information on endogenous ileal amino acid flow in the specific situation where hydrolysed casein is fed to the animal.

The enzyme hydrolysed protein method has the shortcoming that there is a net underestimation of endogenous amino acid flow due to a loss of endogenous small peptides and amino acids in the ultrafiltrate. Studies have attempted to quantify the extent of this loss, by ultrafiltering digesta collected from animals given a protein-free diet and quantifying the amino acids present in the ultrafiltrate. In a controlled study, Moughan and Schuttert (1991) found approximately 10% of the amino nitrogen from total digesta to be present in the ultrafiltrate. It should be noted, however, that feeding protein or enzyme hydrolysed protein to an animal may theoretically affect the composition as well as the amount of endogenous excretion, and that higher or lower than normal amounts of free amino acids and small peptides may occur during protein-free feeding. It is difficult to accurately determine the amounts of small (<10,000 Da) amino molecules in the ileal digesta of animals receiving an EHC based diet, though the concentration of such molecules at the

terminal ileum would be expected to be low. It is concluded that the enzyme hydrolysed protein method applied here may lead to a slight (unquantified) underestimation of total endogenous amino acid flow at the end of the ileum and the results need to be interpreted accordingly.

There is a lot of variation in mean values reported in the literature for endogenous ileal nitrogen and amino acid flow in the rat and pig determined using the enzyme hydrolysed protein method. The results obtained in the present study with the rat (Table 4) are within the range of previous values reported in the literature (Darragh *et al.*, 1990; Butts *et al.*, 1991; Donkoh *et al.*, 1995). The present endogenous ileal nitrogen and amino acid flows in the pig determined using the enzyme hydrolysed protein method (Table 4) are slightly lower than those found in previous studies with pigs (Butts *et al.*, 1993a; Butts *et al.*, 1993b; Moughan *et al.*, 1992a; Schulze *et al.*, 1995). The present values for endogenous ileal nitrogen and lysine flow in the pig determined using the isotope dilution (with labelled diets) and the guanidination methods were also slightly lower than comparable values reported in the literature (Leterme et al, 1994; Nyachoti *et al.* 1997b; Souffrant *et al.*, 1997).

In the present work, the mean endogenous lysine flows were 22-25% lower when determined using the guanidination method in comparison to those determined using the enzyme hydrolysed protein method. This was true for both the rat and pig. This suggests that the guanidination method considerably underestimates endogenous lysine flow. The reason for this is not clear. Most of the assumptions that are made when the guanidination method is used to determine endogenous ileal lysine flow have been tested and appear to be valid. One area that has not been fully investigated is the possible effect of homoarginine on protein digestion and metabolism. Drescher *et al.* (1994) found a lower true nitrogen digestibility for guanidinated diets than for non-guanidinated diets. However, in the present study, any effect of the homoarginine in the diet on protein digestion and metabolism and which could affect endogenous lysine flow determined using the guanidination method, would also have been reflected in the results from the enzyme hydrolysed protein method. A comparison of the endogenous lysine flows determined using the enzyme hydrolysed protein and guanidination methods has not been made previously.

In the rat and pig, endogenous ileal nitrogen flow determined using the isotope dilution method was significantly lower than that determined using the enzyme hydrolysed protein method. Given that the enzyme hydrolysed protein method is expected to

underestimate endogenous ileal nitrogen flow, these results suggest that, as for the guanidination method, the isotope dilution method underestimates endogenous ileal nitrogen flow. When a feed containing protein which is labelled with ^{15}N is fed to a test animal, and the isotope dilution method is used to determine endogenous ileal nitrogen flows, it is assumed that the labelled and unlabelled dietary amino acids are absorbed equally by the animal. It is also assumed that the endogenous protein that is secreted into the gut does not become labelled during the course of the trial. However, it is known that the enterocytes take up a proportion of the labelled dietary nitrogen and resecrete it rapidly into the intestine, which will result in some endogenous protein being mistaken for unabsorbed exogenous protein. Following the ingestion of a single ^{15}N-labelled meal in the growing pig, Leterme *et al.* (1996) found the presence of ^{15}N in pancreatic enzymes within 50 minutes, in the bile within 90 minutes and in the intestinal mucus within 4 hours of the meal. In the present study, some of the labelled dietary amino acids may have been absorbed and resecreted in the period of time between consumption of the labelled dietary amino acids and sampling of the digesta. Resecreted amino acids are endogenous, but would have been labelled with ^{15}N and thus during the analysis would have contributed to the estimate of undigested dietary amino acids. This may explain the underestimation of endogenous ileal nitrogen flow observed in the present study with the isotope dilution method. Digesta were collected five and nine hours after the first meal of the ^{15}N-labelled test diet for the rats and pigs, respectively, in an attempt to minimise the extent of recycling; however, recycling could still have been significant.

Previous comparisons between the isotope dilution technique and other methods such as the enzyme hydrolysed protein or guanidination methods have mostly applied the isotope dilution method by labelling the whole animal with ^{15}N-leucine as opposed to labelling the diet as in the present study. In these previous trials, only leucine is labelled with ^{15}N and it is presumed that other amino acids will be labelled by transamination. However, the ^{15}N label is not transferred to lysine and threonine, and the isotopic enrichment of other amino acids is less than that of the labelled leucine (de Lange *et al.*, 1992; Lien *et al.*, 1997a; 1997b). The use of mixtures of ^{15}N-labelled amino acids has been suggested (Reeds *et al.*, 1997) and may avoid this problem.

Another concern with the use of the isotope dilution method applied by labelling the animal's body nitrogen pool is the choice of an appropriate precursor pool (Moughan *et al.*, 1992b). The TCA-soluble blood plasma is commonly used to indicate ^{15}N

enrichment of the gut endogenous material with the assumption made that the free amino acids in the TCA-soluble pool are used for the synthesis of body protein and thus for most of the endogenous secretions into the digestive tract. For small intestinal mucosa secretions at least, however, absorbed dietary amino acids are used directly for protein synthesis (Alpers, 1972). The ^{15}N-enrichment of the TCA-soluble blood plasma fraction is lower than that in pancreatic secretions and in urinary urea plus ammonia (Souffrant *et al.*, 1993). Thus the use of plasma enrichment may underestimate the enrichment of endogenous nitrogen secreted into the intestinal lumen leading to an overestimation of endogenous protein excretion (Lien *et al.*, 1997b). Leterme *et al.* (1994) compared endogenous nitrogen flows determined for the pig using the isotope dilution method with labelled animals to those obtained using labelled diets. The endogenous nitrogen flow determined using the labelled animals was higher than that using the labelled diets. Schulze *et al.* (1995) working with the young pig, compared the enzyme hydrolysed protein method with the isotope dilution method applied by labelling the animal rather than the diet and found no significant differences between the two methods.

Endogenous ileal total nitrogen flow and flows for the amino acids other than lysine can be calculated using the guanidination method. This is generally done by assuming that endogenous ileal excretions have a constant nitrogen and amino acid composition. Boisen and Moughan (1996b) conducted an extensive review of the literature on the nitrogen and amino acid composition of endogenous ileal excretions, and published a generalised amino acid composition for the pig. The latter estimate of amino acid composition was used in the present work to calculate endogenous nitrogen flows corresponding to the endogenous lysine flows determined directly using the guanidination method for the pig. For the rat, the endogenous ileal flows of nitrogen and lysine reported in previous studies were averaged to give a mean nitrogen to lysine ratio, which was then used to calculate endogenous nitrogen flows corresponding to the endogenous lysine flows determined using the guanidination method. In the rat, the calculated endogenous nitrogen flows (guanidination) were significantly lower ($P<0.05$) than those determined using the enzyme hydrolysed protein method, but significantly greater than those determined using the isotope dilution method. There were no statistically significant differences in endogenous ileal nitrogen flow determined in the pig between all three methods. Souffrant *et al.* (1997) reported endogenous ileal nitrogen flows in the pig determined using both the isotope dilution method with a ^{15}N labelled diet and the guanidination method. The endogenous nitrogen flows calculated with the guanidination method were

quantitatively greater than those determined with the isotope dilution method, although no statistical comparison was reported. Roos *et al.* (1994) determined the true digestibility of casein doubly-labelled with homoarginine and ^{15}N. The digestibility of casein was determined at 3, 6 and 12 hours following the meal. The digestibilities determined with the guanidination method were significantly higher than those determined using ^{15}N at all sampling times. This corresponds to greater endogenous flows determined with the guanidination method than the isotope dilution method. It was suggested that the difference in digestibility between the two methods may have been due to recycling of the ^{15}N label, resulting in an underestimation of the endogenous component with the isotope dilution method (Roos *et al.*, 1994).

It appears that the guanidination method leads to an underestimation of endogenous ileal lysine flow in both the rat and pig. The isotope dilution method (labelled diet) appears to underestimate endogenous ileal nitrogen flow in the rat and pig.

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Chapter 4

Effectiveness of an Ultrafiltration Device for Use with the Enzyme Hydrolysed Protein Method for Determining Endogenous Ileal Amino Acid Excretion in Simple-stomached Animals

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The study reported in the previous Chapter showed that the isotope dilution and guanidination methods may considerably underestimate endogenous ileal protein flows in the pig and rat. The enzyme hydrolysed protein method was, therefore, used for the studies reported in the following Chapters. There were, however, some assumptions inherent in the enzyme hydrolysed protein method that required further examination. Firstly, the assumption is made that all unabsorbed dietary enzyme hydrolysed casein (EHC) moves through the filter of the ultrafiltration device and is discarded with the ultrafiltrate. Thus it is assumed that EHC does not bind to the precipitate plus retentate fraction of digesta, which is analysed to determine endogenous nitrogen and amino acid flows. This assumption has not been tested previously. A further assumption that has not been tested is that the ultrafiltration devices are equally effective when either high or low concentrations of EHC are present in the digesta. The work to be presented in this Chapter involved an *in vitro* study, aimed at testing the effectiveness of disposable Centriprep-10 Concentrator devices for the ultrafiltration of digesta containing EHC.

Effectiveness of an Ultrafiltration Device for Use with the Enzyme Hydrolysed Protein Method for Determining Endogenous Ileal Amino Acid Excretion in Simple-stomached Animals

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ABSTRACT

The aim of the work was to perform an *in vitro* study to determine the effectiveness of Centriprep-10 Concentrator devices for use with the enzyme hydrolysed protein method for the determination of gut endogenous ileal nitrogen and amino acid flows. Different amounts of enzyme hydrolysed casein (EHC) were added to tubes containing digesta collected after pigs had received a protein-free diet for five to eight days. The samples were centrifuged and then ultrafiltered using Centriprep-10 concentrators. The precipitate from the centrifugation step was added to the retentate from the ultrafiltration and this material was analysed for nitrogen and amino acids. The ultrafiltrates were also analysed for nitrogen. The amount of nitrogen that was deemed to have originated from the EHC and remained in the precipitate plus retentate fraction of digesta after processing, expressed as a percentage of the total amount of nitrogen added to the tubes as EHC, ranged from 1.0 to 5.0%. The overall mean amounts of amino acid in the precipitate plus retentate fractions originating from the EHC, expressed as a percentage of the amino acids added to the tubes as EHC ranged from 2.4 to 5.8%. The results demonstrate that with Centriprep-10 concentrators, there is a less than complete separation of nitrogen and amino acids originating from EHC from endogenous material in digesta, but for most amino acids, this is unlikely to be due to binding of the amino acids to digesta. The incomplete separation of EHC from the endogenous fraction of digesta by Centriprep-10 concentrators may lead to a small overestimation (approximately 2%) of endogenous ileal nitrogen and amino acid flows.

INTRODUCTION

The enzyme hydrolysed protein method was first proposed by Moughan *et al.* (1990) as a means for quantifying gut endogenous ileal nitrogen and amino acid losses in simple-stomached animals. The method involves feeding animals a diet containing hydrolysed protein (usually enzyme hydrolysed casein, EHC) as the sole dietary nitrogen source. EHC comprises free amino acids and small peptides less than 5,000 Da in size. Digesta are collected from the animal, centrifuged and the supernatant is ultrafiltered using a disposable ultrafiltration device with a molecular weight cut-off of 10,000 Da. The precipitate from the centrifugation is added to the retentate (>10,000 Da) from the ultrafiltration. This material contains the endogenous protein. Any unabsorbed dietary amino acids are removed in the ultrafiltrate (<10,000 Da fraction). The enzyme hydrolysed protein method has been applied for the determination of gut endogenous nitrogen and amino acid losses in several species of simple-stomached animals (Darragh *et al.*, 1990; Butts *et al.*, 1991; Moughan *et al.*, 1992; Butts *et al.*, 1993; Schulze *et al.*, 1995; Donkoh *et al.*, 1995; Hendriks *et al.*, 1996; Leterme *et al.*, 1996; Rutherfurd and Moughan, 1997).

Centriprep-10 Concentrators (MW exclusion limit 10,000 Da, Amicon Inc., Beverly, MA, USA) have commonly been used in the ultrafiltration of digesta with the enzyme hydrolysed protein method and have been shown to be effective for the ultrafiltration of purified protein, peptide and amino acid solutions (Butts *et al.*, 1991).

A potential criticism of the enzyme hydrolysed protein method is that unabsorbed EHC may covalently bind to substances present in digesta and remain bound to the precipitate plus retentate fraction of digesta throughout the ultrafiltration process. This would result in some dietary amino acids being analysed as part of the endogenous fraction and would cause an overestimation of the endogenous nitrogen and amino acid flows determined using the enzyme hydrolysed protein method. The efficacy of Centriprep-10 Concentrators for the ultrafiltration of unabsorbed free amino acids and small peptides from EHC in digesta has not been examined.

The primary aim of this *in vitro* study was to determine the effectiveness of Centriprep-10 ultrafiltration devices for the separation of free amino acids and peptides originating from EHC, in digesta. A second aim of the study was to determine whether Centriprep-10 ultrafiltration devices are equally effective at

ultrafiltering different concentrations of amino acids and small peptides (<5,000 Da) in digesta.

MATERIALS AND METHODS

Digesta were collected from the terminal ileum of post-valve T-caecum cannulated pigs that had received a protein-free diet for five to eight days (Hodgkinson *et al.*, 1999, refer to Chapter 7 of this thesis). At collection, the digesta were acidified to a pH of 3.5 by the addition of 6 M H_2SO_4 to minimise enzymatic or microbial activity in the digesta, and the digesta were then immediately frozen (-20° C).

Following thawing, a measured amount of well-mixed total digesta (approximately 10 g) was added to each of six centrifuge tubes. Known amounts of EHC (New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand) were added to five of these tubes, with the remaining tube containing digesta only (baseline control, control 1). A further tube was prepared to which was added 450 mg of EHC dissolved in 10 ml of distilled filtered water. This tube did not contain any digesta and acted as a second control (control 2). A duplicate set of the seven tubes (control 1, tubes A-E and control 2) was prepared. All 14 tubes were stirred, covered and then stored at 4° C overnight (16 hours) to ensure that the EHC was completely dissolved. The mean amounts of digesta and added EHC for the treatments and controls are given in Table 1.

Table 1 Digesta and enzyme hydrolysed casein (EHC) added to test tubes before the centrifugation and ultrafiltration steps.

Tube	Digesta (g)[1]	EHC (mg)[1]
Control 1	10.12	0
A	10.08	38
B	10.06	75
C	10.16	113
D	10.07	150
E	9.97	177
Control 2[2]	0	450

[1] Mean of duplicate tubes.

[2] 10 ml of distilled and filtered water.

All of the tubes except control 2 were centrifuged at 7,000 g for 10 minutes. The supernatants were collected and ultrafiltered using Centriprep-10 ultrafiltering devices

(Amicon Inc., Beverly, MA, USA) according to the manufacturer's instructions. The ultrafiltrates (<10,000 Da) were retained. The control 2 samples were ultrafiltered in the same manner but without the centrifugation step.

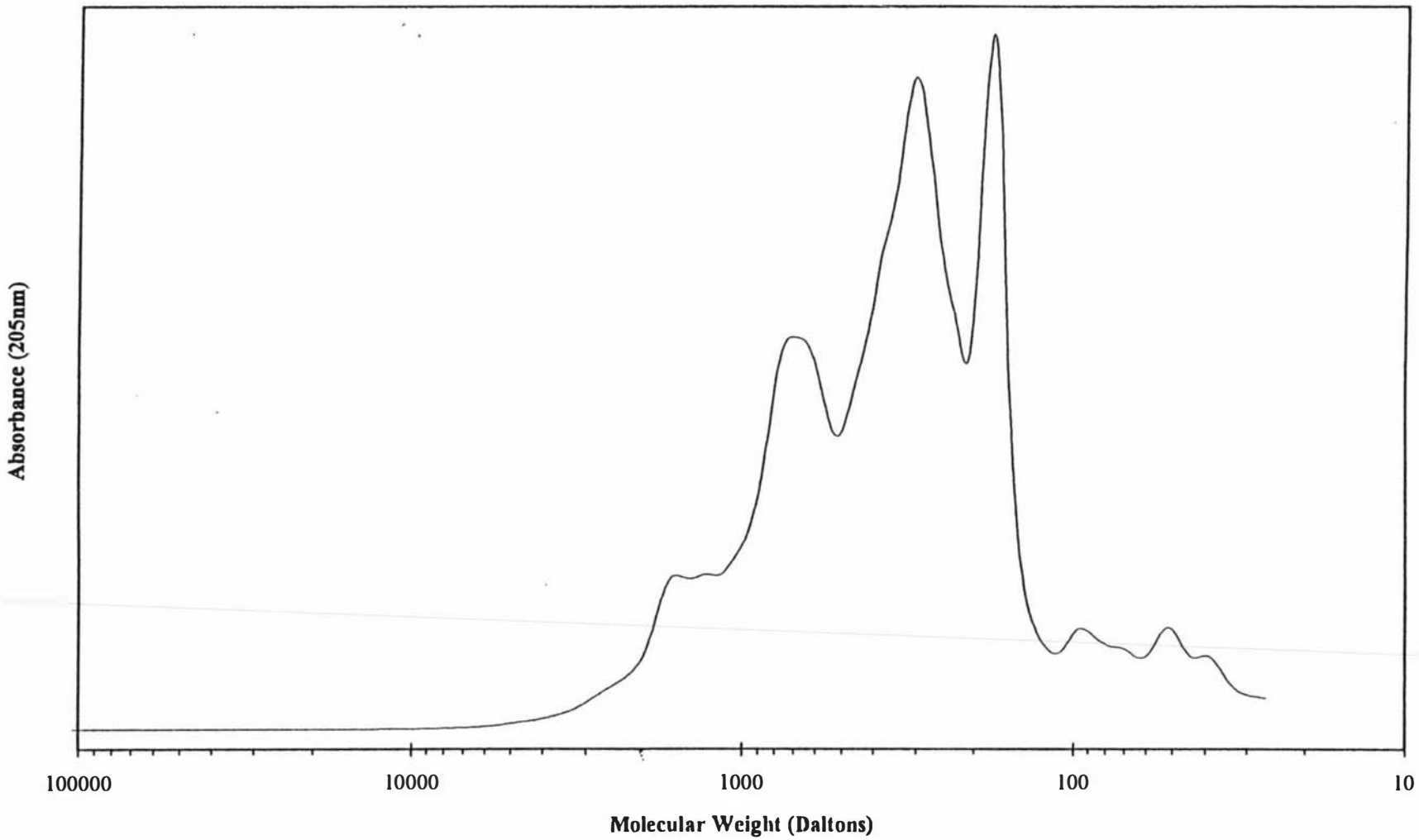
The precipitates from the centrifugation were added to the retentates (>10,000 Da) from the ultrafiltration step, and the material was freeze-dried. The ultrafiltrates were concentrated to a volume of approximately 5 ml using an Automatic SpeedVac (AS290, Savant Instruments Inc., NY, USA). Each ultrafiltrate was then made up to exactly 10 ml with distilled purified water.

The molecular weight distribution of the enzyme hydrolysed casein was determined using an HPLC gel filtration column (Waters Millipore 625 HPLC system and PSK2000SW 60cm column). The eluting solvent contained 0.1% trifluoroacetic acid and 36% acetylnitrile, and the sample was detected using a wavelength of 205 nm. The molecular weight profile of the EHC is shown in Figure 1. All peptides in the EHC were less than 5,000 Da in size. A total of 0.8% of the peptides were in the range 3,000 to 5,000 with 11.3% with a size of 1,000-3,000 Da and the remaining 87.9% were in the range 100-1,000 Da.

Total nitrogen in the EHC sample and precipitate plus retentate and ultrafiltrate fractions of digesta was determined in duplicate using the Kjeldahl method. The samples were digested with concentrated sulphuric acid with potassium sulphate added to increase the digestion temperature. The free ammonia released was determined using the Berthelot reaction (Chaney and Marbach, 1962).

The amino acid compositions of duplicate samples (5-7 mg) of the EHC and precipitate plus retentate fractions were determined as follows. The samples were hydrolysed in 1 ml of 6 mol/L glass-distilled HCl containing 0.1% phenol in glass tubes sealed under vacuum, for 24 hours at 110 $\pm$ 2 °C. Amino acid concentrations were then measured using a Waters ion exchange HPLC system calibrated against a reference amino acid mixture of known concentration. The peaks of the chromatograms were integrated using the dedicated software Maxima 820 (Waters, Millipore, Milford, MA) which identifies the amino acids by retention time against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively, and the weight of each amino acid was calculated using free amino acid molecular weights. No corrections were made for potential losses of amino acids during hydrolysis.

Figure 1 Molecular weight profile of enzyme hydrolysed casein (the molecular weight is plotted on a log scale versus the absorbance at 205 nm).



The determined nitrogen and amino acid contents of the EHC sample were used to calculate the amounts of nitrogen and each amino acid added to each tube in the form of EHC.

Each sample of ultrafiltrate and each precipitate plus retentate fraction was corrected for the amount of nitrogen arising from the digesta, assuming that the relative proportions of nitrogen from digesta in the ultrafiltrates and precipitate plus retentate fractions were the same in each tube as the mean value for control 1. The amount of each amino acid in the precipitate plus retentate fractions was also corrected for the amount of each amino acid arising from the digesta. The remaining amounts of nitrogen and each amino acid (after correction) were deemed to have originated from the added EHC and were expressed as proportions of the total amounts of nitrogen and each amino acid originally added to each tube in the form of EHC.

RESULTS

The variation in the nitrogen content of the duplicate samples (calculated as the difference between the duplicates divided by the mean of the two duplicates and expressed as a percentage), averaged 3.2% for the precipitate plus retentate and 3.7% for the ultrafiltrate fractions. This indicated that the procedure *per se* led to only minor variation in the resulting concentrations.

The total amounts of nitrogen found in the ultrafiltrate and precipitate plus retentate fractions of digesta are presented in Table 2. The amounts of nitrogen that were deemed to have originated from the EHC and were in the ultrafiltrate and precipitate plus retentate fractions of digesta following centrifugation and ultrafiltration, expressed as percentages of the amounts of nitrogen added to the tubes (EHC) are given in Table 3. The amount of each amino acid that was added in the form of EHC and remained in the precipitate plus retentate fractions post-processing, expressed as a percentage of the total amount of the respective amino acid added as EHC, is shown in Table 4.

The amount of nitrogen that was added to the tubes as EHC and remained in the precipitate plus retentate fraction following centrifugation and ultrafiltration ranged from 0.24 to 0.47 mg. This corresponded to 1.0-5.0% of the added EHC (Table 3).

Table 2 Total amounts of nitrogen[1] in the precipitate plus retentate (P+R) and ultrafiltrate fractions of digesta and total amounts of nitrogen added to each tube in the form of enzyme hydrolysed casein (EHC).

