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Gut endogenous protein flows and postprandial  
metabolic utilization of dietary amino acids in  
simple-stomached animals and humans

A thesis presented in partial fulfilment of the requirements for the degree of  
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## ABSTRACT

Dietary protein quality depends on two key measures: true ileal protein digestibility and the metabolic utilization of absorbed amino acids (AA). The objectives of this study were to determine the influence of two dietary factors (antinutritional factors and peptides) on ileal endogenous protein flows; to validate the intubation technique used in humans for ileal digesta sampling; to determine the postprandial metabolic utilization of dietary AA depending on their delivery form and to assess the validity of the growing pig for predicting true ileal protein digestibility in the adult human. Investigations were undertaken in the growing rat, growing pig and adult human. Ileal digesta were collected from euthanised rats, post valve T-caecum cannulated pigs, and naso-ileal intubated conscious adult humans. Ileal endogenous nitrogen (N) and AA were measured using a protein-free (PF) diet, diets containing  $^{15}\text{N}$ -labelled casein in the intact (C) or hydrolysed (HC) form, or a diet based on free AA (diet A), for which some dispensable AA were omitted to allow a direct determination of their endogenous flows. Digesta centrifugation and ultrafiltration (diet HC) allowed for the determination of ileal endogenous protein flows and the extent of tracer ( $^{15}\text{N}$ ) recycling.

Antinutritional factors from a crude extract of kidney beans (*Phaseolus vulgaris*), when given at amounts commonly ingested in practice, enhanced ileal endogenous protein flows (rats, PF diet). After adaptation to the diet, body N balance *per se* did not influence ileal endogenous protein flows (rats, diets PF and A) but dietary peptides led to greater ileal endogenous AA and N flows compared with a protein-free diet. Dietary peptides (HC), compared with peptides naturally released in the gut during protein digestion (C), did not enhance ileal endogenous protein flows (rats, pigs, and humans). The extent of tracer recycling, however, was maximal in frequently-fed rats, lower in meal-fed pigs and minimal in meal-fed humans (65, 21, and 11% of  $^{15}\text{N}$ -labelled ileal endogenous proteins, respectively). Naso-ileal intubation for ileal digesta sampling in humans was shown to be an accurate method and evidence was obtained supporting the growing pig as a valid model for predicting true ileal protein digestibility in the adult human. Finally, the form of delivery of dietary AA (from HC or C) influenced the postprandial metabolic fate of dietary AA, especially in terms of AA catabolism kinetics. However, the overall nutritional value of C and HC were similar.

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# Preface

Proteins are essential components of the body where they play both functional and structural roles, being major cellular structural elements, biochemical catalysts and also important regulators of gene expression. Discussion of protein and amino acid nutrition thus involves virtually every element of mammalian biochemistry and physiology (Reeds & Beckett, 1997). Proteins were first described in 1838 by the Swedish chemist Jöns Jakob Berzelius who named them from the Greek word *πρώτα* or "prota", meaning of primary importance. The central role of proteins in living organisms was appreciated only one century later, in 1926, with James B. Sumner showing that the enzyme urease was a protein (Sumner, 1926). Proteins are macromolecules composed of a linear chain of amino acids (AA) that folds in a variety of ways to form a unique 3-dimensional structure as first observed in the 1960s (Kendrew *et al.*, 1958; Muirhead & Perutz, 1963). Twenty amino acids are involved in protein synthesis. Some of these AA (dispensable AA) can be synthesized by the body from other free AA (by transamination or reductive amination), glucose or ammonia (using the Krebs cycle) whereas others (indispensable AA) have to be imported from an exogenous (dietary) source (Eastwood, 2003a). Body proteins are continually broken down and replaced, with an approximate rate of 3–6 g of protein/kg body weight/day for an adult human, resulting in no apparent change in total body protein content (Bender & Millward, 2005). This is a dynamic equilibrium that can be maintained by replacing losses of body protein by an intake of dietary protein. Not only the quantity but also the quality of dietary proteins matter, hence the importance of dietary protein quality evaluation.