Tube	Nitrogen		
	P+R (mg)	Ultrafiltrate (mg)	Added as EHC (mg)
Control 1	9.00	2.11	0
A	9.25	6.74	5.07
B	9.41	11.31	10.00
C	9.47	16.25	15.06
D	9.46	21.07	20.00
E	9.24	25.05	23.60
Control 2	2.28[2]	55.91	59.99

[1] Means of duplicates.
[2] Retentate only.

Table 3 Percentage recovery[1] of nitrogen (N) added to digesta in the form of enzyme hydrolysed casein (EHC) in the precipitate plus retentate (P+R) and ultrafiltrate fractions following centrifugation and ultrafiltration.

Tube	Recovery (%) of EHC N[2]		
	P+R	Ultrafiltrate	N unaccounted for[3]
A	5.0	91.4	3.6
B	4.1	92.0	3.9
C	3.1	93.9	3.0
D	2.3	94.8	2.9
E	1.0	97.2	1.8
Control 2[4]	3.8[5]	93.2	3.0

[1] Mean of duplicates.
[2] Total N in the P+R and ultrafiltrate fractions arising from EHC expressed as percentages of the total nitrogen added to the digesta as EHC.
[3] The amount of N from EHC unaccounted for in the P+R and ultrafiltrate fractions of digesta expressed as a percentage of the total nitrogen added to the digesta as EHC.
[4] Tube containing 450 mg of EHC dissolved in distilled water and ultrafiltered.
[5] Retentate only.

Table 4 Percentage recovery[1] of amino acids added to digesta in the form of enzyme hydrolysed casein (EHC) in the precipitate plus retentate (P+R) fraction following centrifugation and ultrafiltration[2].

	Tube				
Amino acid	A	B	C	D	E
LYS	1.9	2.5	2.9	1.6	1.0
HIS	3.3	0.6	0.4	2.9	1.7
ARG	4.6	3.5	3.0	1.3	0.9
ASP	1.0	2.2	2.4	1.1	1.8
THR	14.9	7.2	6.6	4.5	8.8
SER	8.8	6.8	6.7	5.0	5.4
GLU	5.5	5.6	5.6	4.4	2.0
PRO	23.7	13.5	8.6	6.2	0.1
GLY	8.7	4.0	0.8	0.1	0.1
ALA	3.9	4.1	2.8	0.1	4.9
VAL	3.3	5.1	4.7	3.3	3.0
ILE	3.4	5.2	4.9	3.3	2.6
LEU	1.7	3.2	3.4	1.8	1.4
TYR	0.4	2.2	2.3	1.4	0.4
PHE	2.0	3.3	3.8	2.2	1.7
Overall Mean[3]	**5.8**	**4.6**	**3.9**	**2.6**	**2.4**

[1] Mean of duplicates.

[2] Amount of each amino acid in P+R fraction arising from EHC, expressed as the percentage of the amino acid added to the digesta as EHC.

[3] Overall means for amino acids from EHC present in the precipitate plus retentate fraction of digesta following centrifugation and ultrafiltration expressed as a percentage of the total amount of the respective amino acid added to the tube in the form of EHC.

The overall mean amount of amino acid in each pair of duplicate tubes originating from the EHC and present in the precipitate plus retentate fraction of digesta, expressed as a percentage of the respective amino acid added to the tubes as EHC, ranged from 2.4 to 5.8% (Table 4). The percentage recoveries for some of the individual amino acids were considerably greater than or lower than the overall mean value.

The ultrafiltrate fractions of digesta contained from 4.6 to 22.9 mg of nitrogen that was deemed to have originated from the added EHC, which corresponds to 91.4-97.2% (Table 3) of the nitrogen added to the tubes in the form of EHC.

For control 2 (added EHC but no digesta), 93.2% of the EHC nitrogen was subsequently found in the ultrafiltrate and 3.8% remained in the retentate.

DISCUSSION

The work reported here involved an *in vitro* preparation, representing the presence of unabsorbed dietary amino acids and small peptides in ileal digesta. There were two issues addressed. Firstly, it was important to determine whether there was any binding of EHC to endogenous material in the precipitate plus retentate fraction of ultrafiltered digesta. If such binding does occur, this would result in overestimation of the endogenous protein flows determined using the enzyme hydrolysed protein method. The second issue was to determine whether Centriprep-10 Concentrators were equally effective at separating unabsorbed dietary amino acids and small peptides from endogenous material when different concentrations of amino acids plus small peptides (EHC) were present in the digesta. This was to determine whether the enzyme hydrolysed protein method can be validly applied to test animals given diets containing concentrations of EHC higher than the usual 10%. This is necessary to allow examination of the effect of dietary peptide concentration on endogenous protein flow.

As shown in Table 3, a small proportion of the nitrogen from the EHC was not accounted for in either the precipitate plus retentate or ultrafiltrate fractions of digesta. This unaccounted-for EHC may have been partially due to minor analytical inaccuracies and/or it may have remained attached to the membrane of the concentrators. Adhesion of EHC to the concentrator membrane would not affect the accuracy of the enzyme hydrolysed protein method, as the EHC is still being removed from the precipitate plus retentate fraction that is analysed for endogenous nitrogen and amino acids.

The amounts of nitrogen present in the precipitate plus retentate fraction of digesta, which were deemed to have originated from the added EHC can be calculated to be 0.25, 0.41, 0.47, 0.46 and 0.24 mg for tubes A, B, C, D and E. These amounts did not increase consistently with increased addition of EHC to the tubes which suggests that the Centriprep-10 Concentrators were equally effective at separating

EHC from digesta when higher concentrations of EHC were present in the digesta. Therefore, Centriprep-10 concentrators can be validly used when test animals are fed diets containing concentrations of EHC higher than 10%. Digesta collected after feeding pigs a diet containing 10% EHC contains a nitrogen concentration close to that found in Tube A while digesta collected following feeding a 20% EHC diet to pigs contains a nitrogen concentration close to that found for Tube B (Hodgkinson SM., Moughan PJ and Reynolds GW, unpublished data).

The results from the present study do highlight, however, that with Centriprep-10 Concentrators, there is a less than complete separation of nitrogen and amino acids, originating from EHC, from the endogenous material in the digesta. Following mixing of the tubes containing the digesta and EHC and before centrifugation and ultrafiltration, the tubes were left overnight. This was to ensure that the EHC was completely dissolved in the digesta, and also to allow for the maximum potential binding between the digesta and EHC. It may also be the case in the live animal that a small proportion of unabsorbed EHC may be present in the precipitate plus retentate (endogenous) fraction of digesta, which would result in an overestimation of endogenous nitrogen and amino acid flows determined with the enzyme hydrolysed protein method. This study involved an *in vitro* preparation and care needs to be exercised in extrapolating the results to the *in vivo* situation.

For many amino acids (such as lysine, histidine, aspartic acid, leucine, tyrosine and phenylalanine), only small amounts of the amino acids added in the form of EHC remained in the precipitate plus retentate fraction of digesta. For most amino acids, the presence of EHC in the precipitate plus retentate is unlikely to have been due to binding of EHC to the precipitate plus retentate fraction of digesta, as the percentage of nitrogen from EHC in control 2, where there were no digesta present, that remained in the retentate fraction was similar to that found for tubes A-E, where digesta were present. This is consistent with the finding of Butts *et al.* (1991) who ultrafiltered solutions containing purified peptides and amino acids with molecular weights less than 10,000 Da and also found a high degree of, but less than complete, ultrafiltration. Therefore, it appears that most "additional" nitrogen, from the added EHC, present in the precipitate plus retentate fraction is present because of incomplete ultrafiltration, as opposed to the binding of amino acids and peptides from EHC to digesta.

The amounts of the amino acids proline and threonine originating from the EHC and present in the precipitate plus retentate fraction of digesta, when expressed as a

percentage of the amounts of these amino acids added as EHC to tube A, were relatively large, in the order of 24 and 15% for proline and threonine, respectively. The absolute amounts of proline and threonine (deemed to have originated from EHC) present in the precipitate plus retentate fractions of digesta for Tubes A-E were relatively constant (0.9, 1.1, 1.0, 1.0 and 0.2 mg of proline in the precipitate plus retentate fractions from Tubes A, B, C, D and E, respectively, and for threonine, 0.3, 0.3, 0.4, 0.4 and 0.8 mg for Tubes A, B, C, D and E). It is possible that binding did occur between the proline and threonine in the EHC and the digesta, but it should be noted that the amount of this binding did not increase when there were greater amounts of EHC added to the digesta. There may be a degree of overestimation of the endogenous amounts of these two amino acids determined using the enzyme hydrolysed protein method.

It can be assumed that a similar proportion of the EHC that remained unabsorbed at the terminal ileum in the *in vivo* situation would remain in the precipitate plus retentate fraction of digesta following ultrafiltration as occurred in the *in vitro* situation of the present study. Having made this assumption, and considering the overall effect of the incomplete removal of unabsorbed EHC from the precipitate plus retentate fraction of digesta on endogenous nitrogen flows determined using the enzyme hydrolysed protein method, it needs to be borne in mind that the digestibility of casein is at least 90% (Kies *et al.*, 1986; Rutherfurd and Moughan, 1997). Therefore only around 10% or less of the dietary intake of EHC will remain in the digesta at the terminal ileum. For example, if an EHC based diet that contained 14 mg nitrogen per g DM (Hodgkinson *et al.*, 1999) was fed to a test animal, there would be approximately 1.4 mg N/g DMI at the terminal ileum arising from unabsorbed dietary EHC. Of this unabsorbed EHC, a maximum of 4-5% may remain in the precipitate plus retentate fraction of digesta following ultrafiltration. This would correspond to 0.06 mg N/g DMI which, given an endogenous ileal nitrogen flow of 2.85 mg/g DMI (Hodgkinson *et al.*, 1999), would comprise only 2% of the endogenous flow. Therefore, the extent of overestimation of endogenous nitrogen and amino acid flows that may occur with the enzyme hydrolysed protein method due to incomplete separation of digesta and EHC is expected to be low. It should also be noted that with the enzyme hydrolysed protein method, there is an underestimation of endogenous nitrogen and amino acid flows due to the presence of some endogenous amino acids in the discarded ultrafiltrate and the two errors are counteracting.

The present results demonstrate that Centriprep-10 Concentrators are equally effective for the removal of higher concentrations of EHC from the precipitate plus retentate fraction of ultrafiltered digesta as low levels of EHC. Except possibly for the amino acids proline and threonine, EHC does not appear to bind to endogenous material in digesta to any significant extent. Therefore, such ultrafiltration devices are suitable for application with the enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid flows.

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Chapter 5

The Enzyme Hydrolysed Protein Method for the Determination of Endogenous Ileal Nitrogen and Amino Acid Flows – A Modification

When the enzyme hydrolysed protein method is used for the determination of endogenous ileal nitrogen and amino acid flows, digesta are ultrafiltered using an ultrafiltration device with a molecular weight (MW) cut-off of 10,000 Da. There may, however, be an underestimation of the endogenous ileal flows determined using a MW cut-off of 10,000 Da for ultrafiltration, due to the presence of endogenous amino acids and small peptides in the ultrafiltrate, which is discarded. The aim of the study reported in this Chapter was to test whether the endogenous ileal flows determined following ultrafiltration with a MW cut-off of 3,000 Da differ greatly from those determined following ultrafiltration with a MW cut-off of 10,000 Da. This work was completed in order to obtain an estimate of the extent of the underestimation of endogenous ileal nitrogen and amino acid flows that occurs due to the loss of endogenous amino acids and small peptides in the ultrafiltrate.

The Enzyme Hydrolysed Protein Method for the Determination of Endogenous Ileal Nitrogen and Amino Acid Flows – A Modification

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ABSTRACT

The aim of the work was to examine the effect of using an ultrafiltration device with a molecular weight (MW) cut-off of 3,000 Da on endogenous ileal amino acid flows determined using the enzyme hydrolysed protein method, as compared with those determined using a MW cut-off of 10,000 Da. Endogenous ileal nitrogen and amino acid flows determined using the enzyme hydrolysed protein method are normally determined using a MW cut-off of 10,000 Da for ultrafiltration. Digesta were sampled from the terminal ileum of male rats (n=24) that had received a diet containing enzyme hydrolysed casein (EHC) at an inclusion level of 10%, for eight days. The digesta samples were divided into six groups, each containing the digesta from four rats. Endogenous ileal nitrogen and amino acid flows were determined on each pool of digesta using the enzyme hydrolysed protein method with ultrafiltration using MW cut-offs of 10,000 Da and 3,000 Da. The endogenous ileal nitrogen and amino acid flows determined using a MW cut-off of 3,000 Da for ultrafiltration were greater than those determined using a MW cut-off of 10,000 Da. The percentage difference in endogenous flow between the results obtained following ultrafiltration at 10,000 Da and those at 3,000 Da was 17% for nitrogen and ranged from 1.7% for arginine and phenylalanine to 26% for serine, with a mean difference of 12% for the amino acids. When determining endogenous ileal nitrogen and amino acid flows using the enzyme hydrolysed protein method, it is recommended that digesta be ultrafiltered using a MW cut-off of 3,000 Da, but the MW cut-off for ultrafiltration must be related to the size of peptides present in the enzyme hydrolysed protein.

INTRODUCTION

The traditional method for determining endogenous ileal nitrogen and amino acid flows involves feeding animals a diet devoid of protein, as under these conditions all of the amino acids present in the digesta must be of endogenous origin. This approach, however, is considered to be unphysiological (Low, 1980). Protein-free feeding has been shown to result in a general decrease in the rate of protein synthesis in the body and gut (Millward *et al.*, 1976; Muramatsu, 1990) and there may be a reduction in the amount of protein secreted into the gut (Snook and Meyer, 1964; Corring and Saucier, 1972; Schneeman, 1982). These effects may lead to a decrease in the endogenous protein present in the digestive tract.

The enzyme hydrolysed protein method has been developed as an alternative method for the determination of endogenous ileal nitrogen and amino acid flows (Moughan *et al.*, 1990; Butts *et al.*, 1991). The latter method allows endogenous flows to be determined while the animal is receiving a diet containing a mixture of peptides and free amino acids, simulating the natural products of protein digestion. The test animal is given a diet that contains an enzyme hydrolysed protein, usually enzyme hydrolysed casein (EHC), as the sole dietary nitrogen source. The EHC contains a mixture of free amino acids and small peptides, with all of the peptides being less than 5,000 Da in size. Digesta are collected from the test animal and centrifuged before being ultrafiltered, which removes any compounds smaller than the filtration cut-off of 10,000 Da, thus removing any unabsorbed dietary amino acids and peptides. The retentate (>10,000 Da fraction of digesta) is added to the precipitate from the centrifugation step and this material is used for the determination of endogenous nitrogen and amino acids. The enzyme hydrolysed protein method has been applied to determine gut endogenous nitrogen losses in several species of simple-stomached animals (Darragh *et al.*, 1990; Butts *et al.*, 1991; Moughan *et al.*, 1992; Butts *et al.*, 1993; Schulze *et al.*, 1995; Donkoh *et al.*, 1995; Hendriks *et al.*, 1996; Leterme *et al.*, 1996; Rutherfurd and Moughan, 1997).

In addition to the unabsorbed dietary amino acids, small peptides and free amino acids of endogenous origin may also be present in the discarded (<10,000 Da) fraction of digesta, and this may lead to an underestimation of endogenous amino acid flow with the enzyme hydrolysed protein method. Disposable ultrafiltration devices with a molecular weight (MW) cut-off of 3,000 Da are now available commercially. Use of the latter ultrafiltration devices may increase the accuracy of the enzyme hydrolysed protein method when used in conjunction with dietary EHC containing peptides smaller

than 3,000 Da in size. The aim of the present study was to ascertain the effect of using an ultrafiltration device with a MW cut-off of 3,000 Da on determined endogenous ileal amino acid flows as compared with using a 10,000 Da cut-off ultrafiltration device.

MATERIALS AND METHODS

Ethics approval for the study was granted by the Massey University Animal Ethics Committee. Sprague Dawley male rats (n=24) with a bodyweight of (mean ± SEM) 179.0 ± 3.0 g were obtained from the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. The rats were housed singly in wire-bottomed cages designed to prevent coprophagy, at 22 ± 2 °C with a 12-hour light/dark cycle. Fresh water was available at all times. Throughout the study the rats were given free access to their food for ten minutes every hour over eight hours each day, with the first meal of the day commencing at 0800h and the final meal commencing at 1500h. The feeders were weighed at the beginning and end of each day, throughout the trial.

Two diets were prepared, a basal casein-based diet (basal diet) and an enzyme hydrolysed casein-based diet (EHC diet). The ingredient composition of the diets is given in Table 1. The molecular weight distribution of the enzyme hydrolysed casein was determined using an HPLC gel filtration column (Waters Millipore 625 HPLC system and PSK2000SW 60cm column). The eluting solvent contained 0.1% trifluoroacetic acid and 36% acetylnitrile, and the sample was detected using a wavelength of 205 nm. All peptides were shown to be less than 5,000 Da in size, with 0.8% between 3,000 and 5,000 Da, 11.3% in the 1,000-3,000 Da range and the remaining 87.9% less than 1,000 Da.

The first seven days of the study were a training period, with the rats given access to the basal diet and trained to eat in eight 10-minute periods per day as described above. The rats then received the EHC diet for a further seven days in the same manner.

On the next day, day 15, the rats were fed the EHC diet following the same procedure (free access to the diet for 10 minutes every hour) and starting five hours after the first meal of the day, the rats were euthanased. Each rat was asphyxiated with CO_2 gas and then immediately decapitated. The body cavity was opened and the final 20 cm of the terminal ileum immediately dissected from the body, rinsed with distilled water to

remove any traces of blood and hair and then gently dried with absorbent paper. Care was taken not to apply pressure to the intestine. The digesta were slowly flushed out with deionised water from a syringe. The pH of the digesta was adjusted to pH 3.5 by the addition of 6 M H_2SO_4 and the digesta were immediately frozen (-20 °C). This procedure was adopted to minimise enzyme and bacterial activity in the digesta, post-collection. The digesta samples were stored at -20 °C until chemical analysis. The maximum time from decapitation until the digesta were collected was four minutes.

Table 1 Ingredient composition (g/kg air dry weight) of the experimental diets.

Ingredient	Basal Diet	EHC diet
Casein	120	-
Enzyme hydrolysed casein (EHC)[1]	-	100
Soya bean oil	35	35
Cellulose	50	50
Sucrose	70	70
Wheaten cornflour	625	639
Vitamin mix[2]	50	50
Mineral mix[3]	50	50
Chromic oxide	-	6

[1] New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand.

[2] Carrier sucrose. Provided (per kg diet): 5.0 mg retinol, 5.0 mg thiamin, 7.0 mg riboflavin, 20.0 mg pantothenic acid, 8.0 mg pyridoxine, 0.05 mg cyanocobalomin, 0.025 mg ergocalciferol, 200.0 mg tocopherol, 1.0 mg biotin, 3.0 mg menadione, 1500.0 mg choline, 2.0 mg folic acid, 200.0 mg inositol, 20.0 mg niacin.

[3] Carrier cellulose. Provided (per kg diet): 6.29 g calcium, 7.79 g chloride, 28.9 µg cobalt, 1.97 mg chromium, 10.68 mg copper, 424.1 mg iron, 151 µg iodine, 5.24 g potassium, 1.06 g magnesium, 78.0 mg manganese, 152 µg molybdenum, 1.97 g sodium, 4.86 g phosphorus, 0.15 mg selenium, 48.2 mg zinc.

The rat digesta samples were randomly divided into six groups each containing the digesta samples from four animals. The digesta were thawed and the digesta from each group of four rats were pooled to give a sufficient amount of digesta for all chemical analyses, thus giving a total of six pooled digesta samples for analysis. Each pooled digesta sample was carefully mixed and divided into two equal portions. The portions of digesta from each pooled sample were centrifuged at 7,000 G for 10 minutes. The supernatant from one portion of digesta from each pooled sample was then ultrafiltered using a Centriprep-3 ultrafiltering device (Amicon Inc., Beverly, MA, USA, 3,000 Da MW cut-off), and the supernatant from the other portion of each pooled digesta sample was ultrafiltered using a Centriprep-10 ultrafiltering device (Amicon Inc., Beverly, MA, USA, 10,000 Da MW cut-off), according to the manufacturer's instructions. Each retentate (>3,000 or >10,000 Da, respectively) from the ultrafiltration

step was added to the corresponding precipitate from the centrifugation step, and the material freeze-dried and finely ground.

The diet and digesta samples were analysed for chromium using an Instrumentation Laboratory Atomic Absorption Spectrophometer according to the method described by Costigan & Ellis (1987).

The EHC diet and precipitate plus retentate fractions of digesta were analysed for dry matter, total nitrogen and amino acids. Total nitrogen was determined in duplicate. Samples were combusted at 1050 °C in oxygen gas. The nitrogen was then reduced to N_2 by a catalyst and this was measured by a thermal conductivity cell using a Leco FP2000 analyser (Leco Corporation, St Joseph, Michigan, USA). Amino acid compositions were determined in duplicate. Samples (5-7 mg) were hydrolysed in 1 ml of 6 mol/L glass-distilled HCl containing 0.1% phenol in glass tubes sealed under vacuum, for 24 hours at 110 $\pm$ 2 °C. Amino acid concentrations were measured using a Waters ion exchange HPLC system calibrated against a reference amino acid mixture. The peaks of the chromatograms were integrated using the dedicated software Maxima 820 (Waters, Millipore, Milford, MA) which identifies the amino acids by retention time against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively, and the weight of each amino acid was calculated using free amino acid molecular weights. No corrections were made for potential losses of amino acids during the hydrolysis step.

Endogenous ileal nitrogen and amino acid flows (related to the ingestion of 1 g of dry matter) were determined using the equation reported by Hodgkinson *et al.* (1999, refer to Chapter 2 of this thesis). The units were µg/g food dry matter intake (DMI).

The differences (%) in endogenous ileal nitrogen and amino acid flow between the results obtained following ultrafiltration at 10,000 Da and those at 3,000 Da were calculated for each pooled digesta sample. The endogenous flows determined for digesta using an ultrafiltration cut-off of 10,000 Da were subtracted from those determined using a MW cut-off of 3,000 Da and the result was divided by the endogenous flow determined using a MW cut-off of 3,000 Da and expressed as a percent.

RESULTS

The growing rats had a daily food intake of (mean ± SEM) 12.0 ± 0.2 g during the trial and over the 8-day period that they were given access to the EHC diet, the growth rate of the rats was (mean ± SEM) 2.8 ± 0.1 g/day.

The mean endogenous ileal nitrogen and amino acid flows determined using the ultrafiltration devices with MW cut-offs of 3,000 or 10,000 Da are given in Table 2. The percentage differences in endogenous ileal amino acid flow (Table 2) ranged from 1.7% for phenylalanine and arginine to 26.0% for serine, with an overall mean percentage difference of 11.7% for amino acid flow and 16.9% for total nitrogen flow.

Table 2 Mean (n = 6) endogenous ileal flows[1] (μg/g dry matter intake) for growing rats receiving an EHC-based semi-synthetic diet and with ultrafiltration of digesta supernatants using two ultrafilter sizes.

	MW cut-off		
Amino acid	3,000 Da	10,000 Da	% difference[2]
LYS	290	269	7.3
HIS	284	265	6.9
ARG	193	190	1.7
ASP	918	787	14.3
THR	660	553	16.2
SER	908	672	26.0
GLU	1574	1188	24.5
PRO	609	553	9.2
GLY	480	419	12.7
ALA	370	315	14.8
VAL	569	475	16.4
ILE	540	421	22.1
LEU	497	436	12.2
TYR	207	202	2.5
PHE	195	192	1.7
Nitrogen	1338	1112	16.9

[1] Digesta supernatants were ultrafiltered using molecular weight (MW) cut-offs of 3,000 or 10,000 Da.
[2] Mean percentage difference in endogenous flows calculated as described in text.

DISCUSSION

A potential source of error with the enzyme hydrolysed protein method used to determine endogenous ileal amino acid flow is that some endogenous amino acids may be present as free amino acids or small peptides in the discarded ultrafiltrate fraction, resulting in an underestimation of endogenous ileal amino acid flow. It is expected that the degree of underestimation would be less when digesta are ultrafiltered using a MW cut-off of 3,000 Da than when a 10,000 Da cut-off is used. Consequently, the endogenous nitrogen and amino acid flows determined using a MW cut-off of 3,000 Da may be greater than when determined using a 10,000 Da cut-off for ultrafiltration.

A molecular weight cut-off of 3,000 Da may only be used for ultrafiltration of digesta, however, if the dietary peptides are less than 3,000 Da in size. In the present study, virtually all of the dietary peptides had a MW lower than 3,000 Da. It was assumed that the small amount of dietary peptide (0.8%) with a MW ranging from 3,000 to 5,000 Da would have been broken down further during digestion, such that all unabsorbed dietary material present at the terminal ileum would have been less than 3,000 Da in size. It is assumed, therefore, that all of the unabsorbed dietary peptides and free amino acids at the terminal ileum were removed by ultrafiltration.

Ultrafiltering ileal digesta using a MW cut-off of 3,000 Da compared to 10,000 Da (Table 2) led to an increase in the determined endogenous ileal flows of nitrogen (17%) and amino acids (12%). It thus appears that the mean endogenous amino acid flow determined using the enzyme hydrolysed protein method and using a MW cut-off for ultrafiltration of 10,000 Da may be underestimated by at least 12%. The total underestimation is potentially greater than this, as there may be free amino acids and peptides of endogenous origin that are less than 3,000 Da in size present in digesta collected from animals receiving an EHC-based diet. Alternatively, there may be little amino acid material in the ultrafiltrate, but the ultrafiltrates were not analysed for amino acids in the present study so this remains unknown and it is not possible to determine the amount of endogenous amino acids present in the <3,000 Da fraction of digesta. It is interesting to note that in a study (Moughan and Schuttert, 1991) where digesta were collected from pigs fed a protein-free diet and were immediately acidified to prevent enzymatic and bacterial action before being ultrafiltered using a MW cut-off of 10,000 Da, some 11% of the total amino nitrogen was found in the ultrafiltrate. This is similar to the percentage increase in the flow of endogenous amino acids found in the present study when the digesta were ultrafiltered at 3,000 Da compared with 10,000 Da.

Leterme *et al.* (1996) ultrafiltered digesta collected from pigs that had received an EHC based diet using ultrafiltration devices with MW cut-offs of 3,000 and 10,000 Da. While Leterme *et al.* (1996) did not lower the pH of the digesta to minimise the action of bacteria and enzymes, and they did not attempt to quantify the amount of endogenous amino acids in the size range of 3,000 to 10,000 Da, they did provide evidence that there may be a significant amount of endogenous peptides lower than 10,000 Da in size.

The endogenous ileal nitrogen and amino acid flows determined in the present study were similar to those reported in previous studies that have used the enzyme hydrolysed protein method in the rat (Butts *et al.*, 1991; Donkoh and Moughan, 1994; Donkoh *et al.*, 1994; Donkoh *et al.*, 1995).

The percentage differences in endogenous ileal amino acid flow (>3,000 Da fraction of digesta compared with the >10,000 Da fraction) differed greatly among the individual amino acids. There were small differences in the endogenous ileal flows for amino acids such as arginine and phenylalanine, but much greater differences for amino acids such as serine, glutamine and isoleucine. The absolute values for the flows of endogenous ileal amino acids in the size range 3,000 to 10,000 Da can be determined by subtracting the flows determined using a MW cut-off of 10,000 Da for ultrafiltration from those determined using a MW cut-off of 3,000 Da (Table 2). There was 226 μg of nitrogen per g DMI in the size range 3,000 to 10,000 Da. The endogenous peptides at the terminal ileum that are between 3,000 and 10,000 Da in size appear to contain relatively high concentrations of aspartic acid, threonine, serine, glutamine and isoleucine but little arginine, methionine, tyrosine or phenylalanine.

Not all of the determined endogenous ileal total nitrogen was accounted for by amino nitrogen. By calculating the moles of amino nitrogen for each amino acid flow, summing these and subtracting this value from the total moles of nitrogen flow, it can be shown that approximately 12% of the nitrogen in the >3,000 Da fraction of digesta and 10% of the nitrogen in the >10,000 Da digesta fraction were from non-amino sources of nitrogen. These non-amino sources of nitrogen could be compounds such as nucleic acids bound in RNA and DNA molecules and nitrogen-containing compounds bound in phospholipids such as choline and ethanolamine.

It appears that the use of a lower MW cut-off (<10,000 Da) may increase the accuracy of the enzyme hydrolysed protein method. It is unknown, however, whether the size of

the dietary peptides *per se* and, therefore, the degree of hydrolysis of the dietary protein, has a significant influence on endogenous ileal amino acid flow. It is also important to note that the MW exclusion limit used in the ultrafiltration step of the enzyme hydrolysed protein method for determining endogenous loss must be related to the size of peptides found in the dietary enzyme hydrolysed protein.

The present study provides the first direct information on the amount of endogenous material flowing through the terminal ileum of the rat in the size range of 3,000 to 10,000 Da. The endogenous ileal nitrogen and amino acid flows, determined using the enzyme hydrolysed protein method with an ultrafiltration exclusion limit of 3,000 Da, were greater than those determined following ultrafiltration at 10,000 Da.

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Chapter 6

The Diurnal Pattern of Ileal Dry Matter and Endogenous Ileal Nitrogen Flows for the Growing Pig

The studies reported in the previous two Chapters of this thesis examined aspects of the enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid flows. The following two Chapters will describe studies using the enzyme hydrolysed protein method. In the study reported in the present Chapter, the enzyme hydrolysed protein method was used to investigate the diurnal pattern of endogenous nitrogen flow in detail. This work was completed as a step towards examination of the mechanisms whereby dietary factors, such as the presence of peptides in the diet, influence endogenous flows of nitrogen at the terminal ileum.

The Diurnal Pattern of Ileal Dry Matter and Endogenous Ileal Nitrogen Flows for the Growing Pig

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ABSTRACT

The aim was to examine the diurnal pattern of endogenous nitrogen flow at the terminal ileum of the pig. Seven 33 kg liveweight entire male pigs had post valve T-caecum (PVTC) cannulae surgically implanted for the collection of ileal digesta. The pigs were fed equal sized meals twice daily (10% of metabolic body weight per day) and were given a 10% enzyme hydrolysed casein diet for an eight-day period, with continuous collection of digesta for 24 hours (0800h - 0800h) on each of the fifth and eighth days. During each hour of digesta collection, 10% (by weight) of the digesta collected for that hour for each pig was sampled. Flows of dry matter and chromium were determined in the digesta and endogenous nitrogen was determined after centrifugation and ultrafiltration (10,000 Da MW cut-off) of the digesta. The concentration of chromium in the digesta expressed on a digesta dry matter basis was relatively constant over the 24-hour periods, with no statistically significant ($P>0.05$) differences from 1200h - 0800h. The ratio of endogenous nitrogen to chromium at the terminal ileum was also relatively constant with no statistically significant ($P>0.05$) differences when digesta were sampled from 1300h - 0800h. The net outcome of endogenous protein secretion and reabsorption in the small intestine may be relatively constant over time in the meal-fed animal, and endogenous ileal nitrogen appears to be proportionally related to dry matter intake.

INTRODUCTION

In the study reported by Hodgkinson *et al.* (1999, refer to Chapter 7 of this thesis), digesta were collected continuously for 24 hour periods from post valve T-caecum (PVTC) cannulated pigs and this study permitted a detailed investigation of the diurnal pattern of endogenous nitrogen flow at the terminal ileum of the growing pig. This aspect of the study is detailed here.

It is of fundamental physiological interest to understand the time course of endogenous nitrogen flow as a step towards elucidating the mechanisms whereby dietary factors, such as the presence of peptides in the diet, influence the amount of endogenous digesta nitrogen. The diurnal pattern of endogenous ileal nitrogen flow was examined here, using the peptide alimentation method, as proposed by Moughan *et al.*, (1990), to determine endogenous nitrogen. Digesta, collected continuously (24-hour periods) from PVTC cannulated meal-fed pigs, were analysed over time for endogenous total nitrogen, dry matter and chromium.

MATERIALS AND METHODS

Seven Large White x (Large White x Landrace) entire male pigs with a common sire and with a liveweight of (mean $\pm$ SEM) 33.3 $\pm$ 1.5 kg at the commencement and 80.0 $\pm$ 3.7 kg at the conclusion of the study were sourced from the Pig Research Unit, Massey University. Palmerston North, New Zealand. The animals were housed individually in smooth-walled steel metabolism crates in a room maintained at 22 $\pm$ 1 °C. Ethics approval for the study was granted by the Massey University Animal Ethics Committee.

Surgery

A PVTC cannula was inserted into the caecum of each pig for the collection of ileal digesta, according to the method of van Leeuwen *et al.* (1991), with anaesthesia and post-operative care as described fully by Hodgkinson *et al.* (1999, refer to Chapter 7 of this thesis). The cannulae were made of medical grade silastic tubing with an ID of 25 mm and OD of 29 mm.

Diets and Feeding

A casein-based diet (basal) and an enzyme hydrolysed casein (EHC) based diet were prepared. The dietary ingredient compositions are given in Table 1. Chromic oxide was included as an indigestible marker in the EHC diet.

Table 1 Ingredient compositions of the experimental diets (g/kg air-dry).

	Diet	
Ingredient	Basal	EHC
Casein	120	-
Enzyme hydrolysed casein (EHC)	-	100
Soya bean oil	35	35
Cellulose	50	50
Sucrose	70	70
Wheaten cornflour	719	733
Vitamin/mineral mix[1]	2.5	2.5
Dicalcium phosphate	2.5	2.5
Sodium chloride	0.3	0.3
Potassium carbonate	0.5	0.5
Magnesium sulphate	0.2	0.2
Chromic oxide	-	6

[1] Pig grower/finisher vitamin, mineral premix, Danmix, Nutritech, Auckland, New Zealand, containing (per kg diet) 4,000,000 IU vitamin A, 800,000 IU vitamin D, 12,000 IU vitamin E, 800 mg vitamin K, 400 mg vitamin B1, 1 g vitamin B2, 800 mg vitamin B6, 4 mg vitamin B12, 84 mg folic acid, 4 g pantothenic acid, 6 mg biotin, 6 g niacin, 20 g choline, 120 mg selenium, 200 mg cobalt, 400 mg iodine, 50 g copper, 40 g iron, 18 g manganese, 48 g zinc and 4 g zinc bacitracin.

For the first 10 days post-operation, all pigs were fed a standard barley-based grower diet to appetite. The following day, meal feeding commenced with the pigs receiving the diets at 0.10 metabolic body weight ($kg^{0.75}$) in two equal portions (08:00 and 17:00h). This feeding regimen was maintained for the remainder of the trial. The basal diet was gradually introduced over the following four days. The diets were mixed with water (1:1 w/v) immediately prior to feeding and water was freely available between meals. Each pig was weighed and the level of food intake adjusted accordingly each time it was given access to a new diet.

The experiment commenced 14 days after the surgery. The pigs were fed the EHC-based diet for 8-day periods (along with other protein-free and EHC-based diets) in a Latin Square design as described by Hodgkinson *et al.* (1999). The basal diet was given to the pigs for 6-day periods between each test diet. Each pig received the EHC-based diet for a single 8-day period. On the fifth and eighth days that the pigs were given access to the EHC diet, ileal digesta were collected continuously for 24 hours using plastic bags attached to the cannulas. The bung was removed from each cannula an hour before the collection commenced as suggested by van Leeuwen *et al.* (1991), to allow the ileocaecal valve to move so that it was protruding

into the lumen of the cannula instead of the intestinal lumen. The plastic bags were emptied at least hourly and the digesta adjusted to pH 3.5 by the addition of 6 M H_2SO_4. This procedure was adopted to reduce enzyme and bacterial activity in the digesta. At the end of each hour, 10% (by weight) of the digesta collected from each pig during the previous hour was sampled and frozen until analysis (-20 °C).

Chemical Analysis

Each digesta sample was separately centrifuged at 7,000 G for 10 minutes. Each supernatant was then ultrafiltered using Centriprep-10 ultrafiltering devices (Amicon Inc., Beverly, MA, USA, 10,000 Da MW cut-off) according to the manufacturer's instructions. The precipitate from the centrifugation step was added to the retentate (>10,000 Da) from the ultrafiltration, and the material freeze-dried. The dry matter contents of the diets and digesta samples were determined after drying the duplicate samples at 80 °C overnight. The chromium (Cr) contents of the diets and digesta samples were determined using an Instrumentation Laboratory Atomic Absorption Spectrophometer according to the method described by Costigan & Ellis (1987).

The diets and precipitate+retentate fractions of digesta were analysed in duplicate to determine their total nitrogen concentration. The samples were combusted at 1050 °C in oxygen gas. The nitrogen was then reduced to N_2 by a catalyst and this was measured by a thermal conductivity cell using a Leco FP2000 (Leco Corporation, St Joseph, Michigan, USA).

Data Analysis

The dry matter (DM) and Cr contents of the digesta and the nitrogen contents of the precipitate+retentate fractions of the digesta were used to calculate the total amounts of DM, Cr and endogenous nitrogen that were present in the digesta samples collected each hour. These values were then used to calculate the total amounts of dry matter, Cr and endogenous nitrogen that flowed through the terminal ileum for each hour of the two 24-hour collection periods for each pig (mg/hour). The ratio of endogenous nitrogen to Cr in each digesta sample was also calculated.

All statistical analyses were performed using the statistical programme SAS (version 6.12, 1997). The data were firstly analysed using a model for a split-plot design, to determine whether there was a statistically significant effect of day of digesta collection (day 5 vs day 8) on digesta flows of DM, Cr, total endogenous N and endogenous N/g digesta DM. The model used for the analysis was:

$$y_{ijk} = \mu + \alpha_i + \pi_j + \alpha\pi_{ij} + \beta_k + \alpha\beta_{ik} + \varepsilon_{ijk}$$

where: α_i, π_j, $\alpha\pi_{ij}$, β_k and $\alpha\beta_{ik}$ represented the collection day, pig, the collection day-pig interaction, hour of collection, and the collection day-collection hour interaction, respectively. The type III mean square error for the collection-pig interaction was used as the error term to determine whether day of collection had a significant effect on the digesta DM, Cr or endogenous N flows.

An analysis of covariance was also performed to determine whether pig bodyweight had a significant effect on the digesta DM, Cr or endogenous N flows.

During some of the hourly collection periods of the 24-hour collection, there was no digesta flow for some pigs resulting in no sample for that hour. Periods where there was no sample for an animal were treated as missing data points for the statistical analysis of the concentrations of Cr and the endogenous N to Cr ratio, and statistical corrections were made for these points. A single factor repeated measures analysis was conducted to determine whether the Cr concentration or the endogenous N to Cr ratio differed from hour to hour during the collection periods. The model used was:

$$y = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where α_i and β_j represented the effects due to the pig and time (hour of collection), respectively. Significant differences in the digesta concentrations of Cr and the endogenous N to Cr ratio were determined by conducting a multiple range test using least square means following Bonferroni adjustment for multiple comparisons.

RESULTS

The pigs appeared healthy and consumed their diets readily. The mean daily liveweight gain determined over the 8 day experimental periods was (mean $\pm$ SEM) 613 $\pm$ 75 g/day. The Cr recovery in the digesta was (mean $\pm$ SEM) 72 $\pm$ 3%.

There was no statistically significant ($P>0.05$) effect of day of collection, or interaction between collection time and day of collection for digesta flows of DM, Cr or endogenous N, therefore the results from the two collection days were pooled for each pig, before the conduct of other statistical analyses.

Pig bodyweight was not found to be a significant ($P>0.05$) covariate for the digesta flows of DM, Cr or endogenous N. Therefore, no correction for bodyweight was made.

The amounts of Cr that flowed through the terminal ileum during each hour of collection are given in Figure 1. The mean amounts of digesta DM and endogenous ileal nitrogen for each hour of the collection periods (with and without correction to 100% Cr recovery) are given in Figures 2 and 3. Least squares means and standard errors for the concentrations of Cr in the digesta collected each hour and the ratios of endogenous N to Cr in the digesta are given in Tables 2 and 3, respectively.

DISCUSSION

It was expected that the experimental preparation would allow a near complete quantitative collection of digesta over the 24-hour collection periods. This did not occur with an average marker recovery of only 72%. These recoveries, however, fall within the range of those reported by den Hartog *et al.* (1988), Köhler *et al.* (1990) and Köhler *et al.* (1991), who found mean Cr recoveries of 71-106% in PVTC cannulated pigs. It is assumed that the remaining 28% of the digesta in the present study by-passed the cannula and entered the large intestine, as chromic oxide can be quantitatively recovered in pig faeces (Lloyd *et al.*, 1955; Ehle *et al.*, 1982; Saha and Gilbreath, 1993).

Given that a proportion of the digesta was not collected through the cannulas during the digesta collection periods, the first point to consider is whether the digesta that were collected were representative of the total digesta. When samples of digesta are collected through a PVTC cannula, the percentage of digesta collected will depend on the diet, but with the PVTC cannulation procedure, the digesta collected should be representative of total digesta (van Leeuwen *et al.*, 1996). The representativeness of digesta collection can be tested for by determining whether there is a correlation between the recovery of the indigestible marker and apparent ileal digestibility of crude protein and dry matter when the same diet is fed to the animals. A correlation between Cr recovery and apparent ileal crude protein or dry matter digestibility would signify that fractionation had occurred and the digesta collections were not representative. In the present study, the apparent ileal nitrogen digestibilities could only be determined using the values from the total 24-hour digesta collections as the nitrogen contents of the complete digesta collected each hour were not determined, but dry matter digestibilities were calculated for each hour of the 24-hour collection periods. The correlations between Cr recovery and nitrogen and DM digestibility, respectively, were determined and no significant

Figure 1 Means (n=7) and standard errors for the amount of chromium that flowed through the terminal ileum each hour (mg) in a continuous 24 hour collection of ileal digesta in the growing pig.

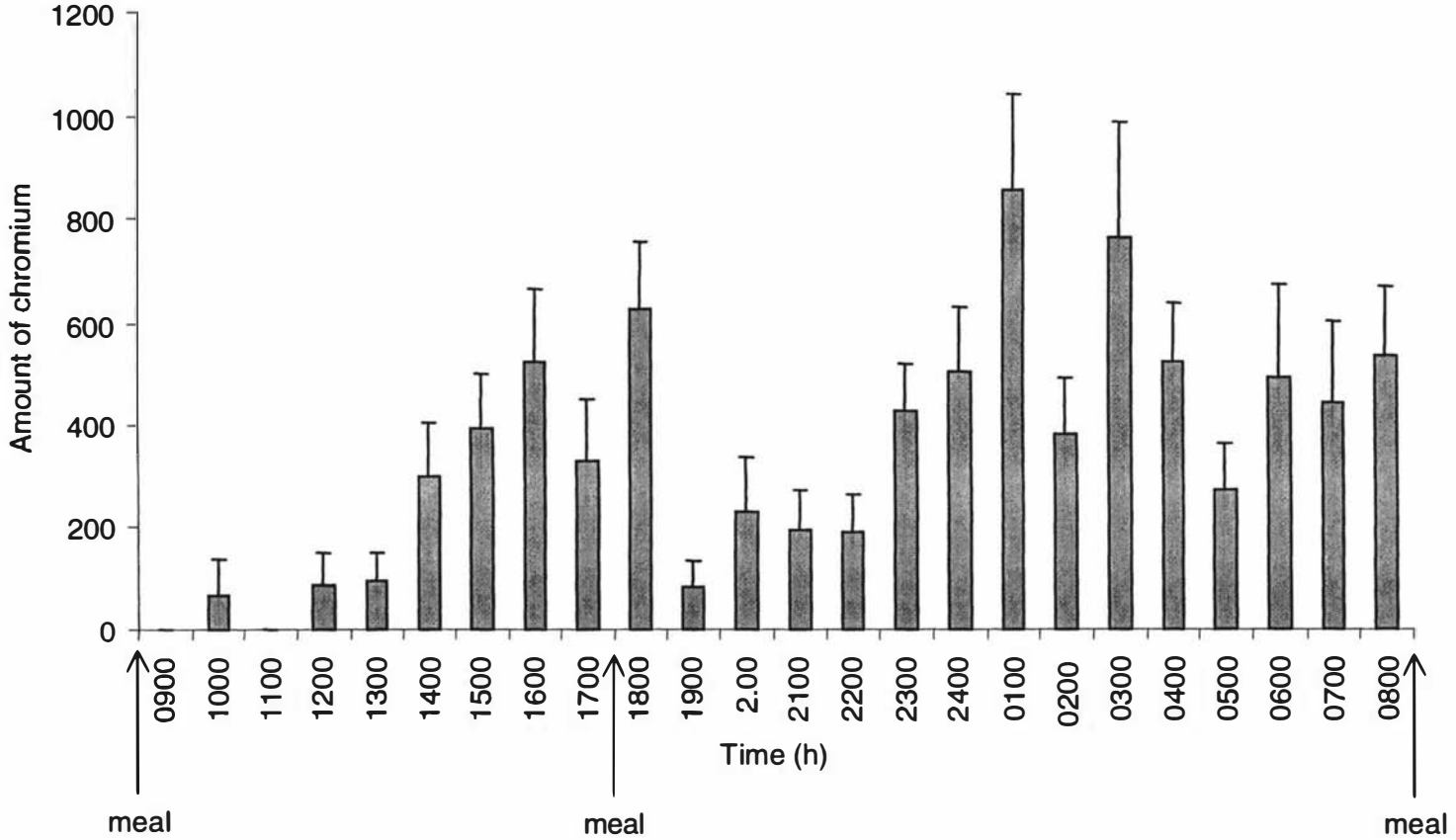

Figure 2 Means (n=7) and standard errors for the amount of dry matter (DM, g) that flowed through the terminal ileum each hour (mg) in continuous 24 hour collections of ileal digesta in the growing pig, uncorrected or corrected to 100% marker recovery.