The nutritional quality of dietary protein depends on protein bioavailability and metabolic utilization. Protein bioavailability can be estimated in most cases by true protein digestibility determined after correction for intestinal endogenous protein losses, which are more accurate when measured at the ileal level, *i.e.* before being subjected to the prolific microbial metabolism occurring in the colon. Dietary factors can modulate ileal endogenous protein losses; and this has implications for the methodology used for the measurement of ileal endogenous protein losses. It is thus important to gain further knowledge in this area, as this was the overall objective of the work presented here. Dietary factors can also influence protein metabolic utilization, which, in turn, may modulate the overall nutritional value of dietary protein. Further investigations in this direction are urgently required.

# CHAPTER I

## Review of the literature

## Introduction

Nutrition research has focused on the capacity of food to meet the metabolic demand for nitrogen (N) and indispensable amino acids (IAA) (FAO/WHO/UNU, 2007). The evaluation of protein quality aims to determine how well dietary protein is digested and absorbed and how well absorbed amino acids (AA) are utilized by the body. The protein digestibility-corrected AA score (PDCAAS) is the current recommended protein quality index (FAO/WHO/UNU, 2007) despite some important limitations. This index takes into account the digestibility of dietary protein and its AA content relative to the requirement pattern (FAO/WHO/UNU, 2007). To date, a level of consensus within the scientific community has been reached for the protein and IAA requirements of man (FAO/WHO/UNU, 2007), although further re-evaluation can still be expected. In addition, whereas the measurement of AA content in dietary protein has significantly improved over the last decades, the accurate determination of digestibility is still critical. Protein digestibility is a determining factor for protein bioavailability (Fuller & Tomé, 2005; FAO/WHO/UNU, 2007), as it indicates the extent of intestinal digestion and absorption of food protein as AA that can subsequently be utilized by the body. Protein digestibility has been reported to differ substantially among diets, in particular between those from developing and developed countries (Gilani *et al.*, 2005). Protein digestibility is indirectly determined from the amount of N flowing at the end of the digestive tract. Gut N flows arise both from undigested dietary N and from non-dietary (endogenous) nitrogenous compounds that have escaped digestion and/or (re)absorption, and are referred to as N losses when determined at the end of the digestive tract. Endogenous N losses account for a substantial proportion of total N losses and thus need to be distinguished from undigested dietary N to correct apparent digestibility coefficients (derived from total N losses) to true digestibility coefficients. True digestibility represents the specific fate of dietary N in the digestive tract (Fuller & Tomé, 2005). Measurement of digestibility at the ileal level is now recognized as being more accurate than measurement of digestibility at the faecal level (Darragh & Hodgkinson, 2000; Moughan, 2003; Fuller & Tomé, 2005), especially for individual AA for which the profile can be modified by the prolific microbial metabolism in the hindgut. In addition, most dietary AA are absorbed in the small intestine (Krawielitzki *et al.*, 1990; Fuller & Reeds, 1998). It remains unclear if the colon can absorb AA to a nutritionally significant extent (Blachier *et al.*, 2007).



After their intestinal absorption, dietary AA are involved in biosynthetic reactions including synthesis of protein or of other nitrogenous compounds or are subject to oxidative deamination. The balance between catabolic and anabolic pathways determines the overall dietary N retention, which can be modulated by dietary factors, especially dietary protein quality. It is thus important to improve our understanding of the dietary modulations of postprandial protein metabolism, which in turn influence net body N retention.

In this review, firstly, the concept of protein quality evaluation is discussed. The different sources of N flowing along the gastrointestinal tract are then examined, the methods available to distinguish endogenous N and dietary N in ileal digesta are discussed and the dietary factors influencing ileal endogenous N losses are addressed. Finally, the postprandial metabolic fate of dietary N and its modulation by dietary factors are explored.

## **I. Evaluation of dietary protein quality in adult humans**

Evaluation of protein quality aims to assess the contribution of dietary protein in satisfying the metabolic requirement for N and AA (FAO/WHO/UNU, 2007). Both the estimation of protein requirements and aspects of quality evaluation still include unresolved issues, which are raised in this section.

### **1. Dietary protein and amino acid requirements**

#### **1.1. Concept of requirement**

Protein requirement has historically been difficult to estimate, thus generating wide debate (Young *et al.*, 1989; Fuller & Garlick, 1994; Millward, 2001; Reeds, 2001; Millward & Jackson, 2004). The word requirement, may, in itself, be confusing as it can refer to: (1) metabolic requirement, *i.e.* biological demand for the quantity of N and IAA consumed in various metabolic pathways; (2) dietary requirement (or estimated average requirement) for the minimum amount of dietary N and IAA that satisfies the biological demand for N and IAA at the individual level; (3) safe level of intake at the population level (recommended daily allowance or dietary allowance), which takes into account individual variations in requirement [dietary requirement + 2 standard deviations (SD) of

intake] and represents the upper range (97.5<sup>th</sup> centile) of distribution of the requirements (Reeds & Beckett, 1997; FAO/WHO/UNU, 2007). Metabolic requirement includes needs for maintenance of body protein equilibrium, which represent the largest part of the requirement in the adult human, and needs for growth (protein deposition), plus extra needs for reproduction or lactation. Whereas the growth requirement can be defined from the AA composition of newly deposited proteins and the efficiency with which bioavailable AA support protein deposition (Reeds & Garlick, 2003), the maintenance requirement is more complex to assess. The N balance approach, which is the historically used, but not completely satisfactory, method to determine protein requirement, is now complemented by isotopic techniques that provide new insights to the determination of IAA requirement.

The difficulty in determining protein and AA requirements lies in the numerous influencing factors arising from the individual's biology (genotype, reproductive/development stage), his environment (*e.g.* injury, infection), his lifestyle (especially level of physical activity) and his nutrition (especially energy intake).

An unresolved and dividing issue is the adaptation mechanisms of body protein metabolism to low protein intake (Nicol & Phillips, 1976; Waterlow, 1990; Hegsted, 2000; Millward, 2003). Authors such as Millward (2003) and Millward & Jackson (2004) strongly argue that there is a long-term and slow adaptive behaviour of AA oxidation, such as illustrated by previous studies in which from 7 to 40 days are required to achieve N balance after variations in the protein intake level. Thus, the adaptation factor should be included in the definition of protein and AA requirements (Millward & Jackson, 2004). Conversely, authors such as Young & Borgonha (2000) and Rand *et al.* (2003) consider that metabolic adaptation is not an important issue, as studies have reported that an adaptation period of 4–5 days (usually used in N balance studies) is sufficient for the body to reach a relatively steady-state of N balance (Rand *et al.*, 1976; Young & Borgonha, 2000). This is the current position of the international authorities (FAO/WHO/UNU, 2007). Additionally, there are scarce data regarding the influence of high-protein diets, which might modulate the body's ability to recycle endogenous AA, thus modifying the pattern of AA required (Bos *et al.*, 2002). All these factors contribute to the complexity of protein and AA requirement evaluation, as emphasized by the long time period (22 years) required to update the earlier international protein and AA requirements (FAO/WHO/UNU, 1985), which, in turn, were based on studies undertaken in the 1960s.