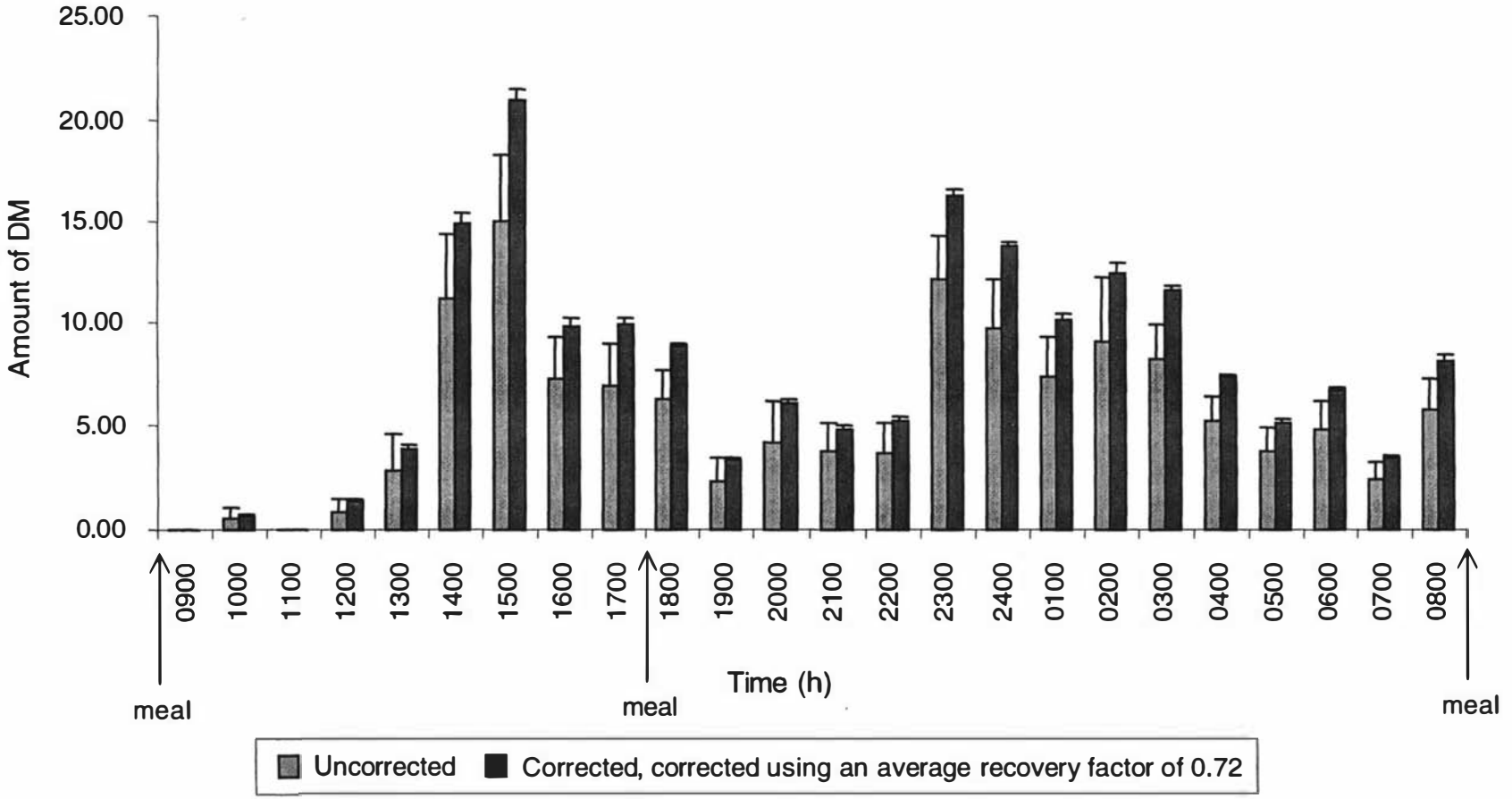

Figure 3 Means (n=7) and standard errors for the amount of endogenous nitrogen (N, mg) that flowed through the terminal ileum each hour (mg) in continuous 24 hour collections of ileal digesta in the growing pig, uncorrected or corrected to 100% marker recovery.

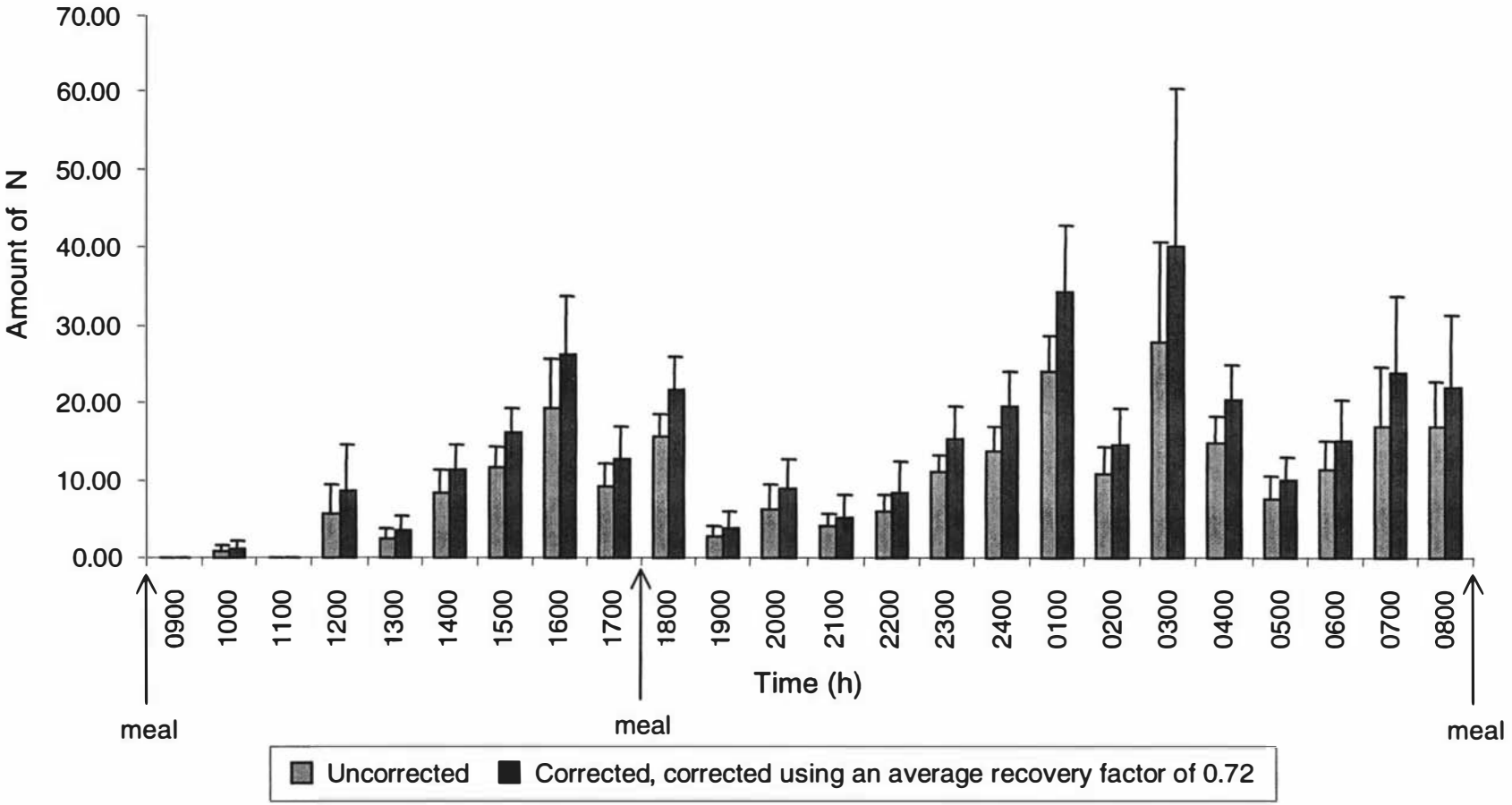

Table 2 Least square means and standard errors for the concentration of chromium (mg Cr/g digesta DM) in the digesta collected each hour for 24 hours in growing pigs given an enzyme hydrolysed casein-based diet.

Time (h)[1]	Chromium concentration[2]	SEM[3]	n[4]
0900	-	-	0
1000	69.5	6.8	1
1100	-	-	0
1200	36.5	4.8	2
1300	46.3	3.9	3
1400	46.3	2.2	6
1500	48.0	1.9	7
1600	48.1	2.1	6
1700	47.5	2.4	5
1800	52.0	2.0	7
1900	39.9	3.9	3
2000	50.8	3.0	5
2100	51.1	3.0	3
2200	48.0	3.0	4
2300	52.5	1.9	7
2400	53.7	2.0	7
0100	53.8	1.9	6
0200	49.6	2.2	6
0300	50.1	1.9	7
0400	47.2	2.1	6
0500	50.6	2.7	5
0600	49.8	2.4	6
0700	50.9	2.7	5
0800	46.7	2.3	5

[1] Time at end of collection period.

[2] There were no statistically significant differences in chromium concentration except that the sampling period finishing at 1000h significantly ($P<0.05$) differed from that finishing at 1200h.

[3] The SEM's were estimated taking into consideration missing data points (refer Data Analysis Section).

[4] Represents the number of pigs for which a digesta sample was collected during the hour-long collection period.

Table 3 Least square means and standard errors for the ratio of endogenous nitrogen to chromium (N:Cr, mg N/g Cr) in ileal digesta collected each hour for 24 hours in the growing pig given an enzyme hydrolysed protein-based diet.

Time (h)[1]	N:Cr[2]	SEM[3]	n
0900	-	-	0
1000	107.9	100.7	1
1100	-	-	0
1200	611.4	71.2	2
1300	280.5	57.9	3
1400	316.9	33.3	6
1500	332.7	28.6	7
1600	329.5	31.6	6
1700	326.6	35.5	5
1800	262.0	29.9	7
1900	356.3	57.9	3
2000	267.8	44.5	5
2100	234.4	45.3	3
2200	286.1	44.9	4
2300	254.3	28.6	7
2400	271.7	29.9	7
0100	307.7	28.8	6
0200	290.5	33.3	6
0300	320.3	28.6	7
0400	300.0	31.6	6
0500	275.2	40.8	5
0600	259.0	35.3	6
0700	384.5	40.9	5
0800	348.1	33.5	5

[1] Time at end of collection period.

[2] There were no statistically significant differences in N:Cr ratio except that the samples collected during the period finishing at 1000h significantly ($P<0.05$) differed from those collected from the period finishing at 1200h and the samples for the period finishing at 1200h significantly ($P<0.05$) differed from those in the collection period finishing at 1800h and all those finishing from 2000-0600h.

[3] The SEM's were estimated taking into consideration missing data points (refer Data Analysis Section).

[4] Represents the number of pigs for which a digesta sample was collected during the hour-long collection period.

(P>0.05) correlations were found suggesting that there was no fractionation of digesta. Based on the observations of van Leeuwen *et al.* (1996), which are supported by our own above reported observations, it is assumed that there was no fractionation between the digesta that flowed through the cannula and that which bypassed the cannula over the collection periods. Therefore, the digesta collected appear to have been representative of the total ileal digesta. It is noteworthy that the samples that were collected comprised 0.72 of the total digesta, which is a sizeable sample.

The second point to consider regarding the amount of digesta that was collected is whether the proportion of digesta that by-passed the cannula was constant over time. This could not be confirmed in the present study. If it is assumed that the same proportion of digesta by-passed the cannula whenever there were digesta flowing through the ileo-caecal valve, the data for each hourly collection can be justifiably corrected to an assumed 100% total Cr recovery. Figures 2 and 3 show the amounts of DM and endogenous N collected in the digesta with and without correction to 100% marker recovery.

Due to the less than complete marker recovery care must be taken in the interpretation of the absolute results, although certain patterns were evident. During the first three hours after the first meal of the day, the flows of Cr, DM and endogenous N were very low. The flows then increased sharply, with maximum flows found in the period 1400h to 1800h, corresponding to 6 to 10 hours after feeding. The maximum flows following the second feeding were in the period 2300h to 0400h, 6 to 11 hours after the second meal. These periods of maximum flow are similar to those reported by other workers. Darcy *et al.* (1980) measured DM flows in digesta collected from pigs fitted with post-valvular ileocolic fistulas and fed protein-free diets or diets containing 8 or 16% crude protein, and reported maximum dry matter flows in the period 4 to 10 hours after the meal, with flows decreasing thereafter. Van Leeuwen *et al.* (1997) measured the ileal passage of digesta in PVTC cannulated pigs fed barley/wheat gluten and rapeseed meal diets. The maximum digesta flows at the end of the ileum in animals fed twice daily were found 3 to 8 hours after the meals. The results of the present study show that sampling ileal digesta over the period starting 5 hours after feeding should provide adequate sample sizes.

Due to the incompleteness of digesta collection encountered in the present study, the remainder of the discussion will centre on the diurnal pattern for the concentration of Cr in the digesta and the ratio of endogenous N relative to Cr. The Cr concentration of the digesta, when expressed on a digesta DM basis, was very constant from the 1300h collection onwards, which corresponds to 4 hours after the first feed, with no statistically significant ($P>0.05$) differences in digesta Cr concentration from this time onwards for the remainder of the collection period. There were statistically significant ($P<0.05$) differences in the concentration of Cr in the digesta collected at 1000 and 1200h but very little digesta were collected at these times, with a collection of digesta from only one pig at 1000h, and only two pigs at 1200h. Van Leeuwen *et al.* (1996) also found a nearly constant concentration of Cr in digesta collected continuously for 12-hour periods from PVTC cannulated pigs fed a soyabean meal based diet, but reported more variation when the pigs received a wheat-bran-gluten meal based diet. Jørgensen *et al.* (1997) reported relatively large variations over time in the Cr concentration of digesta (expressed on a dry matter basis) collected from PVTC cannulated pigs receiving alfalfa meal- and white clover meal-based diets. Graham and Åman (1986) reported fluctuations in the Cr concentration of digesta collected from T-cannulated pigs but found a significant correlation between dry matter concentration and Cr concentration. An assumption that is made when an indigestible marker is used, is that the marker moves through the intestines in synchrony with the dry matter. The nearly constant concentration of Cr when expressed on a dry matter basis in the present work lends support to the validity of this assumption at least in the case where hydrolysed casein based diets were used. It also appears that the net effect of dry matter absorption and secretion throughout the upper tract is relatively constant post-feeding.

Accepting that the digesta that by-passed the PVTC cannulas did not differ in composition from that which was collected via the cannulas, the ratio of endogenous N to Cr in the sampled digesta can be examined as an indicator of the pattern of endogenous N flow through the terminal ileum (Table 3). The first sample collected during the collection period (0900-1000h) had a low endogenous N:Cr ratio, and the second sample (1100-1200h) a rather higher one, but this may have been an artefact of the small amount of digesta collected during these sampling times. From the collection finishing at 1300h and onwards for the remainder of the 24 hours, the endogenous N:Cr ratio was relatively constant, with no statistically significant differences found. This relatively constant N:Cr ratio suggests a rather constant

amount of endogenous N moving through the lower small intestine with food DM flow. The main sources of endogenous nitrogen secretion are digestive enzymes and bile acids, mucus, epithelial cells and serum albumen. Other sources, such as bacteria and hair, that are not strictly endogenous, are also included in the endogenous component. The resulting endogenous N flow is the net effect of secretion and reabsorption of these materials. Over a 24-hour period, the amount of nitrogen secreted into the gut will not be constant (eg. digestive enzymes are not continuously secreted), but rather secretion is stimulated by factors such as the presence of dietary components within the digestive tract. Yet, in the present study, a strong constancy was found in the endogenous N to Cr ratio over the 24-hour collection periods. This may mean that the net outcome of endogenous secretion and reabsorption in the small intestine is relatively constant over time post-feeding, at least in the meal fed animal, and may be proportionally related to dry matter flow through the small intestine. This suggests that there may be mechanisms that regulate the readsorption of endogenous nitrogen, the result of which is a relatively constant endogenous nitrogen flow through the terminal ileum.

When pigs were given two EHC-based meals nine hours apart and following 15 hours without food, the concentration of the indigestible dietary marker, chromic oxide, and the ratio of endogenous nitrogen to Cr in ileal digesta showed variation for the first 4 hours following the first meal, but remained remarkably constant for the following 20 hours.

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Chapter 7

The Effect of Dietary Peptide Concentration on Endogenous Ileal Amino Acid Loss in the Growing Pig

Previous studies have demonstrated that endogenous ileal nitrogen and amino acid flows are greater in animals that receive a diet that contains protein or its breakdown products (peptides) than those animals receiving a diet devoid of protein and peptides. The aim of the study presented in this Chapter was to use the enzyme hydrolysed protein method to determine the effect of dietary peptide concentration on endogenous ileal amino acid flows in the growing pig in order to determine whether the effect of dietary protein/peptide concentration on endogenous ileal amino acid flows is an "all-or-nothing" or "dose-dependent" relationship.

The Effect of Dietary Peptide Concentration on Endogenous Ileal Amino Acid Loss in the Growing Pig

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SYNOPSIS

The aim was to determine whether dietary peptide concentration had an effect on endogenous ileal amino acid flow in the growing pig. Eight 33 kg liveweight entire male pigs had post valve T-caecum (PVTC) cannulae surgically implanted for the collection of ileal digesta. The pigs were fed twice daily at 10% of metabolic body weight per day and were given the diets (0, 5, 10 and 20% enzyme hydrolysed casein, EHC) in a Latin Square design. A basal casein-based diet was fed to the pigs for six-day periods between receiving the experimental diets. The pigs received the experimental diets for eight-day periods, with continuous collection of digesta for 24 hours on each of the fifth and eighth days. The endogenous ileal amino acid flows were determined with reference to recovery of the marker, chromium, directly for pigs receiving the protein-free diet or after centrifugation and ultrafiltration (10,000 Da MW cut-off) for pigs on the EHC based diets. Mean endogenous ileal nitrogen flows were 1753, 1948, 2851 and 5743 µg/g dry matter intake when the pigs received diets containing 0, 5, 10 and 20% EHC, respectively. There was a significant ($P<0.05$) effect of dietary peptide concentration on the endogenous ileal flows of nitrogen and all of the amino acids, with an increase in endogenous ileal amino acid flow with increasing dietary EHC concentration.

Keywords: pig, amino acid, endogenous, ileal, enzyme hydrolysed protein, PVTC cannula.

INTRODUCTION

The "apparent" ileal digestibility of a dietary amino acid is based on a determination of the total flow of amino acids at the terminal ileum. With the apparent measure, no correction is made for the flow of endogenous protein; protein that is secreted into the digestive tract and including digestive enzymes, mucus and desquamated cells (Fauconneau & Michel 1970, Snook 1973). Taking the endogenous component into account results in "true" digestibility. Theoretically, this is a better measure for representing amino acids absorbed from the gut (Darragh *et al.* 1995) and is important in both animal and human nutrition. Simple stomached animals are often used as nutritional models for the human, and the growing pig is a particularly suitable model for protein digestion (Rowan *et al.* 1994).

The traditional approach to determining endogenous protein loss from the small bowel is to feed an animal a diet devoid of protein, and assume that all of the protein present in the terminal ileal digesta is endogenous. This method has been criticised, however, as being unphysiological. Protein-free feeding may result in a reduction in the amount of gastric and pancreatic enzymes secreted (Fauconneau & Michel 1970, Schneeman 1982) and a general decrease in the rate of protein synthesis in the body and gut (Millward *et al.* 1976).

The enzyme hydrolysed protein (peptide alimentation) method for determining endogenous protein loss was proposed by Moughan *et al.* (1990) and allows gut endogenous nitrogen and amino acid losses to be determined in animals fed a diet containing peptides. With this method, the animal is fed a semi-synthetic diet containing an enzyme hydrolysed protein, (usually enzyme hydrolysed casein, EHC) comprising free amino acids and peptides (MW < 5,000 Da), as the sole nitrogen source. The EHC is commonly included at a level of 10% in the diet. Ileal digesta are collected, centrifuged and ultrafiltered. The precipitate plus the high molecular weight fraction (MW > 10,000 Da) resulting from the ultrafiltration contains the endogenous material. Any unabsorbed dietary amino acids or peptides are discarded in the low molecular weight fraction. The dietary enzyme hydrolysed protein is considered to mimic the natural products of gastric digestion with the assumption that the peptides entering the small intestine are similar in size to those that would enter the small intestine if the non-hydrolysed protein had been fed to the animal. An assumption of the method is that the dietary amino acids and peptides maintain a physiologically normal state in the digestive tract.

Comparison between the protein-free and enzyme hydrolysed protein methods in the pig and other simple-stomached species has shown that endogenous ileal amino acid loss is significantly higher for animals given a peptide containing diet (Darragh *et al.* 1990, Butts *et al.* 1991, Moughan *et al.* 1992, Butts *et al.* 1993*a*, Donkoh *et al.* 1995, Hendriks *et al.* 1996, Leterme *et al.* 1996). Higher endogenous ileal amino acid flows when animals receive diets containing peptides or intact protein have also been reported using other methods for the measurement of endogenous ileal amino acid flow, such as the isotope dilution (de Lange *et al.*, 1990) and guanidination methods (Moughan and Rutherfurd, 1990). With the enzyme hydrolysed protein method, however, it is not known whether the dietary concentration of amino acids and peptides influences endogenous ileal amino acid flow.

Furthermore, most comparisons made to date involving the peptide alimentation method have involved a sampling of digesta from the euthanased animal. Whereas this is a well accepted technology, it is possible that such sampling could lead to a bias in results due to possible unrepresentativeness of the sample of digesta collected, as with this approach the sample size is relatively small. Recently a new method for digesta collection in pigs has been developed (post valve T-caecum, PVTC cannulation, van Leeuwen *et al.* 1991) which allows much larger samples of digesta to be collected in conscious pigs.

The primary aim of the present study was to determine whether the concentration of dietary peptides in an enzyme hydrolysed casein based diet had an effect on the flow of endogenous nitrogen and amino acids at the terminal ileum of the pig. An additional aim of the study was to provide, using the PVTC cannulation technique, corroborative evidence for an overall effect of peptide alimentation on endogenous ileal amino acid flow.

MATERIALS AND METHODS

Animals and Housing

Eight Large White x (Large White x Landrace) entire male pigs with a common sire, and with an overall mean liveweight of (mean $\pm$ SEM) 33.3 $\pm$ 1.5 kg at the commencement of the study were sourced from the Pig Research Unit, Massey University, Palmerston North, New Zealand. The animals were housed individually in smooth-walled steel metabolism crates in a room maintained at 22 ± 1°C. The Massey University Animal Ethics Committee granted ethics approval for the study.

Surgery

A PVTC cannula was inserted into the caecum of each pig for the collection of ileal digesta, according to the method of van Leeuwen *et al.* (1991). The cannulae were made of medical grade silastic tubing with an ID of 25 mm and OD of 29 mm. The pigs were not fed for 24 hours before surgery. Anaesthesia was induced with an intramuscular injection of Zoletil (Zoletil 50, Techvet Laboratories Ltd., Auckland, New Zealand, 4 mg/kg bodyweight) and Xylozine (Xylaze 100, Parnell Laboratories, Takanini, New Zealand, 2.2 mg/kg bodyweight) and maintained via inhalation of halothane (Fluothane, Imperial Chemical Industries Ltd., Cheshire, UK) in oxygen. For the first three days following surgery, each pig was given an injection of antibiotic (2 ml of Engemycin 10%, Intervet International B.V., Boxmeer, The Netherlands) intramuscularly twice daily and antibiotic powder was dusted on the wound site daily. The site where the cannula was exteriorised was washed with water, and zinc cream was applied daily throughout the experiment. The pigs regained consciousness within 1 hour of surgery and were standing 4-5 hours after surgery.

Diets and Feeding

Five diets were prepared, including a basal diet and test diets that contained 0 (protein-free diet), 5 (EHC5), 10 (EHC10) and 20% (EHC20) enzyme hydrolysed casein. The dietary ingredient compositions are given in Table 1 and the determined nitrogen and amino acid compositions of the "protein-free" and EHC diets are given in Table 2. The "protein-free" diet contained 0.63 g N/kg diet, which corresponds to 0.36% crude protein.

For the first 10 days post-operation, all pigs were fed a standard barley-based grower diet to appetite. During the following four days, meal feeding (08:00 and 17:00h) commenced. This feeding regimen was maintained for the remainder of the trial. The basal diet was gradually introduced over these four days.

The pigs were then fed a test diet in two equal portions at a level of 0.10 metabolic body weight ($W^{0.75}$) per day. Each pig was weighed and the level of food intake adjusted accordingly whenever the animal was introduced to another diet. The diets were mixed with water (1:1 w/v) immediately prior to feeding and water was freely available between meals. Chromic oxide was included in each diet except the basal diet as an indigestible marker.

Table 1. Ingredient compositions of the experimental diets (g/kg, air-dry basis).

	Diet				
Ingredient	Basal	Protein-free	EHC5	EHC10	EHC20
Casein	120	-	-	-	-
Enzyme hydrolysed casein*	-	-	50	100	200
Soya bean oil	35	35	35	35	35
Cellulose	50	50	50	50	50
Sucrose	70	70	70	70	70
Wheaten cornflour	719	833	783	733	633
Vitamin/mineral mix†	2.5	2.5	2.5	2.5	2.5
Dicalcium phosphate	2.5	2.5	2.5	2.5	2.5
Sodium chloride	0.3	0.3	0.3	0.3	0.3
Potassium carbonate	0.5	0.5	0.5	0.5	0.5
Magnesium sulphate	0.2	0.2	0.2	0.2	0.2
Chromic oxide	-	6	6	6	6

* New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand. The molecular weight distribution of the enzyme hydrolysed casein was determined using an HPLC gel filtration column (Waters Millipore 625 HPLC system and PSK2000SW 60cm column). The eluting solvent contained 0.1% trifluoroacetic acid and 36% acetylnitrile, and the sample was detected using a wavelength of 205 nm. All peptides were less than 5,000 Da in size, with 0.8% between 3,000 and 5,000 Da, 11.3% in the 1,000-3,000 Da range and the remaining 87.9% less than 1,000 Da.

† Pig grower/finisher vitamin, mineral premix, Danmix, Nutritech, Auckland, New Zealand, containing (per kg diet) 4,000,000 IU vitamin A, 800,000 IU vitamin D, 12,000 IU vitamin E, 800 mg vitamin K, 400 mg vitamin B1, 1 g vitamin B2, 800 mg vitamin B6, 4 mg vitamin B12, 84 mg folic acid, 4 g pantothenic acid, 6 mg biotin, 6 g niacin, 20 g choline, 120 mg selenium, 200 mg cobalt, 400 mg iodine, 50 g copper, 40 g iron, 18 g manganese, 48 g zinc and 4 g zinc bacitracin.

Table 2 Nitrogen and amino acid compositions of the experimental diets (g/kg dry matter).

	Diet			
Amino acid	Protein-free	EHC5	EHC10	EHC20
LYS	0.13	4.69	9.59	19.07
HIS	0.03	1.69	3.35	6.35
ARG	0.08	2.07	4.37	8.87
ASP	0.19	4.81	10.08	19.79
THR	0.08	2.62	5.24	10.42
SER	0.10	3.27	6.41	12.84
GLU	0.31	12.98	26.39	53.09
PRO	0.07	5.30	10.85	20.79
GLY	0.12	1.14	2.23	4.37
ALA	0.12	1.81	3.63	7.23
CYS	0.10	0.32	0.47	0.79
VAL	0.10	3.48	7.12	14.34
MET	0.08	1.50	2.80	6.23
ILE	0.08	2.90	5.85	11.96
LEU	0.14	5.18	10.54	21.33
TYR	0.07	1.52	3.00	6.32
PHE	0.08	2.74	5.57	11.47
Nitrogen	0.63	7.43	13.72	27.99

Experimental Design

The diets were administered using a Latin Square design (4x4 Latin Square, duplicated) such that every test diet followed every other test diet once only. The pigs were randomly allocated to the Latin Square and received their respective diets for eight days. At the end of this period, the basal diet was given for a period of six days to allow re-equilibration of body nitrogen balance for the pigs fed the protein-free and low protein diets. Following this, the pigs were given their next test diet. Each pig received each of the four test diets once only.

On the fifth and eighth days of each test period, ileal digesta were collected continuously for 24 hours using plastic bags attached to the cannulae. The bung was removed from the cannula an hour before the collection commenced as suggested by van Leeuwen *et al.* (1991), to allow the ileocaecal valve to move so that it was protruding into the lumen of the cannula instead of the intestinal lumen.

The plastic bags were emptied at least hourly, and the digesta were frozen (-20 °C) hourly after adjustment to pH 3.5 by the addition of 6 M H_2SO_4. This procedure was adopted to reduce enzyme and bacterial activity in the digesta.

Chemical Analysis

Digesta were thawed and pooled for each diet and pig over the two 24 hour collection periods. Each pool of digesta was then weighed and homogenised.

A subsample of approximately 60 g was taken from each digesta pool from the pigs receiving the EHC based diet, and centrifuged at 7,000 G for 10 minutes. The supernatants were then ultrafiltered using Centriprep-10 ultrafiltering devices (Amicon Inc., Beverly, MA, USA, 10,000 Da MW cut-off) according to the manufacturer's instructions. The precipitate from the centrifugation step was added to the retentate from the ultrafiltration, and the material freeze-dried and finely ground. A subsample (approximately 60 g) was taken from each digesta pool from the pigs receiving the protein-free diet. This subsample was immediately freeze-dried and finely ground.

The diets, non-fractionated digesta collected after feeding the protein-free diet and the digesta precipitate+retentate (MW > 10,000 Da) fractions from pigs fed the EHC based diets were analysed for total nitrogen, amino acids, chromium and dry matter.

Total nitrogen was determined in duplicate. The samples were combusted at 1050 °C in oxygen gas. The nitrogen was then reduced to N_2 by a catalyst and this was measured by a thermal conductivity cell using a Leco FP2000 (Leco Corporation, St Joseph, Michigan, USA).

The amino acid composition of the samples was determined as follows: Duplicate samples (5-7 mg) were hydrolysed in 1 ml of 6 mol/L glass-distilled HCl containing 0.1% phenol in glass tubes sealed under vacuum, for 24 hours at 110 $\pm$ 2 °C. Amino acid concentrations were then measured using a Waters ion exchange HPLC system calibrated against a reference amino acid mixture of known concentrations. The peaks of the chromatograms were integrated using the dedicated software Maxima 820 (Waters, Millipore, Milford, MA, USA) which identifies the amino acids by retention time against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively, and the weight of each amino acid was calculated using free amino acid molecular weights. No corrections were made for losses of amino acids during hydrolysis.

Cysteine and methionine are destroyed during hydrolysis, so were determined after oxidation of duplicate samples (3-4 mg) with 1 mL of performic acid (1 part 30% H_2O_2 to 9 parts 88% formic acid) for 16 hours at 0 °C. The samples were then neutralised with 0.15 mL of 50% (w/w) HBr prior to acid hydrolysis. Tryptophan, which is also destroyed during acid hydrolysis, was not determined.

The chromium contents of the diet and ileal digesta were determined using an Instrumentation Laboratory Atomic Absorption Spectrophometer according to the method described by Costigan & Ellis (1987).

Data Analysis

Endogenous nitrogen and amino acid flows (related to the ingestion of 1 g of dry matter) were determined using the following equation:

Endogenous flow =

$$\frac{\text{Concentration of compound in digesta x Diet chromium concentration}}{\text{Digesta chromium concentration}}$$

The units were µg/g dry matter intake.

The data were tested for homogeneity of variance using Bartlett's Test (Snedecor & Cochran 1980). All statistical tests were carried out using the computer programme SAS (version 6.12, 1997). The data were analysed using a general linear model for a Latin Square design adjusted for the loss of one pig. The model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \delta_k + \varepsilon_{ijk}$$

Where α_i, β_j and δ_k represented the effects due to the treatment (diet), collection period and pig, respectively.

Differences in endogenous nitrogen and amino acid loss between the pigs fed the protein-free and EHC10 diets were determined by conducting a multiple range test using least square means following Bonferroni adjustment for multiple comparisons.

The endogenous ileal nitrogen and amino acid flows were plotted against the EHC concentration in the diet. Linear and curvilinear functions were fitted (SAS version 6.12, 1997) to the data obtained after all four test diets were fed to the pigs, and a linear function was also fitted to the data pertaining to the 5, 10 and 20% EHC based diets.

RESULTS

One pig was removed from the trial due to complications with its cannula. The remaining pigs appeared healthy and consumed their diets readily. The mean daily liveweight gains (± SEM) of the pigs determined over the 8 day periods throughout the study were 238 (± 138), 575 (± 100), 613 (± 75) and 925 (± 75) g/day for pigs fed the PF, EHC5, EHC10 and EHC20 diets, respectively. The mean liveweight of the pigs at the completion of the experiment was 80.0 kg. There was no leakage of digesta around the cannulas during digesta collections and minimal leakage at other times during the experiment. The chromium recoveries averaged (mean ± SEM) 73.2 ± 4.4%.

All variances were found to be homogeneous. The endogenous ileal amino acid and total nitrogen flows for the animals given the PF and EHC10 diets are shown in Figure 1. The endogenous flows of total nitrogen, aspartic acid, threonine, serine, glutamine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine and methionine were all statistically significantly ($P<0.05$) higher for pigs receiving the EHC10 diet compared to pigs receiving the PF diet.

The mean endogenous ileal nitrogen and amino acid flows pertaining to the diets containing different amounts of EHC are shown in Table 3. There was a significant ($P<0.05$) effect of dietary EHC concentration on total nitrogen and amino acid flow for all of the amino acids.

Figure 2 shows curvilinear and linear relationships between endogenous ileal nitrogen flow and dietary EHC concentration. The curve (Endogenous N flow = $1508.6 + e^{0.0665 \times \text{EHC concentration}}$) had an R^2 value of 0.88, whereas the linear relationship pertaining to the 5, 10 and 20% dietary EHC concentrations (Endogenous N flow = 501.9 + [258.2 x EHC concentration]) had an R^2 of 0.87. The R^2 value for a linear relationship between the endogenous ileal nitrogen flow and 0, 5, 10 and 20% dietary EHC (Endogenous N flow = 1252 + [208 x EHC concentration]) was 0.82. Only the data for total nitrogen are given in Figure 2, but the shapes of the relationships shown are representative of those found for the individual amino acids.

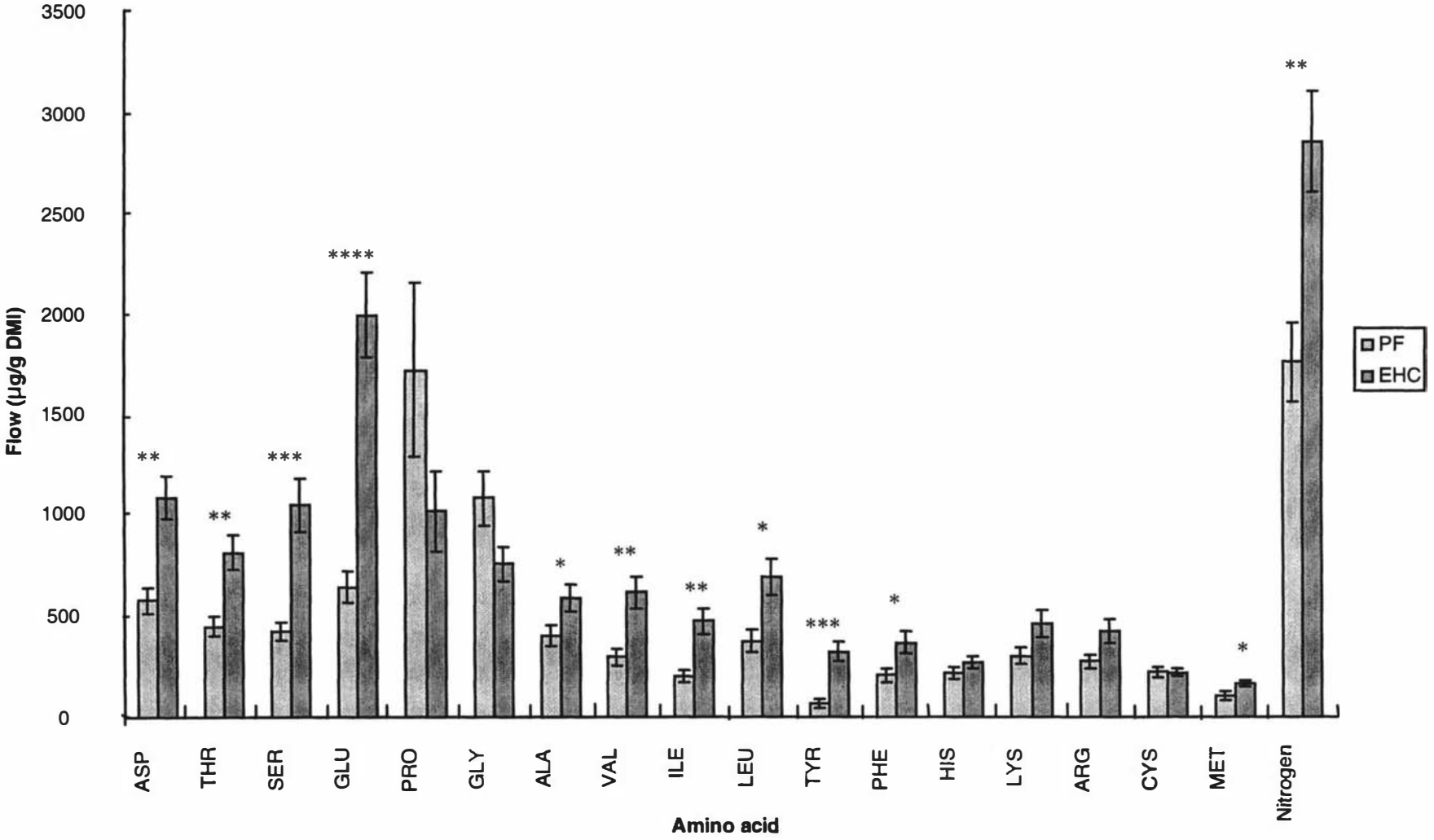


Figure 1 Mean (± SEM) endogenous ileal amino acid flows in pigs fed protein-free (PF) or enzyme hydrolysed casein (EHC, 10%) -based diets.
* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$

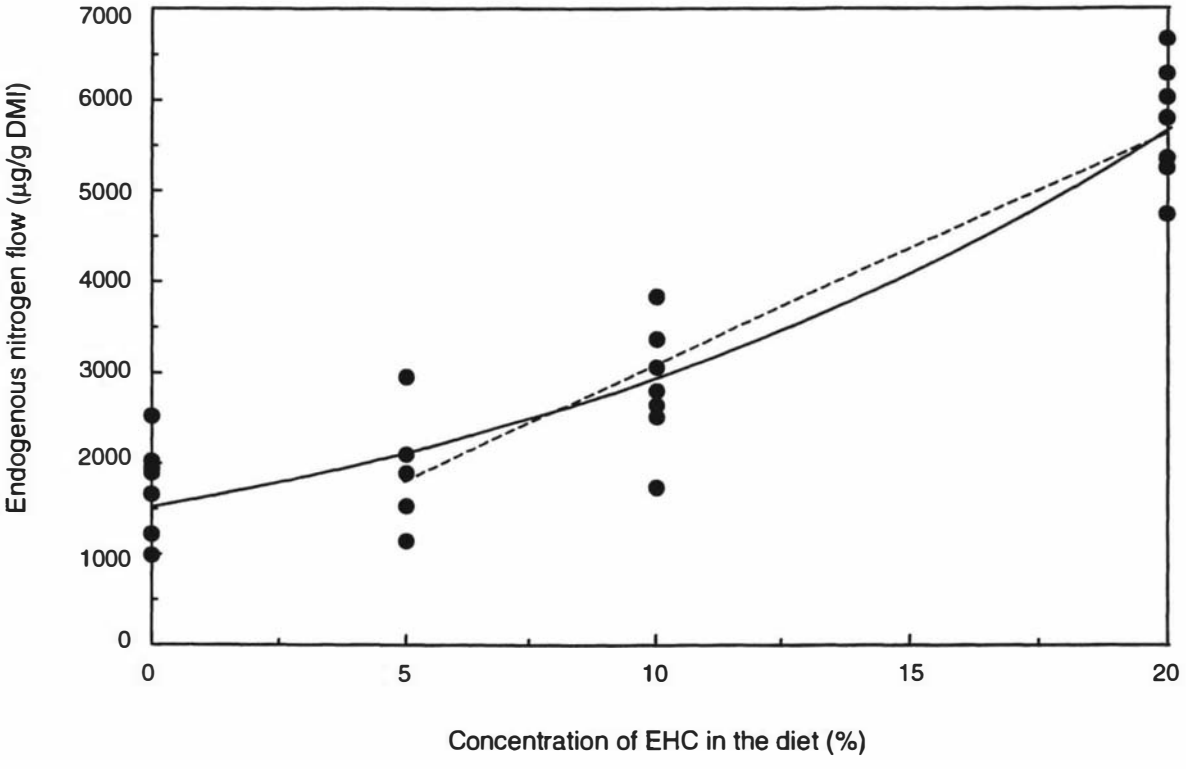
Table 3. Mean (n = 7) endogenous flows (µg/g DMI) for pigs receiving semi-synthetic diets containing 5 (EHC5), 10 (EHC10) or 20% (EHC20) enzyme hydrolysed casein.

	Diet				
Amino acid	EHC5	EHC10	EHC20	Overall SEM	Significance[†]
LYS	262	456	523	74.4	****
HIS	141	264	379	56.1	****
ARG	232	419	456	74.5	***
ASP	502	1078	1509	182.7	****
THR	382	807	1097	168.7	****
SER	502	1040	1288	204.6	****
GLU	820	1983	3000	392.1	****
PRO	462	1009	1253	628.8	*
GLY	477	749	696	248.2	**
ALA	302	583	674	106.0	****
CYS	130	217	254	47.3	****
VAL	308	610	760	109.4	****
MET	88	162	267	50.6	***
ILE	237	469	540	85.8	****
LEU	377	686	804	104.5	****
TYR	164	319	353	70.1	****
PHE	201	363	406	61.2	****
Nitrogen	1948	2851	5743	524.7	****

[†] Significance of effect of level of EHC in the diet on endogenous ileal flows. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Table 4 gives linear regression equations for the relationships between endogenous nitrogen or amino acid flow and EHC concentration in the diet for pigs receiving the diets containing 5, 10 and 20% EHC. The R^2 values ranged from 0.06 to 0.87. The slopes were statistically significantly different from 0 for nitrogen and all of the amino acids measured except for glycine. The intercepts were also significantly greater than 0 for all amino acids except for glutamine, proline and methionine, and for nitrogen.

Figure 2 Endogenous nitrogen flow in growing pigs given diets containing 0, 5, 10 or 20% enzyme hydrolysed casein (EHC). —Nonlinear regression for data from all diets, Endogenous N flow = 1508.6 + $e^{0.0665 \times \text{EHC concentration}}$, R^2 = 0.88. - - Linear regression for data from diets containing 5, 10 or 20% EHC, R^2 = 0.87.



DISCUSSION

One of the aims of this study was to determine endogenous ileal amino acid flows to corroborate earlier findings of a significantly higher loss of amino acids and nitrogen at the terminal ileum in the growing pig when a mixture of peptides and free amino acids is added to a protein-free diet (Moughan *et al.* 1992, Butts *et al.* 1993a, Leterme *et al.* 1996). Most earlier studies relied upon the "slaughter method" for the collection of ileal digesta, whereas the present study used PVTC cannulated pigs. The slaughter method, which involves removal of a terminal section of small intestine from the anaesthetised animal, with manual collection of the contents, is a straightforward method for the collection of ileal digesta in pigs and other simple-stomached animals. The slaughter technique has the advantages of simplicity and ethical acceptability compared to cannulation and anastomosis techniques and there is minimal interference with the animal's digestive tract prior to sampling. However, because of the relatively small amount of digesta collected with this method, possibility exists of a bias in results due to an unrepresentativeness of the sample of digesta collected.

Table 4 Linear regression relationships between endogenous ileal amino acid or nitrogen flows and dietary concentration of enzyme hydrolysed casein (EHC).

	Regression equation*	R^2 †	$P_{intercept}$ ‡	P_{slope} §
LYS	y = 228(61) + 16(5)x¶	0.35	0.05	0.01
HIS	y = 83(32) + 15(2)x	0.65	0.05	0.0001
ARG	y = 213(58) + 13(4)x	0.29	0.01	0.01
ASP	y = 286(112) + 64(8)x	0.73	0.05	0.0001
THR	y = 236(92) + 45(7)x	0.67	0.05	0.0001
SER	y = 378(131) + 49(10)x	0.54	0.01	0.0001
GLU	y = 312(212) + 139(16)x	0.79	NS	0.0001
PRO	y = 339(176) + 49(13)x	0.38	NS	0.01
GLY	y = 504(103) + 12(8)x	0.06	0.0001	NS
ALA	y = 257(70) + 23(5)x	0.46	0.01	0.001
CYS	y = 111(23) + 8(2)x	0.49	0.0001	0.001
VAL	y = 233(76) + 28(6)x	0.53	0.01	0.001
MET	y = 36(29) + 12(2)x	0.58	NS	0.0001
ILE	y = 201(62) + 18(5)x	0.42	0.01	0.001
LEU	y = 318(90) + 26(7)x	0.41	0.01	0.01
TYR	y = 146(45) + 11(3)x	0.34	0.01	0.01
PHE	y = 179(51) + 12(4)x	0.32	0.01	0.01
Nitrogen	y = 501(298) + 258(23)x	0.87	NS	0.0001

* Where y is the endogenous ileal flow (µg/g dry matter intake) and x is the concentration of EHC in the diet (5, 10, 20%).

† Adjusted for the number of variables in the model.

‡ The probability that the intercept is equal to 0.

§ The probability that the slope is equal to 0.

¶ The number in brackets following the parameter estimate is the SEM.

PVTC cannulation (van Leeuwen *et al*. 1991) offers a practical alternative that allows a quantitatively greater collection of ileal digesta. The major advantage with the PVTC cannula over several other cannulation methods is that the small intestine is not transected, so there are minimal effects on ileal muscle function. Higher and more representative recoveries of digesta are expected in comparison with simple T cannulation. The effect on the animal of long-term PVTC cannulation (12 weeks) has been examined by Köhler *et al*. (1992a, b) who concluded that the PVTC cannula does not significantly alter the metabolism of the pig and is suitable, therefore, for the collection of digesta in metabolic studies. In the present study, the PVTC cannulated pigs appeared healthy and grew normally. At *post mortem* there were no signs of any adverse effects of the cannulation procedure. Theoretically, a complete collection of

digesta is possible with the PVTC cannula. Although this was not found in the present work, the chromium recoveries were relatively high and in line with values reported following continuous collections of digesta for 24-hour periods from PVTC cannulated pigs. Mean chromium recoveries of 71.4% (Köhler *et al.*, 1991), 71.6, 71.9, 90.8, and 106.4% (den Hartog *et al.*, 1988; Köhler *et al.*, 1990) have been reported previously. The mean chromium recovery of 73.2% found in the present work is within this range of reported values. As chromic oxide is believed to be quantitatively recovered in pig faeces (Ehle *et al.* 1982, Mroz *et al.* 1996) it appears that some of the digesta bypassed the cannula and entered the large intestine. The degree of recovery of digesta in the present study was, however, considered to be sufficient to provide representative digesta samples. The representativeness of digesta collection can be tested by determining whether there is a correlation between marker recovery and apparent ileal crude protein digestibility when the same diet is fed to the animals (van Leeuwen *et al.* 1996). A correlation between chromium recovery and apparent ileal crude protein digestibility would signify that the digesta collections are not representative. These correlations were calculated using the results from the present study for each diet, and no significant correlation was found, which supports the contention that the digesta collected should be representative of ileal digesta.