## 1.2. Dietary protein requirement

Although the N balance approach includes a number of shortcomings, especially a lack of precision and accuracy (Young *et al.*, 1989), this approach is still the only valid method available for N requirement evaluation (FAO/WHO/UNU, 2007). The meta-analysis performed by Rand *et al.* (2003) on 19 N balance studies served for the current protein requirement estimate (FAO/WHO/UNU, 2007). Briefly, an N balance study consists in regressing the body N balance on intake to predict the protein intake (dietary requirement) that would produce a zero N balance. This is derived from the intercept (obligatory N losses, 48 mg/kg/d) and the slope (efficiency of dietary protein utilization, 0.47) of the linear regression obtained (Rand *et al.*, 2003). The determination of the total obligatory N losses, determined after feeding the subjects with a low-protein diet for approximately 5–6 days, is critical. Whereas urinary and faecal losses can be directly measured, dermal and miscellaneous losses (saliva, hair, nails, breath, nasal mucous, semen) are usually estimated and might vary among studies. Rand *et al.* (2003) estimated that the dermal and miscellaneous losses were 4.8 and 11 mg N/kg/d for temperate and tropical regions, respectively. As a result, the current dietary protein requirement and allowance have been established at 0.66 and 0.83 g good-quality protein/kg/d, respectively, for healthy adult humans. These values are 10% higher than those determined earlier (FAO/WHO/UNU, 1985). It should be noted that these values are also valid for the elderly, as no consensus has yet been reached regarding the possible need for a higher intake (FAO/WHO/UNU, 2007), despite several reviews indicating the necessity to define higher safe protein intakes for elderly subjects (Campbell & Evans, 1996; Kurpad & Vaz, 2000). Children, as well as lactating or pregnant women, have a higher requirement on a body weight (BW) basis, but this is not discussed here.

The criterion on which the N requirement definition is based (achievement of N equilibrium or zero balance) has been questioned, as, although it is assumed to reflect health maintenance, it might not necessarily reflect the optimal conditions of specific physiological functions such as immune function, bone health or muscle mass (Millward, 1999; Wolfe, 2002; Millward, 2003; New & Millward, 2003). However, to date, there is no quantifiable indicator of the dietary N requirement for achieving such goals (FAO/WHO/UNU, 2007).

In summary, the current dietary protein requirement has to be considered within the limited context of the N balance approach and has thus been defined recently as “the

lowest level of dietary protein intake that will balance the losses of N from the body, and thus maintain the body protein mass, in persons at energy balance with modest levels of physical activity, plus, in children or in pregnant or lactating women, the needs associated with the deposition of tissues or the secretion of milk at rates consistent with good health” (FAO/WHO/UNU, 2007).

### 1.3. Indispensable amino acid requirements

There is an absolute metabolic need for every AA, with the difference that some can be synthesized by the body (dispensable AA), whereas others cannot (indispensable AA). The AA categorization is complex as some dispensable AA can become conditionally indispensable under specific pathological or physiological conditions (**Table 1**). Also, the rate of formation of dispensable AA in the body appears to be determined by the total intake of N. Thus, the requirement for IAA is not an absolute value, but should be expressed in relation to the total N intake (FAO/WHO/UNU, 2007).

**Table 1.** Amino acid classification. From Reeds *et al.* (2000b).

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**Indispensable amino acids**

*(nutritionally indispensable under all conditions)*

Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine

**Conditionally indispensable amino acids**

*(indispensable under specific pathological or physiological conditions)*

Cystine, Tyrosine, Taurine, Glycine, Arginine, Glutamine, Proline

**Dispensable amino acids**

Aspartic acid, Asparagine, Glutamic acid, Alanine, Serine

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Stable isotopic methods have enabled a dynamic approach to the estimation of IAA requirements. Two main approaches have been developed. Firstly, the “indicator AA balance”, or “carbon balance” method, consists of feeding a subject graded levels of the AA of interest (<sup>13</sup>C-labelled) over a 6-day adaptation period and determining dietary requirement of the AA of interest in terms of the intake allowing for a zero carbon balance (intake - oxidation) of this <sup>13</sup>C-amino acid (Young *et al.*, 1989). Secondly, the “indicator AA oxidation” consists in feeding a subject with graded levels of the AA tested without adaptation and determining the dietary requirement of this AA from the minimum level of oxidation (“breakpoint”) reached by an indicator AA, usually <sup>13</sup>C-