In the present work, the endogenous flows of total nitrogen, aspartic acid, threonine, serine, glutamine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine and methionine were statistically significantly ($P<0.05$) higher for the EHC10-fed pigs compared to those fed the protein-free diet. This is in agreement with previous studies involving a controlled comparison of peptide alimentation and the protein-free approach in the growing pig (Moughan *et al.*, 1992, Butts *et al.* 1993*a*, Leterme *et al.* 1996) and it would appear that dietary peptides exert a positive influence on endogenous ileal protein loss.

An advantage of the peptide alimentation method is that it allows the endogenous flows of total nitrogen and all of the amino acids to be determined directly, as opposed to the homoarginine (Hagemeister & Erbersdobler 1985, Rutherfurd & Moughan 1990) and ^{15}N methods (Souffrant *et al.* 1982, Souffrant 1991) where only the lysine or nitrogen flows, respectively, are determined directly. With the peptide alimentation method, endogenous free amino acids or peptides that are less than 10,000 Da in size are discarded with the ultrafiltrate leading to some underestimation of the endogenous flows determined with this method. Attempts have been made to quantify the extent of

this underestimation by ultrafiltering digesta collected from pigs receiving a protein-free diet, and determining the amino acids present in the ultrafiltrate. Moughan & Schuttert (1991) in a carefully controlled study showed that amino nitrogen present in the ultrafiltrate comprised only 11% of the nitrogen in the total ileal digesta. In contrast, Butts *et al.* (1992) found that the ultrafiltrate (MW < 10,000 Da) of digesta obtained after feeding rats a protein-free diet contained some 13-24% of each amino acid in the total ileal digesta, except for glycine (46%), histidine (36%) and lysine (28%) where higher amounts were found. Leterme *et al.* (1996) also ultrafiltered the soluble fraction of digesta obtained after feeding pigs a protein-free diet and found that some 22% of the total digesta N was present in the < 10,000 Da fraction. In the latter two studies, however, the pH of the digesta was not adjusted to prevent autolysis in the digesta, thus these values may be overestimates. Further, it needs to be recognised that the feeding of peptides may affect the composition as well as the amount of endogenous excretion, and higher than normal amounts of free amino acids and small peptides may occur with protein-free feeding. Physiologically, it seems unlikely that there would be a high proportion of unabsorbed small peptides in the digesta at the terminal ileum and this is reflected by the observations of Moughan and Schuttert (1991). The molecular weight profile of the EHC used in the present study showed that only 0.83% of the total peptides had a molecular weight greater than 3,000 Da. The peptide alimentation method could, therefore, be modified by ultrafiltering the digesta using ultrafiltration devices with a molecular weight cut-off of 3,000-5,000 Da as opposed to 10,000 Da. This would be expected to substantially decrease the proportion of endogenous nitrogen that may be discarded with the ultrafiltrate, thereby increasing the accuracy of the method. To conclude, the removal of endogenous material in the ultrafiltrate will lead to some underestimation of endogenous amino acid loss with the peptide alimentation method, but such underestimation only serves to make the protein-free, EHC diet comparison more conservative.

The ultrafiltration devices used in the present study have been shown to be effective for the ultrafiltration of purified protein, peptide and amino acid solutions. Butts *et al.* (1991) demonstrated that only negligible amounts of proteins that are smaller than 10,000 Da remain in the retentate and equally small amounts of proteins that are larger than 10,000 Da move through the filter during ultrafiltration. The effectiveness of the ultrafiltration devices that were used in the present study has also been tested by adding increasing amounts of EHC to digesta, ultrafiltering the digesta and quantifying the nitrogen and amino acids in the fractions of digesta (Hodgkinson & Moughan unpublished information, refer Chapter 3). In the latter work, the nitrogen

and amino acid concentration of the precipitate plus retentate fraction of digesta did not consistently increase as more EHC was added to the digesta. This demonstrates that any binding of EHC to the retentate of the ultrafiltered digesta is negligible and that the ultrafiltration devices are effective for the ultrafiltration of digesta which contains EHC.

The main aim of the present study was to determine whether the concentration of peptides in the diet had an effect on endogenous ileal amino acid flow in the growing pig. As shown in Table 3, increasing the EHC concentration of the diet from 5 to 20% significantly increased the endogenous ileal flows of nitrogen and all of the amino acids. The endogenous flows of nitrogen and amino acids for pigs given the EHC10 diet were numerically similar to those found in previous studies where pigs were fed a diet containing 10% EHC (Butts *et al*. 1993a, b, Moughan *et al*. 1992). Schulze *et al*. (1995) fed growing pigs a diet containing 18% EHC and the endogenous amino acid flows were generally greater than those determined in the studies by Butts *et al*. (1993a, b) and Moughan *et al*. (1992). The flows reported by Schulze et al (1995) are very similar to those recorded here for the EHC20 diet. The present finding confirms the preliminary result of Butts *et al*. (1998), who examined the effect of dietary peptide intake on endogenous ileal lysine flow using the enzyme hydrolysed protein method in the growing rat, and found a statistically significant effect of dietary peptide concentration on endogenous ileal lysine flow. In contrast, Souffrant *et al*. (1997) have reported preliminary results using the homoarginine and isotope dilution methods in piglets, where there was no difference in endogenous ileal nitrogen flows dependent upon dietary casein concentration.

When functions were fitted to the data overall (Figure 2), a curve (Endogenous N flow = 1508.6 + $e^{0.0665 \times \text{EHC concentration}}$) gave the best overall fit (R^2 = 0.88). However, if it is assumed that pigs given the protein-free diet are effectively in a different physiological state compared to those receiving protein-containing diets, it may be more appropriate to fit functions over the range of 5 to 20% EHC inclusion. When this was done (Figure 2) a linear function gave a similar fit to the data (R^2 = 0.87).

The slopes of the linear regression equations for ileal amino acid flow related to dietary EHC concentration were positive and generally significantly different from zero (Table 4). The present result demonstrates that the stimulatory effect of dietary peptides on endogenous amino acid flows is not an "all or nothing" effect, but is rather "dose-

dependent". This has important implications for the practical determination of true digestibility coefficients in diet formulation.

Previous studies have demonstrated that increasing dietary protein concentration at a set dry matter intake results in an increase in apparent ileal amino acid digestibility in the growing pig (Sauer *et al.* 1980, Bell *et al.* 1983, Furuya & Kaji 1989). It has been suggested that the endogenous ileal amino acid losses remain constant with increasing dietary protein concentration and, therefore, constitute a greater proportion of the protein present at the terminal ileum at lower dietary protein concentrations (Taverner 1979, Sauer *et al.* 1980). Studies that have investigated the relationship between dietary protein concentration and true ileal digestibility at a constant level of dietary dry matter intake have assumed a constant value for endogenous ileal amino acid flows in their calculations (Furuya & Kaji 1989, Donkoh & Moughan 1994), and have found no difference in true digestibility with increasing dietary protein concentration. Donkoh & Moughan (1994) examined the effect of protein concentration in the diet on true ileal digestibility coefficients for meat and bone meal. If the endogenous nitrogen and amino acid flows determined in the present study are used with the values of Donkoh & Moughan (1994), it can be shown that the true digestibility of meat and bone meal increases with increasing inclusion of peptides in the diet. There is no obvious explanation for this observation. When true digestibility coefficients are to be compared among different protein sources, the coefficients may need to be determined at a standard inclusion level of dietary protein.

There is now a considerable body of work accumulated on endogenous ileal nitrogen and amino acid flows in the growing pig determined using the protein-free and enzyme hydrolysed protein approaches. Table 5 presents a summary of selected published results for endogenous ileal lysine and total nitrogen flows determined using these two methods in the growing pig. The data presented in Table 5 are taken only from studies where growing pigs were fed semi-synthetic diets containing 3-5% cellulose as the sole source of fibre. In spite of this basis for comparability, the reported endogenous ileal nitrogen and lysine flows determined under protein-free alimentation are highly variable, ranging from 1360 to 3168 and 250 to 630 mg/kg DMI, respectively. The mean flows under protein-free alimentation (2132 and 370 mg/kg DMI for nitrogen and lysine, respectively) are notably lower than those obtained under peptide alimentation (3417 and 524 mg/kg DMI for nitrogen and lysine, respectively).

Table 5 Published endogenous ileal flows of nitrogen (μg/g DMI) and lysine (μg/g DMI) in the growing pig (liveweight 10-115 kg) determined using the protein-free or peptide alimentation methods.

	Flow		
Method	Nitrogen	Lysine	Reference
Protein-free alimentation*	1753	298	Present study
	1790	284	Souffrant *et al.* 1997
	1500	252	Butts *et al.* 1993a
	2970	530	Furuya & Kaji 1992
	1360	350	Leterme *et al.* 1992
	~2300	312	Moughan *et al.* 1992
	3168	530	de Lange *et al.* 1989a
	2960	630	de Lange *et al.* 1989b
	1710	260	Furuya & Kaji 1989
	1810	250	Taverner *et al.* 1981
Mean:	2132	370	
Enzyme hydrolysed protein†	2851	456	Present study
	3700	448	Butts *et al.* 1993a
	2746	591	Butts *et al.* 1993b
	NR	461	Moughan *et al.* 1992
Mean:	3417	524	

NR This figure was not reported in the publication.

* Diets were protein-free, with nitrogen and amino acids present in ileal digesta assumed to be of endogenous origin.

† Pigs fed diets with 10% enzyme hydrolysed casein (MW<5,000 Da) as the sole protein source. Ileal digesta ultrafiltered (MW cut-off 10,000 Da) to separate endogenous protein.

The EHC based endogenous ileal amino acid flows determined in the present study were statistically significantly higher for most of the amino acids determined, compared to those found with protein-free alimentation. This confirms, using a different approach to digesta collection, other reported findings that the traditional protein-free method markedly underestimates endogenous loss in the growing pig. Increasing the peptide concentration of the diet had a major effect on endogenous ileal amino acid losses in the growing pig.

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Chapter 8

Effect of the Concentration of Dietary Protein (Zein) on Endogenous Ileal Lysine Flow in the Growing Rat

In the previous Chapter, it was shown that the dietary concentration of amino acids and peptides has a positive effect on endogenous ileal nitrogen and amino acid flows in the growing pig. In the study described in the present Chapter, rats were fed diets containing different amounts of the protein zein, which contains virtually no lysine, and the endogenous ileal flow of lysine was determined directly. The aim of the work was to use a different approach to measure endogenous ileal protein flows to corroborate the results reported in Chapter 7.

Effect of the Concentration of Dietary Protein (Zein) on Endogenous Ileal Lysine Flow in the Growing Rat

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ABSTRACT

The aim of the study was to determine the effect of the amount of protein in the diet on endogenous ileal lysine flow in the growing rat. Male rats (n=108; mean bodyweight 170 g) were given free access to experimental diets for ten minutes each hour for eight hours each day. The rats received diets containing 5, 10, 15, 20, 25 or 30% zein for eight days. Zein is nearly devoid of lysine and tryptophan, so the diets were supplemented with lysine and tryptophan for the first six days and lysine and tryptophan were given to the rats via intraperitoneal injections over the final two days of this period. Digesta were sampled from the terminal ileum of the rats on day eight following euthanasia. Digesta were pooled to give a total of six samples for each experimental diet. The flow of endogenous lysine through the terminal ileum was determined in each sample. The linear regression equation was: Endogenous ileal lysine flow (μg/g DMI) = 317.1 + (13.24 x dietary zein concentration, %). R^2 = 0.33, residual standard error = 26.6. Increasing the amount of zein in the diet led to a significant ($P < 0.0001$) increase in the flow of endogenous lysine through the terminal ileum.

INTRODUCTION

Protein-containing material of body origin is released into the alimentary tract during the digestion of food and whereas 70-80% of this material is reabsorbed before the end of the small intestine (Souffrant, 1991), the remaining material enters the large intestine. Microbes are present in high numbers in the large intestine and these microbes metabolise the amino acids, changing the amino acid profile of the digesta. Also, amino acids are not absorbed, as such, to any significant extent in the large intestine (Just *et al.*, 1981; Schmitz *et al.*, 1991; Darragh *et al.*, 1994) and the nitrogen that is absorbed from the large intestine (in the form of ammonia, amines and amides) is normally of no nutritional value to the animal, and is metabolised and excreted in the urine as urea (Zebrowska, 1973; Just, 1983; McNeil, 1988). Measurements of endogenous amino acid loss based on faecal collection are misleading, therefore, and for accuracy, endogenous nitrogen and amino acids need to be quantified at the end of the small intestine, the terminal ileum (Sauer and Ozimek, 1986).

An approach that has been used to directly quantify the amount of endogenous amino acids that flow through the terminal ileum is to feed animals a diet containing a natural protein that is devoid of specific amino acids (Butts *et al.*, 1993a). Any amino acid, not found in the dietary protein, but present in the digesta must be of endogenous origin. An advantage of this method over several other approaches used to determine endogenous ileal amino acid flow is that the animals are in a positive body nitrogen balance throughout the study and the tissues of the digestive tract have a direct supply of amino acids and peptides from the digestion of dietary protein. Zein is a naturally occurring protein in maize and is almost completely lacking in the amino acids lysine and tryptophan. It can be used, therefore, to directly measure endogenous lysine and tryptophan flows (Butts *et al.* 1993a).

It appears, based on a study involving feeding pigs an enzymatic hydrolysate of casein, that the dietary concentration of amino acids and peptides has an effect on endogenous ileal nitrogen and amino acid flow (Hodgkinson *et al.*, 1999a). The present study was conducted to confirm the latter observation, using a different approach (zein based diets) to determine endogenous ileal protein flows.

MATERIALS AND METHODS

The Massey University Animal Ethics Committee granted ethics approval for the studies reported here, which involved feeding semi-synthetic diets to rats with collection of ileal digesta, following euthanasia. Seven diets were prepared, a basal casein-based diet and semi-synthetic diets containing 5 (Z5), 10 (Z10), 15 (Z15), 20 (Z20), 25 (Z25) and 30 (Z30) % zein, a purified protein from maize. The ingredient compositions of the diets are given in Table 1.

Given that zein is virtually devoid of lysine and tryptophan, the animals receiving the zein-based diets received supplementary lysine and tryptophan. It was calculated based on NRC (1995) that the diets needed to contain 85 mg of lysine and 12.5 mg of tryptophan per gram of protein to ensure that neither lysine nor tryptophan was the first limiting amino acid for growth. The amounts of synthetic lysine and tryptophan added to the diets are given in Table 2.

Table 1 Ingredient composition (g/kg air dry weight) of the basal and experimental diets.

	Diet						
Ingredient	Basal	Z5	Z10	Z15	Z20	Z25	Z30
Lactic casein	120	0	0	0	0	0	0
Zein[1]	0	50	100	150	200	250	300
Corn starch	625	689	639	589	539	489	439
Soyabean oil	35	35	35	35	35	35	35
Cellulose[2]	50	50	50	50	50	50	50
Vitamin mix[3]	50	50	50	50	50	50	50
Mineral mix[4]	50	50	50	50	50	50	50
Sucrose	70	70	70	70	70	70	70
Chromic oxide	0	6	6	6	6	6	6

[1] Sigma Chemical Company, St Louis, USA. Contained (g/100g protein): 0.10 lys, 1.22 his, 1.32 arg, 5.01 asp, 2.53 thr, 4.56 ser, 22.96 glu, 10.02 pro, 1.14 gly, 10.04 ala, 3.46 val, 1.47 met, 3.66 ile, 19.26 leu, 4.86 tyr, 6.90 phe.

[2] Microcrystalline Cellulose, FMC Corporation, Newmark Delaware, USA.

[3] With sucrose as the carrier. Provided (per kg diet): 5.0 mg retinol, 5.0 mg thiamin, 7.0 mg riboflavin, 20.0 mg pantothenic acid, 8.0 mg pyridoxine, 0.05 mg cyanocobalamin, 0.025 mg ergocalciferol, 200.0 mg tocopherol, 1.0 mg biotin, 3.0 mg menadione, 1500.0 mg choline, 2.0 mg folic acid, 200.0 mg inositol, 20.0 mg niacin.

[4] With cellulose as the carrier. Provided (per kg diet): 6.29 g calcium, 7.79 g chloride, 28.9 µg cobalt, 1.97 mg chromium, 10.68 mg copper, 424.1 mg iron, 151 µg iodine, 5.24 g potassium, 1.06 g magnesium, 78.0 mg manganese, 152 µg molybdenum, 1.97 g sodium, 4.86 g phosphorus, 0.15 mg selenium, 48.2 mg zinc.

Table 2 Amounts (g/kg diet) of synthetic lysine[1] and tryptophan[2] added to the experimental diets.

	Diet					
Amino acid	Z5	Z10	Z15	Z20	Z25	Z30
Lysine	4.25	8.5	12.75	17.0	21.25	25.5
Tryptophan	0.625	1.250	1.875	2.500	3.125	3.765

[1] L-lysine monohydrochloride USP, Sigma Aldrich Pty. Ltd., St Louis, USA.
[2] L-tryptophan SigmaUltra, Sigma Aldrich Pty. Ltd., St Louis, USA.

For a period of time at the end of the study, the diets that the rats received were not supplemented with synthetic lysine and tryptophan and thus lysine and tryptophan were administered to the rats by intraperitoneal injection. The amounts of lysine and tryptophan given to the rats each day by intraperitoneal injection were calculated at a rate of 85 mg of lysine and 12.5 mg of tryptophan per gram of dietary protein consumed (NRC, 1995). The amounts of lysine and tryptophan given to the rats in each of two intraperitoneal injections per day are given in Table 3. Sterile solutions containing lysine and tryptophan were prepared in sterile isotonic (0.9%) saline and distilled water that had been filtered (NANOpure II filtering system, Barnstead, USA) to have a resistance of 18 MOhms. The solutions had osmolarities slightly greater than an iso-osmotic solution. Each solution was passed through 0.2 µm filters (Sterile Acrodisc, Gelman Sciences) into sterile containers, with each container holding sufficient solution for one set of injections. The containers were sealed and the material immediately frozen until use.

The study was conducted in two parts. A preliminary study was undertaken to demonstrate that rats are able to adequately metabolise lysine and tryptophan when these amino acids were supplied by intraperitoneal injection (twice daily) at a rate deemed to be sufficient to support a positive body nitrogen balance. The preliminary study was followed by a main study, the objective of which was to determine whether the concentration of protein (zein) in the diet had an effect on endogenous ileal lysine flow.

Table 3 Amounts of lysine (mg) and tryptophan (mg) administered to rats in each of two daily intraperitoneal injections while the rats received diets containing zein as the sole source of protein but with no additional lysine or tryptophan, and the volumes (ml) of the solutions administered.

Diet	Lysine[1]	Tryptophan[2]	Volume given at each injection
Z5	32.00	4.7	1.0
Z10	63.75	9.4	1.0
Z15	95.65	14.1	1.5
Z20	127.65	18.8	2.0
Z25	159.65	23.5	2.0
Z30	191.65	28.2	2.5

[1] L-lysine monohydrochloride USP, Sigma Aldrich Pty. Ltd., St Louis, USA.
[2] L-tryptophan SigmaUltra, Sigma Aldrich Pty. Ltd., St Louis, USA.

Preliminary Study

Twenty Sprague Dawley male rats with a body weight of (mean ± SEM) 142.7±1.53 g were placed singly in metabolism cages which allowed a complete and separate collection of faeces and urine and were held at 22 ± 1 °C with a 12 hour light/dark cycle. Water was available at all times. The rats were given free access to their food for ten minutes each hour for eight hours each day throughout the study. The first meal each day commenced at 0800h and the last meal at 1500h. The food intakes of the rats were recorded each day.

The first seven days of the study were a training period, with the rats given access to the basal casein-based diet (Table 1) and trained to eat in eight 10-minute periods per day as described above.

Following this, the rats were randomly divided into two groups each of ten rats. One group was given access to the diet containing 5% zein and the other group the diet containing 30% zein (the two extremes of the dietary treatments to be used in the main study) for a total of ten days, in the manner described above. For the first two days of this ten-day period, the diets were supplemented with synthetic lysine and tryptophan (Table 2).

For the remaining eight days of this ten-day period, the diets that the rats received were not supplemented with lysine and tryptophan, but lysine and tryptophan were

administered to the rats via intraperitoneal injection as described. Before administration to the rats, the solutions were thawed and warmed to 37 °C in a water bath. The rats were given two injections per day with the first injection given following the first meal of the day and the second injection following the second to last meal of the day.

A complete and separate collection of urine and faeces was made over the final six days of this 8-day period that the rats received the intraperitoneal injections. The urine was collected over acid (10% sulphuric acid with a ratio of acid to urine of 0.025 v/v). The urine and faeces in the collection vessels from each cage were removed twice each day (0745 and 1515h) and were frozen.

Upon completion of the urine and faecal collection periods, the rats were asphyxiated with CO_2 gas and then decapitated and a *post mortem* examination of the abdomen was carried out.

The urine collections over the six-day period were pooled to give one urine sample per rat. The faeces were pooled in a similar manner and each faecal sample was freeze-dried and finely ground.

The Z5 and Z30 diets, urine and faeces were analysed for total nitrogen using the Kjeldahl method. The sample was digested with concentrated sulphuric acid with potassium sulphate added to increase the digestion temperature. The free ammonia released was determined using the Berthelot reaction (Chaney and Marbach, 1962).

The mean daily intakes and outputs of total nitrogen were used to determine nitrogen balances for each animal.

Main Study

A total of 108 Sprague Dawley male rats with a bodyweight of (mean $\pm$ SEM) 169.5 $\pm$ 1.0 g were placed in individual wire-bottomed cages that were designed to prevent coprophagy, and were held at 22 $\pm$ 1 °C with a 12 hour light/dark cycle. The rats had free access to water at all times, and were given free access to their diet for ten minutes each hour for eight hours each day as described for the preliminary study. The food intakes of each rat were recorded daily.

For the first six days, the rats were given access to the basal casein-based diet in eight meals per day in the manner described for the preliminary trial.

The rats were then randomly divided into six groups, each of 18 rats. Each group of rats was given access to one of the six zein-based diets (Table 1). The rats were fed their test diet for a total of eight days. For the first six days of this eight-day experimental period, the diets were supplemented with synthetic lysine and tryptophan. On the second to last day of the eight-day period, following the first meal and second to last meal of the day, and again after the first meal on the final day of this period, the rats were given lysine and tryptophan via intraperitoneal injection as described for the preliminary trial. On the final day of the eight-day period, starting five hours after the first meal of the day, the rats were asphyxiated with CO_2 gas and then decapitated. The terminal 20 cm of the ileum was dissected from the body, rinsed with distilled water and gently dried with absorbent paper, with care taken not to apply pressure to the intestines. The digesta were then flushed out of the section of intestine with distilled water and the digesta sample was immediately frozen (-20 °C).

To obtain sufficient digesta for the chemical analyses, the digesta samples collected from each group of 18 rats that received a particular experimental diet were divided at random into six groups each of three digesta samples. The digesta from each group of three rats were pooled to give a total of six pooled digesta samples for each dietary treatment.

The diets and pooled digesta samples were analysed for chromium, dry matter, nitrogen and lysine. The amino acid composition of the zein was also determined.

The chromium contents of the diet and ileal digesta samples were determined using an Instrumentation Laboratory Atomic Absorption Spectrophometer according to the method of Costigan & Ellis (1987).

The total nitrogen concentrations of the diets and digesta samples were determined in duplicate. The samples were combusted at 1050 °C in oxygen gas. The nitrogen was then reduced to N_2 by a catalyst and this was measured by a thermal conductivity cell using a Leco FP2000 (Leco Corporation, St Joseph, Michigan, USA).

The amino acid composition of the zein (see Table 1), and lysine concentrations of the digesta samples were determined as follows. Duplicate samples (5-7 mg) of the digesta and quadruplicate samples of zein (10 mg) were hydrolysed in 1 ml of 6 mol/L

glass-distilled HCl containing 0.1% phenol, in glass tubes sealed under vacuum, for 24 hours at 110 $\pm$ 2 °C. Amino acid concentrations were then measured using a Waters ion exchange HPLC system calibrated against a reference amino acid mixture. The peaks of the chromatograms were integrated using the dedicated software Maxima 820 (Waters, Millipore, Milford, MA) which identifies the amino acids by retention time against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively, and the weight of each amino acid was calculated using free amino acid molecular weights. No corrections were made for potential losses of amino acids during hydrolysis.

The endogenous ileal flows of lysine were calculated as described by Hodgkinson *et al.* (1999b, refer to Chapter 2 of this thesis).

All statistical analyses were conducted using the software package SAS (version 6.12, 1997). ANOVA (Ott, 1993) was conducted to determine whether there was an overall effect of diet on endogenous ileal lysine flow. The endogenous lysine flows were plotted against the concentration of zein in the diet, and linear and curvilinear functions were fitted to the data.

The apparent digestibilities of nitrogen (N) and those for all of the amino acids except lysine were calculated using the equation below (where DMI = dry matter intake):

Apparent digestibility =

$$\frac{\text{Dietary N or amino acid (µg/g DMI)} - \text{Digesta N or amino acid (µg/g DMI)}}{\text{Dietary N or amino acid (µg/g DMI)}}$$

The apparent digestibility data were subjected to ANOVA. The apparent digestibility coefficients for nitrogen were plotted against the concentration of zein in the diet and a linear function was fitted to the data.

RESULTS

Preliminary Study

Two rats were removed from the Z5 group, because of difficulties arising from the intraperitoneal injections. The remaining rats appeared healthy throughout the study and at *post mortem*, no adverse effects of the injection regimen were observed. During the two days that the rats received the Z5 and Z30 diets, where the diets were supplemented with lysine and tryptophan, the mean (± SEM) dietary intakes were 8.5 (± 0.44) and 8.9 (± 0.51) g/day for the Z5 and Z30 diets, respectively. During the 8-day period that the rats received the intraperitoneal injections of lysine and tryptophan, the mean (± SEM) dietary intakes for the rats on the Z5 and Z30 diets were 10.5 (± 0.81) and 10.8 (± 1.78) g/day, respectively. During this 8-day period, the rats on the Z5 diet lost an average of (mean ± SEM) 0.4 ± 0.14 g/day bodyweight and those on the Z30 diet gained 3.0 ± 0.12 g/day bodyweight.

Table 4 shows the body nitrogen balance data. The rats that received the Z5 diet were in a negative body nitrogen balance, losing an average of 6 mg of nitrogen per day over the six-day period that faeces and urine were collected. The rats that received the Z30 diet were in a positive body nitrogen balance, retaining on average 153 mg of nitrogen each day of the six-day faeces and urine collection.

Table 4 Mean dietary nitrogen (N) intakes, losses of N in faeces and urine, total N excreted by the rats and N retention (mg/24 hours) for rats receiving diets containing 5 (Z5) and 30 (Z30) % zein.

Diet	n[1]	N intake	N faecal loss	N urinary loss	Total N excreted	N retention
Z5	8	70 (4.7)[2]	25 (1.8)	51 (2.6)	76 (1.6)	-6 (4.4)
Z30	10	431 (70.4)	136 (48.3)	142 (44.4)	278 (70.2)	153 (66.3)

[1] Number of rats

[2] Numbers in brackets represent SEM's.

Main Study

The rats appeared healthy throughout the main study. The mean feed intakes over the experimental periods were 7.3, 8.3, 7.5, 9.5, 7.3 and 12.8 g/day for the rats receiving the diets containing 5, 10, 15, 20, 25 and 30% zein, respectively. The mean growth rates for the rats over the final 8 days of the study were –0.9, -0.1, 0.43, 1.06, 1.07 and 3.18 g/day for the rats receiving the diets containing 5, 10, 15, 20, 25 and 30% zein, respectively.

The mean endogenous ileal lysine flows are presented in Table 5. There was a

significant (P<0.0001) effect of diet on endogenous ileal lysine flow. A linear model (Figure 1) gave the best fit to the endogenous ileal lysine flows as related to dietary zein concentration. The equation for the linear model was:

Endogenous ileal lysine flow (µg/g DMI) = 317.1 + (13.24 x dietary zein concentration, %). R^2 = 0.33, residual standard error = 26.6.

The probability that the slope was zero was P=0.0002 and that the intercept was zero was P=0.0001.

Table 5 Mean[1] endogenous ileal lysine flows (µg/g DMI) in rats given semi-synthetic diets containing different amounts of zein.

	Dietary zein concentration (%)							
	5	10	15	20	25	30	SEM	P^2
Lysine flow	390.6	340.3	594.8	558.0	859.3	550.0	112.01	****

[1] n = 6.

[2] **** P < 0.0001.

The mean apparent ileal digestibilities of nitrogen and the amino acids are shown in Table 6. There was a significant (P<0.01) effect of diet on the apparent digestibility of nitrogen and the apparent amino acid digestibilities except for that of glycine. The equation for the linear function relating dietary zein concentration and apparent nitrogen digestibility (Figure 2) was:

Apparent nitrogen digestibility (%) = 56.51 + (0.66 x dietary zein concentration, %). R^2 = 0.55, residual standard error = 0.88.

The probability that the slope of the line was zero was P = 0.0001 and that the intercept was zero was P=0.0001.

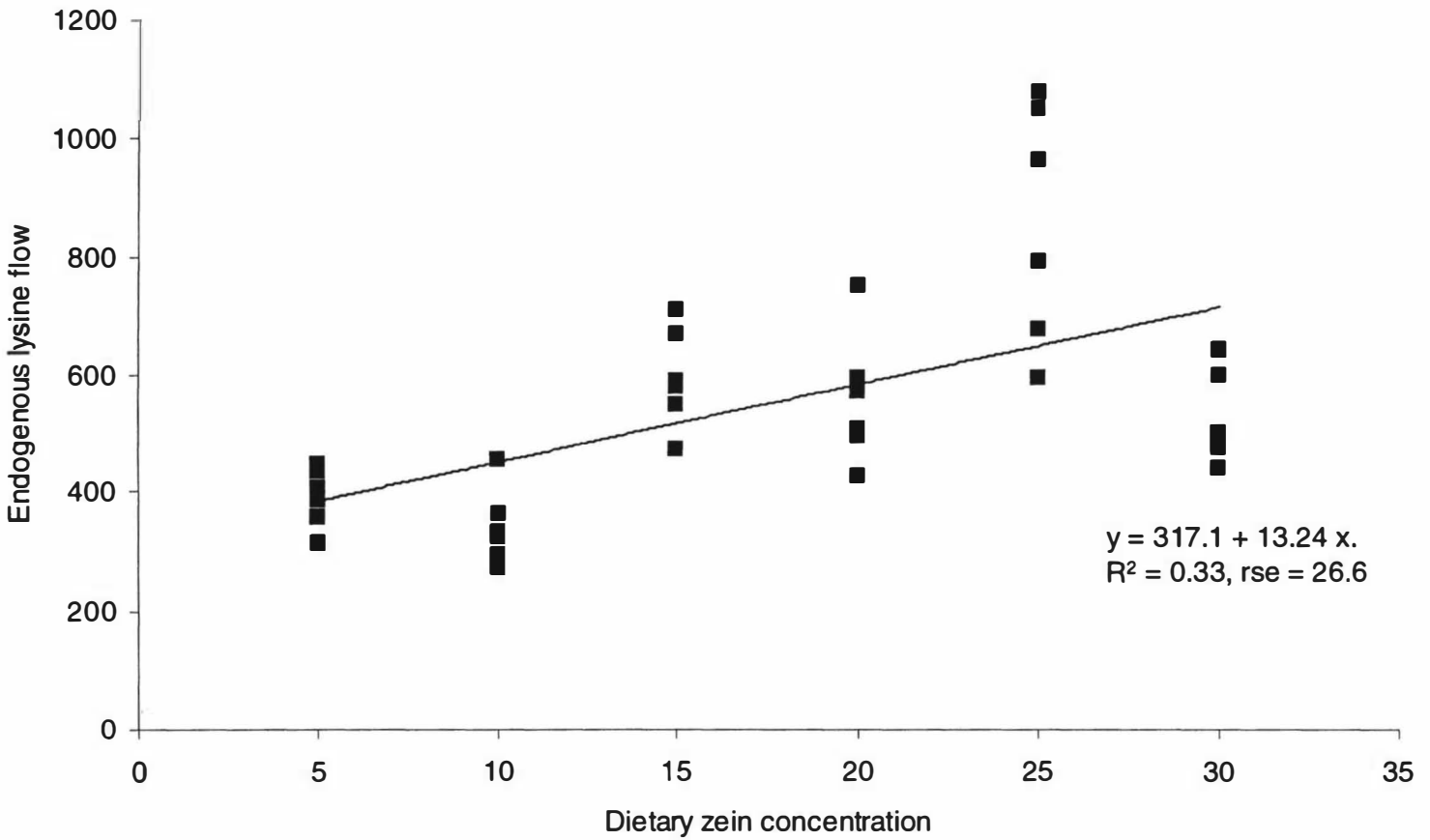

Figure 1 Endogenous ileal lysine flows (µg/g dry matter intake) in growing rats (n=6) receiving diets containing different amounts of zein (g zein/100g diet) as the sole nitrogen source.

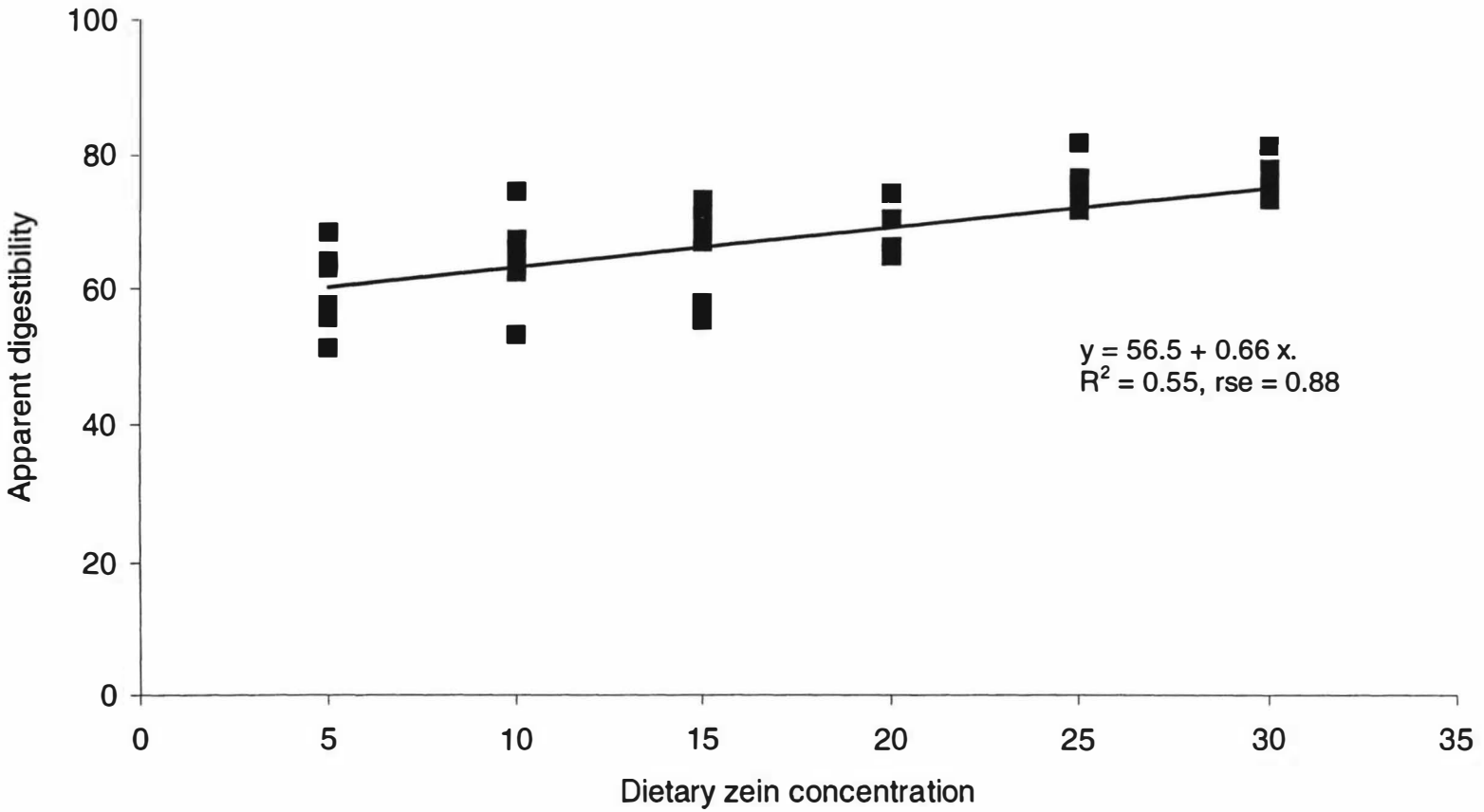

Figure 2 Apparent ileal digestibility (%) of nitrogen determined in the growing rat (n=6) receiving diets containing different amounts of zein (g zein/100 g diet) as the sole nitrogen source.

Table 6 Mean[1] apparent ileal amino acid and nitrogen digestibilities (%) in zein for the growing rat fed diets containing different amounts of zein.

	Dietary zein concentration (%)							
	5	10	15	20	25	30	SEM	P[2]
HIS	57.7	60.2	60.0	65.4	69.2	75.1	6.08	***
ARG	54.8	56.3	61.5	66.4	72.6	76.2	5.66	****
ASP	58.2	61.6	63.9	69.8	70.0	75.8	4.79	****
THR	54.7	56.7	60.3	67.5	68.5	76.0	5.26	****
SER	56.9	62.0	63.1	68.5	72.1	75.7	5.31	****
GLU	67.6	70.9	71.1	72.5	78.3	77.4	4.75	**
PRO	66.0	69.6	72.4	75.5	76.8	78.7	4.81	***
GLY	39.2	42.1	45.2	49.1	51.0	54.8	10.10	NS
ALA	68.7	69.1	71.3	72.1	79.1	78.3	4.97	**
VAL	63.5	65.6	66.6	70.9	74.6	78.6	4.72	****
MET	61.5	65.6	67.5	68.8	75.2	77.4	5.41	****
ILE	62.4	66.7	67.8	70.3	75.5	77.4	4.86	****
LEU	73.0	73.3	73.6	74.0	81.2	80.2	4.73	**
TYR	64.4	67.5	69.3	70.4	76.5	77.0	5.51	**
PHE	69.2	69.8	69.6	70.6	77.2	78.7	5.00	**
Nitrogen	60.0	64.2	65.5	67.5	74.9	76.3	5.32	****

[1] n = 6.

[2] NS not significant; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

DISCUSSION

The aim of the study reported here was to examine the effect of dietary protein concentration on endogenous ileal lysine flow. Growing rats were given access to diets that contained different concentrations of the protein zein, which is almost devoid of lysine and tryptophan, and the flow of lysine at the terminal ileum was determined. Because there was very little lysine and tryptophan in the diets, and these are dietary essential amino acids, the rats had to receive supplements of these amino acids to be in a positive body nitrogen balance. In the main study and until the final two days of the experimental period, the diets were supplemented with lysine and tryptophan. This was not appropriate for the final two days of the study, however, as the supplemental lysine may not have been completely absorbed. Therefore the rats were given lysine and tryptophan via intraperitoneal injection over the final two days of the study. Firstly,

however, it had to be confirmed that the rats were able to adequately metabolise lysine and tryptophan received via intraperitoneal injection at a rate to support a positive body nitrogen balance. In this case, a preliminary study was conducted and nitrogen balances were determined with rats receiving lysine and tryptophan via intraperitoneal injection.

In the preliminary trial, the rats that received the diet containing the lowest concentration of zein (5%) were in a slightly negative body nitrogen balance. The amount of body weight lost, expressed as a percentage of the average bodyweight while the rats were receiving the intraperitoneal injections, was 0.3% per day. During this period, the feed intake of the rats receiving the Z5 diet did not decrease compared to when the rats received the test diet supplemented with synthetic lysine and tryptophan, with the rats consuming an average of 10.5 g of feed per rat each day. The rats consuming the Z30 diet during the preliminary study were in a positive body nitrogen balance and grew at a rate of 2% per day (average gain in bodyweight expressed as a percentage of the average bodyweight of the rats while receiving the Z30 diet and intraperitoneal injections of lysine and tryptophan) while consuming an average of 10.8 g of feed per rat per day. These results demonstrate that the rats were able to metabolise lysine and tryptophan, when supplied via intraperitoneal injection at a rate required to support a positive body nitrogen balance.

Increasing the concentration of zein in the diet from 5 to 30% led to a significant ($P<0.0001$) increase in the endogenous flow of lysine at the terminal ileum (Table 5). This result is in agreement with that found by Hodgkinson *et al.* (1999a, refer Chapter 7 of this thesis) and the preliminary results presented by Butts *et al.* (1998). These earlier studies used the enzyme hydrolysed protein method to determine endogenous lysine flows. The enzyme hydrolysed protein method involves feeding the animal a diet where the sole source of nitrogen is a protein that has been hydrolysed so that all of the peptides have a molecular weight of < 5,000 Da. Ileal digesta are collected and then centrifuged and ultrafiltered (MW cut-off of 10,000 Da). The retentate (>10,000 Da fraction of digesta) from the ultrafiltration is added to the precipitate from the centrifugation step and this total fraction contains the endogenous material. This method, however, relies on several assumptions that have not been completely validated (Moughan *et al.*, 1998). Firstly, it is assumed that the peptides that enter the intestine when the hydrolysed protein is fed to the animals are of a similar size to those that would enter if the non-hydrolysed protein had been fed to the animals. If this is not so, and peptide size affects endogenous

protein flow, then the results that are obtained with the enzyme hydrolysed protein method may not be accurate. Secondly, it is assumed that there are only low amounts of endogenous peptides in ileal digesta that are smaller than 10,000 Da in size. The approach used in the present work does not rely upon these assumptions and is a more direct approach to determining endogenous lysine flow.

The use of zein, however, also relies on certain criteria being met. Firstly, it is assumed that lysine and tryptophan supplied to the rats via intraperitoneal injection, as opposed to oral ingestion with food, are absorbed by the animals and used for metabolism at a rate sufficient to support a positive body nitrogen balance. This assumption was confirmed by the results from the preliminary study. The second assumption that was made in the present study is that all of the lysine present in the digesta was of endogenous origin, as opposed to dietary origin. However, the zein used in the present work was found to contain a small amount of lysine (Table 1), which is in accord with the observations of Landry and Moureaux (1982), Schönhaus and Sgarbieri (1983), Butts *et al.* (1993a) and Sarwar, (1997). At least 60-80% of the dietary lysine would be expected to be absorbed before the terminal ileum (based on the determined apparent digestibility of nitrogen). It can be calculated that the presence of unabsorbed lysine of dietary origin may account for up to 20% of the increase in the endogenous ileal lysine flow predicted by the regression equation fitted to the data relating dietary zein intake and endogenous lysine flow. Therefore, the possible presence of some unabsorbed dietary lysine at the terminal ileum would not have explained all of the observed changes in endogenous lysine flow that occurred as the amount of zein in the diet was increased.

It should be noted that the mean feed intakes differed between the rats receiving the different experimental diets. Dry matter intake has been shown to be linearly related to endogenous ileal amino acid flow (Butts *et al.*, 1993b). Therefore, the endogenous ileal lysine flows were calculated on a dry matter intake basis, which would be expected to have accounted for the differences in food intake between the rats receiving the different diets.

As the amount of zein in the diet increased, the apparent ileal nitrogen digestibility and the apparent digestibilities of all of the amino acids except for glycine increased significantly ($P<0.01$). This increase in apparent nitrogen and amino acid

digestibility is in agreement with the work of Sauer *et al.* (1980), Furuya and Kaji (1989) and Bell *et al.* (1983).

Previous studies that have examined the effect of dietary protein concentration on true ileal nitrogen digestibility (Furuya and Kaji, 1989; Donkoh and Moughan, 1994) have used a constant value (per unit DMI) for the endogenous nitrogen and amino acid losses at each level of dietary protein intake, assuming that dietary protein concentration does not influence endogenous protein flow at the terminal ileum. When a constant value for endogenous nitrogen flow is used in the calculation of true ileal digestibility, the ratio of endogenous nitrogen flow to dietary intake of nitrogen and thus the proportion of the total ileal digesta nitrogen that is of endogenous origin, will decrease as the protein content of the diet is increased. If this decrease in the ratio is equal to the increase in apparent digestibility, the values for true digestibility will not change with increasing intake of dietary nitrogen, as has been reported to be the case (Furuya and Kaji, 1989; Donkoh and Moughan, 1995). However, the results of the present work negate the assumption that dietary protein intake has no effect on the flow of endogenous nitrogen and amino acids at the terminal ileum. When true ileal digestibility coefficients are calculated using the increasing endogenous nitrogen flow with increased dietary nitrogen intake, the ratio of endogenous nitrogen to dietary nitrogen (and therefore the proportion of the total nitrogen in ileal digesta that is of endogenous origin) will change with increasing dietary nitrogen intake. Therefore the true digestibility of nitrogen would be expected to change somewhat with increased dietary nitrogen intake. For the comparison of true nitrogen and amino acid digestibility coefficients among different protein sources, it appears that the effect of dietary protein concentration on endogenous ileal amino acid flow needs to be accounted for.

When diets containing a range of concentrations of zein were given to growing rats, there was a significant increase in endogenous ileal lysine flow with increasing dietary zein concentration indicating that there is an effect of dietary protein content on endogenous ileal lysine flow.

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CHAPTER 9

GENERAL DISCUSSION

Knowledge concerning the amount of nitrogen and amino acids of endogenous origin that flows through the terminal ileum of animals is necessary for several reasons. Firstly, it is necessary to allow for the determination of "true" ileal nitrogen and amino acid digestibility coefficients, which give an estimation (for unprocessed feedstuffs at least) of the bioavailability of amino acids present in the feedstuff. In this case, "bioavailability" is defined as the amount of an amino acid that is absorbed by the animal in a chemical form that can be utilised by the animal for body protein synthesis. It is also important to have knowledge of the amount of endogenous ileal nitrogen and amino acids for the factorial determination of an animal's requirement for nitrogen and amino acids.

Several approaches have been used to determine endogenous ileal nitrogen and amino acid flows in simple-stomached animals. The traditional approach involves measuring the amount of nitrogen and amino acids present in ileal digesta collected from an animal receiving (for seven to ten days) a diet that is devoid of protein. This approach has been criticised as being unphysiological (Low, 1980) as the animal is in a negative body nitrogen balance when digesta are collected, and it appears that the animal makes a metabolic adjustment to the protein-free diet. The result of this adjustment may be underestimation of determined endogenous ileal nitrogen and amino acid flows. The aim of the study described in Chapter 2 of this dissertation was to determine whether the duration of feeding of a protein-free diet to the pig had an effect on the determined endogenous ileal nitrogen and amino acid flows. It is possible that digesta could be collected from animals fed a protein-free diet for a shorter period of time than the standard seven to ten days, thus minimising or avoiding any effects of metabolic adjustment to the protein-free diet. This could result in more physiologically meaningful estimates of endogenous ileal nitrogen and amino acid flow. It is clear from the results given in Chapter 2 that endogenous ileal nitrogen and amino acid flows determined using digesta collected only 30 hours after the last protein-containing meal did not differ significantly ($P>0.05$) from those determined when an animal has received a protein-free diet for a period of eight days. This suggests that the metabolic adjustment to a protein-free diet may occur within 30 hours of receiving the last meal containing protein. Alternatively, the

metabolic adjustment may not be reflected in the endogenous ileal nitrogen and amino acid flows. Given that adjustment by the animals to a protein-free diet includes a decrease in the amount of protein secreted into the digestive tract (Snook and Meyer, 1964; Buraczewska, 1979; Schneeman, 1982) and may include an increased breakdown and reabsorption of enzymes secreted into the digestive tract (Snook and Meyer, 1964; Fauconneau and Michel, 1970), the latter possibility appears unlikely. If digesta were collected from animals less than 30 hours after receiving their last protein-containing meal, there could be no guarantee that all of the dietary protein had been absorbed before the terminal ileum. The protein-free approach, therefore, does not appear to be a suitable method for the determination of endogenous ileal nitrogen and amino acid flows in the simple-stomached animal.

The regression method for determining endogenous ileal nitrogen and amino acid flows involves giving the animal increasing amounts of dietary protein (at a constant dry matter intake), and mathematically extrapolating the ileal nitrogen and amino acid flows to a zero dietary protein intake, to determine the endogenous flows. This method relies on the assumption that there is no change in endogenous ileal nitrogen and amino acid flows with changes in dietary protein content, and this assertion has now been disproved (refer Chapters 7 and 8). Therefore, the regression method is not valid for the determination of endogenous ileal nitrogen and amino acid flows.

The feeding of natural proteins that are devoid of specific amino acids, the enzyme hydrolysed protein, isotope dilution and guanidination methods are all approaches that allow the determination of endogenous ileal protein flows while the test animal receives a diet that contains protein or peptides. Comparisons of the endogenous ileal nitrogen and/or amino acid flows determined using these methods with those determined following the feeding of a protein-free diet have demonstrated that endogenous ileal nitrogen and amino acid flows are greater when the animal is fed a diet containing protein or peptides (Darragh *et al.*, 1990; de Lange *et al.*, 1990; Moughan and Rutherfurd, 1990; Butts *et al.*, 1991; Moughan *et al.*, 1992; Butts *et al.*, 1993; Donkoh *et al.*, 1995; Hendriks *et al.*, 1996; Leterme *et al.*, 1996; Rutherfurd and Moughan, 1997) and this has been confirmed in the present work (see Chapter 7, Figure 1).

An important advantage of the enzyme hydrolysed protein method is that it allows a determination to be made of the endogenous ileal flows of nitrogen as well as all of

the amino acids, as opposed to the isotope dilution and guanidination methods and feeding of natural proteins that are devoid of specific amino acids which only allow nitrogen or specific amino acids to be determined. With the latter three methods, endogenous flows of nitrogen and/or amino acids can be calculated by assuming that the nitrogen and amino acid composition of endogenous proteinaceous excretions from the terminal ileum remains constant. This assumption has not been fully tested. Boisen and Moughan (1996) reviewed the amino acid composition of endogenous ileal protein excretions and concluded that the composition can vary considerably although it was relatively constant in most of the studies examined.

The enzyme hydrolysed protein method involves giving animals a diet containing an enzyme hydrolysed protein (usually enzyme hydrolysed casein, EHC) as the sole nitrogen source. EHC contains free amino acids and peptides that are less than 5,000 Da in size. Digesta are collected from the animal and centrifuged before being ultrafiltered, to remove any unabsorbed dietary amino acids and peptides. The precipitate from the centrifugation is combined with the retentate from the ultrafiltration, and this material contains the endogenous fraction. The molecular weight (MW) cut-off used for ultrafiltration is normally 10,000 Da. However, the use of an ultrafiltration cut-off of 10,000 Da will result in underestimation of endogenous nitrogen and amino acid flows, due to the known presence of endogenous free amino acids and peptides that are less than 10,000 Da in the ultrafiltrate that is discarded. The endogenous ileal amino acid flows determined when an ultrafiltration MW cut-off of 3,000 Da was used were increased by approximately 12% compared with those determined using a MW cut-off of 10,000 Da (refer Chapter 5, Table 2). The use of 3,000 Da as a MW cut-off for ultrafiltration with the enzyme hydrolysed protein method may increase the accuracy of this method. However, a MW cut-off of 3,000 Da can only be used when the peptides in the dietary enzyme hydrolysed protein are less than 3,000 Da in size. The possibility also exists that even with an ultrafiltration cut-off of 3,000 Da, endogenous ileal amino acid flows may be underestimated, due to the presence in the ultrafiltrate of endogenous free amino acids and peptides that are less than 3,000 Da. Ultrafiltration devices with a MW cut-off of 1,000 Da are also available commercially. These devices could, however, only be used with the enzyme hydrolysed protein method if all of the dietary peptides were less than 1,000 Da. Of the peptides present in the EHC used in the work described in this thesis, 12.1% were greater than 1,000 Da in size and there would be no surety that all of these peptides would be digested such that they were less than 1,000 Da at the terminal ileum.

Therefore, for the valid use of an ultrafiltration device with a MW cut-off of 1,000 Da, further hydrolysis of the dietary casein would be required. It should be noted that the effect of peptide size and thus the extent of hydrolysis of the dietary protein on endogenous ileal nitrogen and amino acid flows is unknown, and the extent of hydrolysis of the dietary casein may influence endogenous ileal nitrogen and amino acid flows. The effect of peptide size on endogenous ileal nitrogen and amino acid flows requires thorough investigation.

A further aspect of the enzyme hydrolysed protein method that was examined in this study was whether amino acids and peptides present in EHC bind to the precipitate plus retentate fraction of digesta and remain bound throughout the ultrafiltration step (refer Chapter 4). The results demonstrated that the ultrafiltration devices used in this work were not completely effective, and did not remove all EHC from the precipitate plus retentate fraction of digesta. Except possibly in the case of the amino acids proline and threonine, this incomplete separation of the EHC from the precipitate plus retentate fraction of digesta did not appear to be due to binding between amino acids present in the EHC and the precipitate plus retentate fraction of digesta. From the results presented in Chapter 4, it appears that endogenous ileal nitrogen flows are overestimated by approximately 2% due to incomplete ultrafiltration of the digesta, leading to the presence of some nitrogen from the EHC in the precipitate plus retentate fraction of digesta. This overestimation of endogenous ileal nitrogen flow due to incomplete separation of EHC from digesta would be expected to at least partially offset the underestimation of endogenous ileal flows that may occur when digesta are ultrafiltered using a MW cut-off of 3,000 or 10,000 Da, due to the presence of endogenous nitrogen in the ultrafiltrate (<3,000 or <10,000 Da fraction of digesta).

Despite the widespread use of the enzyme hydrolysed protein, isotope dilution and guanidination methods for the determination of endogenous ileal protein flows, no controlled experimental comparison has been previously carried out to determine whether the three methods give similar estimates of endogenous ileal nitrogen and/or lysine flows. Such comparisons were conducted in both the pig and the rat in the study reported in Chapter 3 of this work. The endogenous ileal lysine flows determined in both the rat and pig using the guanidination method were significantly lower than those determined using the enzyme hydrolysed protein method (Chapter 3, Table 3). In the rat, the endogenous ileal nitrogen flows determined using the isotope dilution method were significantly lower than those determined using the

enzyme hydrolysed protein method. In the pig, the endogenous ileal nitrogen flows determined using the isotope dilution method did not differ significantly ($P>0.05$) from those determined using the enzyme hydrolysed protein method, although the mean endogenous ileal nitrogen flow determined using the isotope dilution method was lower than that determined using the enzyme hydrolysed protein method. Given that the enzyme hydrolysed protein method is known to somewhat underestimate endogenous ileal nitrogen and lysine flows, especially when a MW cut-off of 10,000 Da is used for the ultrafiltration of digesta, as was the case in the study reported in Chapter 3, these results suggest that the isotope dilution and guanidination methods also underestimate endogenous ileal nitrogen and lysine flows, respectively, to a greater extent than the enzyme hydrolysed protein method. Therefore, the isotope dilution and guanidination methods were not used in further studies reported here. The reason why the guanidination method underestimates endogenous lysine flows is not clear. The isotope dilution method most likely underestimates endogenous ileal nitrogen flows due to recycling of the labelled amino acids. Dietary amino acids, which are labelled with ^{15}N, may be absorbed from the intestine and resecreted. The resecreted amino acids are, by definition, endogenous, but are labelled with ^{15}N so that during the chemical analysis they will be analysed as dietary unabsorbed amino acids. The extent of recycling and thus the underestimation of endogenous nitrogen flows with the isotope dilution method may be decreased by the use of a shorter time period between feeding the ^{15}N-labelled diet and the collection of ileal digesta.

The enzyme hydrolysed protein method was used to examine the diurnal pattern of endogenous ileal nitrogen flows in the growing pig (Chapter 6). This work was completed as the first step towards studying the mechanisms whereby dietary factors, such as the presence of peptides in the diet, influence the amount of endogenous ileal nitrogen and amino acids. If these mechanisms are determined, they may be able to be manipulated to result in a reduction of endogenous amino acid loss from the animal. This would be expected to result in the presence of less nitrogen in the faeces, which is important environmentally. Manipulation of these mechanisms may also afford an increase in production efficiency. A decrease in the loss of endogenous protein may also be of benefit to patients with renal diseases. A low protein diet can slow down the progression of chronic renal failure (Fouque *et al.*, 1992; Steinman, 1996; Soroka *et al.*, 1998; Jovanovic and Djukanovic, 1999) and diabetic nephropathy (Zeller *et al.*, 1991). In children especially, however, this low protein diet may lead to malnutrition and have negative effects on growth

(Coppo *et al.*, 1998; Zadik *et al.*, 1998). If it is possible to decrease the amount of endogenous protein lost from the intestines, this may increase metabolic efficiency, allowing the consumption of a diet containing low levels of protein, while minimising the negative effects of the low protein diet on nutrition and growth.

The study of the diurnal pattern of endogenous ileal nitrogen flows made use of the post-valve T-caecum (PVTC) cannula for the collection of ileal digesta. It should be noted that the recovery of the marker, chromic oxide, averaged only 72%. This indicates that a complete collection of digesta was not accomplished in this study, although this marker recovery is in line with recoveries reported in other studies that have used the PVTC cannula for the collection of ileal digesta (den Hartog *et al.*, 1988; Köhler *et al.*, 1990; Köhler *et al.*, 1991). Therefore it appears that the use of the PVTC cannula for the collection of ileal digesta does not always result in a complete quantitative collection of ileal digesta. As there was little leakage of digesta around the cannula during the digesta collection periods, the remaining digesta that did not enter the cannula are assumed to have by-passed the cannula and entered the large intestine. Steered ileo-caecal valve (SICV) cannulation has recently been described as a method that may allow a more complete collection of ileal digesta (Mroz *et al.*, 1994; Mroz *et al.*, 1996). This method involves the surgical insertion of a cannula into the caecum, opposite the ileo-caecal valve. Two rings are placed, with one inside and one outside the terminal ileum, and a cord is attached to the inner ring. When digesta collection is to commence, the cord is gently pulled to move the ileo-caecal valve into the mouth of the cannula. Digesta can then drain through the cannula. The presence of rings in and around the terminal ileum with SIVC cannulation could affect the motility of the intestine, whereas with PVTC cannulation there is no surgical interference with the terminal ileum. PVTC cannulation requires the removal of most of the caecum, however, and the metabolic effects of this removal are not clear. As well as an expected more complete collection of digesta with SIVC cannulation, an advantage of this method over PVTC cannulation is that the caecum is not removed.

The major finding of the study of the diurnal pattern of endogenous ileal nitrogen flow (Chapter 5) was that the ratio of endogenous nitrogen to chromium in digesta collected from the terminal ileum of the pig was relatively constant, with no significant differences ($P>0.05$) in the ratio of endogenous nitrogen to chromium for digesta collected from 1300 to 0800h. This suggests that in the meal-fed animal, the net outcome of endogenous protein secretion and reabsorption up to the

terminal ileum may be relatively constant over time. The net outcome of endogenous protein secretion and reabsorption up to the terminal ileum may also be proportionally related to dry matter intake. It is not known, however, what proportions of the endogenous amino acids at the terminal ileum originated from each of the sources of endogenous secretion, that is, what proportions originated from digestive enzymes, bile acids, mucus, cells sloughed from the gut walls and serum albumin.

The next step towards determining the mechanisms by which dietary peptides influence the amount of endogenous nitrogen and amino acids that flow through the terminal ileum would be to examine the origin of the endogenous nitrogen and amino acids that remain unabsorbed at the terminal ileum while animals receive a peptide-containing diet and to compare the results with those obtained when a protein-free diet is given to the animals. Mucus has been suggested to be a major source of endogenous ileal amino acids (Holmes *et al.*, 1974; Tavemer, 1979; Fuller, 1991; Lien *et al.*, 1997). Given that proteolytic enzymes such as pepsin, chymotrypsin, trypsin and papain are not very effective at degrading mucus (Hashimoto *et al.*, 1963) and that both mucins (Pigman *et al.*, 1963; Horowitz, 1967; Lien *et al.*, 1997) and endogenous ileal amino acid excretions (Chapter 6 Table 3) contain relatively high proportions of the amino acids serine, threonine, glycine and proline, this suggestion appears reasonable. Lien *et al.* (1997), however, determined the amount of mucin present in ileal digesta collected from pigs fed a protein-free diet and concluded that mucin represented a relatively small proportion (5-10%) of the total endogenous ileal amino acids, although the contributions from mucin of threonine, serine and proline to endogenous ileal flows were markedly higher. The study by Lien *et al.* (1997) does rely on some assumptions that have not been confirmed. For example, it is assumed that the concentration of the carbohydrates that were measured in this work do not differ between soluble mucus (which was determined) and insoluble mucus. If a difference does exist, the results of this study may not be correct. The quantitative contribution of mucins to endogenous ileal nitrogen and amino acid flows when animals are fed a protein or peptide-containing diet is not known. There is also evidence to suggest that bacteria may be significant contributors to endogenous ileal nitrogen and amino acid flows (Caine *et al.*, 1999). Although bacteria are not, strictly speaking, of endogenous origin, they are analysed as part of the endogenous (non-diet) contribution to the amino acids present in digesta at the terminal ileum. A comparison of the proportions of endogenous amino acids deriving from the various

endogenous sources when an animal receives a peptide-containing diet as compared with those found when an animal receives a protein-free diet, would provide valuable information.

The aim of the studies reported in Chapters 7 and 8 of this thesis was to examine whether there is an effect of the concentration of peptides and protein in the diet on endogenous ileal protein flows. Chapter 7 involved the use of the enzyme hydrolysed protein method to determine endogenous ileal nitrogen and amino acid flows when diets containing 0, 5, 10 and 20% EHC were fed to growing pigs. Endogenous ileal nitrogen and amino acid flows were shown to significantly ($P<0.05$) increase with increasing concentration of EHC in the diet (Chapter 7, Table 3). In Chapter 8, rats received diets containing 5, 10, 15, 20, 25 and 30% zein. Zein is almost devoid of lysine, thus it was possible to directly determine the endogenous flow of lysine through the terminal ileum in rats that had received the zein-based diets. The latter study confirmed that endogenous protein flows are positively influenced by the concentration of peptides and protein in the diet.

The linear regression relating endogenous lysine flow to dietary EHC concentration in the pig (Figure 1) was: endogenous lysine flow (μg/g DMI) = 228 + 16 x dietary concentration of EHC (%) (refer Chapter 7, Table 4). For the rats receiving a zein-based diet (see Chapter 8), a linear regression was fitted to the data pertaining to the diets containing from 5 to 20% zein to allow comparison with the results obtained after pigs had received diets containing 5 to 20% EHC (Chapter 6). The resultant regression equation was: endogenous lysine flow (μg/g DMI) = 282 + 15 x dietary concentration of zein (%). Even though endogenous ileal lysine flow was determined using two distinct methods, the slopes of the equations relating endogenous lysine flow and dietary concentration of zein and EHC were very similar, being (slope $\pm$ SEM) 16 ($\pm$ 5) and 15 ($\pm$ 4). Extrapolation of the regression lines to a zero protein intake gives endogenous lysine flows of 228 and 282 μg/g DMI. This is close to the value of (mean $\pm$ SEM) 298 $\pm$ 43 for endogenous ileal lysine flow determined when a protein-free diet was fed to growing pigs (Chapter 7 Figure 1). These values provide a base estimate of the endogenous ileal flow of lysine, that is, the flow that will occur in the absence of the stimulatory effects of dietary protein or peptides.

The studies reported in this thesis (Chapters 7 and 8) provide strong and important new evidence that the concentration of peptides and protein in the diet has a

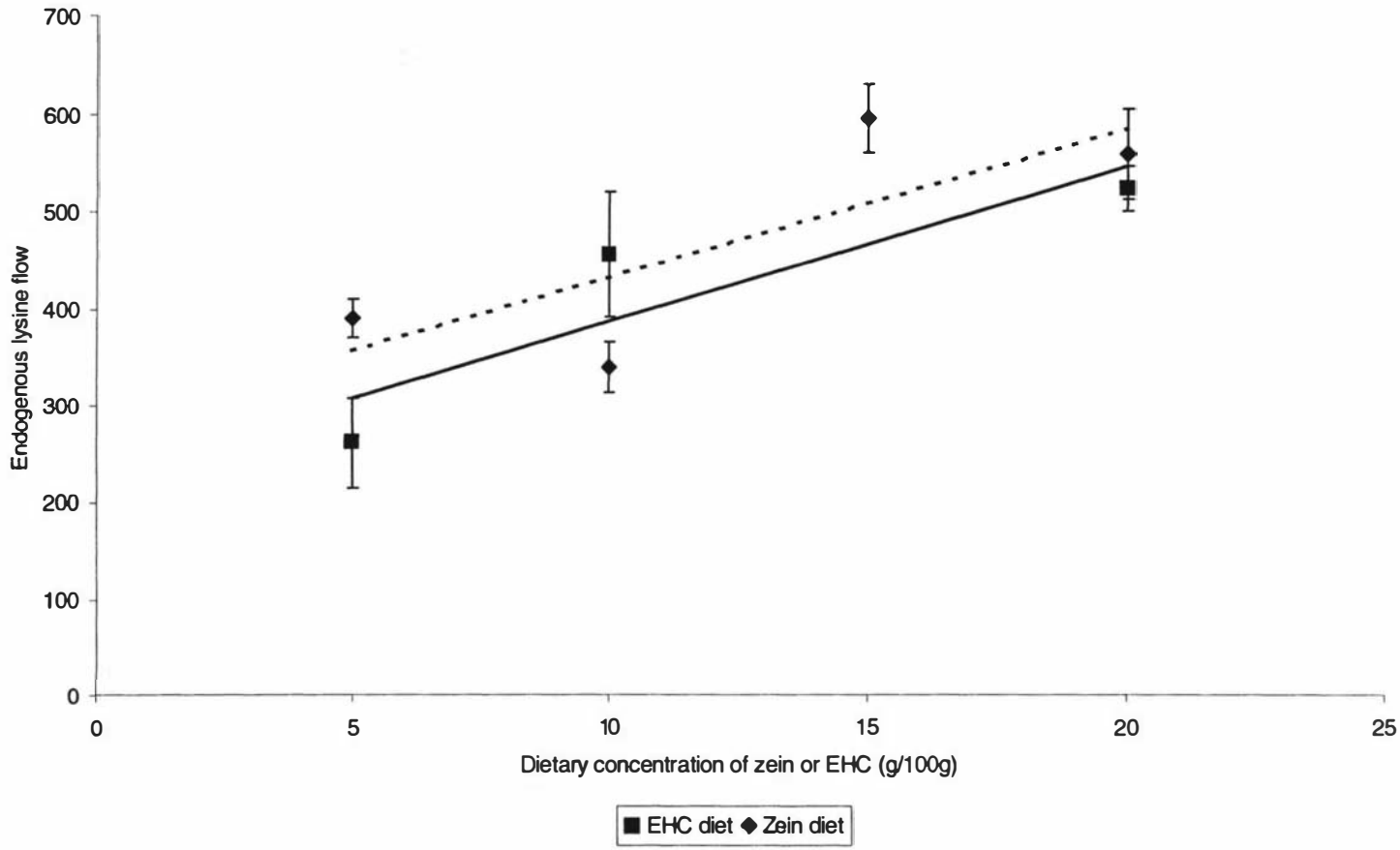

Figure 1 Endogenous ileal lysine flows (μg/g dry matter intake) in growing rats (n=6) receiving diets containing different amounts of zein, and growing pigs (n=7) receiving diets containing different amounts of enzyme hydrolysed casein (EHC). A linear model is fitted to the EHC data (—) and the zein data (- - ·).

positive effect on the flows of endogenous nitrogen and amino acids through the terminal ileum. One of the main practical reasons that it is important to have information on the flow of endogenous protein through the terminal ileum is for the generation of true ileal digestibility coefficients. Previous studies that have examined the effect of dietary protein concentration on true digestibility coefficients have assumed that endogenous ileal nitrogen and amino acid flows do not change with changes in the amount of protein in the diet (Furuya and Kaji, 1989; Donkoh and Moughan, 1994). This assumption has been negated in Chapters 7 and 8, and true ileal digestibility coefficients would be expected to change with changes in the concentration of protein in the diet. This effect of dietary protein concentration on true ileal digestibility coefficients needs to be accounted for when using true ileal digestibility coefficients for comparison of the protein quality of different protein sources.

The components of the endogenous protein flow that may change with changes in the amount of dietary protein or peptides are not known. The endogenous amino acids that flowed through the terminal ileum while pigs received a diet containing 20% EHC were abundant in the amino acids serine, threonine, glycine and proline (Chapter 7 Table 3). These amino acids are also abundant in mucus. Therefore at the terminal ileum, the amount of amino acids that originated from mucus may increase with increasing amounts of dietary protein/peptides. The increase in mucus may be related to the increase in the amount of digestive enzymes such as pancreatic enzymes that occurs with an increase in the amount of protein in the diet (Corring *et al.*, 1989). As well as potentially adding directly to the endogenous flow of amino acids, the digestive enzymes may partially degrade mucins lining the walls of the intestines, resulting in the loss of more mucus.

As the amount of protein/peptides in the diet increases, there will be greater amounts of amino acids flowing through the terminal ileum. The greater amounts of amino acids may support an increase in the number of bacteria present in the small intestine resulting in an increase in the amount of amino acid originating from bacteria flowing through the terminal ileum.

The protein-free diet and regression approaches appear to be invalid for the determination of endogenous nitrogen and amino acid flows through the terminal ileum of simple-stomached animals. The isotope dilution and guanidination methods appear to underestimate endogenous ileal nitrogen and amino acid flows.

The enzyme hydrolysed protein method may allow a valid direct determination of endogenous nitrogen and amino acid flows. Lowering the MW cut-off for ultrafiltration may increase the accuracy of the endogenous ileal nitrogen and amino acid flows determined with the enzyme hydrolysed protein method, but necessitates the use of smaller dietary peptides. The range in size of the peptides that stimulate endogenous flows when an animal receives a protein-containing diet is not known.

A significant new finding of this work is that the concentration of protein and peptides in the diet has a major influence on the flow of endogenous nitrogen and amino acids through the terminal ileum of simple-stomached mammals. It now remains to elucidate the mechanisms that control this dietary stimulated increase in endogenous flow.

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