phenylalanine or  $^{13}\text{C}$ -lysine (Brunton *et al.*, 1998). Both methods present shortcomings, as reviewed elsewhere (Bos *et al.*, 2002). To limit methodological shortcomings, the “24-h indicator AA oxidation” approach has emerged (Kурpad *et al.*, 1998), based on  $^{13}\text{C}$ -leucine as the indicator AA. Subjects are adapted to increased levels of the AA tested for 6 days and its dietary requirement is determined from the zero leucine balance, determined over the entire day, rather than from the minimum leucine oxidation rate. Although the 1985 IAA requirement values, based on N balance studies, were clearly underestimated (Young *et al.*, 1989; Fuller & Garlick, 1994; Millward *et al.*, 2000a), isotope studies need to be considered with caution as they rely on many assumptions (FAO/WHO/UNU, 2007). The 24 h-indicator AA oxidation approach appears to give the most reliable values for most AA (FAO/WHO/UNU, 2007). The current recommended dietary IAA requirements, which are two or three times higher than the 1985 IAA requirement values, are reported in **Table 2**.

**Table 2.** Estimates of dietary amino acid requirements for adult humans.

	FAO/WHO/UNU (1985)	FAO/WHO/UNU (2007)
<i>Indispensable amino acids</i>		mg/kg/d
Threonine	7	15
Valine	10	26
Isoleucine	10	20
Leucine	14	39
Phenylalanine + tyrosine	14	25
Lysine	12	30
Histidine	8–12	10
Methionine + cysteine	13	15
Tryptophan	3.5	4
<b>Total indispensable amino acids</b>	<b>93.5</b>	<b>184</b>

## 2. *In vivo* protein quality index

A protein quality index has to be representative and needs to be determined routinely. Although protein efficiency ratio and net protein ratio were previously determined in young rats, these criteria have been criticized because of discrepancies between the human and the rat for their AA requirements, in particular for sulphur-containing AA (National Research Council, 1978) (**Table 3**), and their growth rates. In addition, these criteria were not additive between protein sources, making their use difficult for assessing

the quality of mixed protein sources. As a result, other approaches have been proposed.

**Table 3.** Amino acid scoring pattern across species.

Amino acid	Adult human <sup>1</sup>	Laboratory rat <sup>2</sup>	Growing pig <sup>3</sup>
	<i>mg/g protein</i>		
Threonine	23	41	34
Valine	39	49	36
Isoleucine	30	41	28
Leucine	59	71	50
Phenylalanine + tyrosine	38	68	48
Lysine	45	61	52
Methionine + cysteine	22	65	30
Tryptophan	6	13	10

<sup>1</sup> (FAO/WHO/UNU, 2007).

<sup>2</sup> (National Research Council, 1995).

<sup>3</sup> (National Research Council, 1998).

## 2.1. Protein digestibility-corrected amino acid score

The PDCAAS, based on human IAA requirements, is recognized as the most suitable method for the routine determination of protein quality for humans (FAO/WHO, 1991; FAO/WHO/UNU, 2007). The PDCAAS takes into account protein digestibility and the AA score based on the following equation (Schaafsma, 2000):

$$\text{PDCAAS} = \text{digestibility} \times \frac{\text{mg of first limiting AA in 1 g of test protein}}{\text{mg of the same AA in requirement pattern}}$$

where digestibility refers to true faecal N digestibility as assessed in the growing rat. There have been criticisms of this method (Darragh *et al.*, 1998; Reeds *et al.*, 2000b; Hegsted, 2000; Tomé *et al.*, 2002; Bos *et al.*, 2002; Millward, 2003; Schaafsma, 2005).

An important concern relates to the digestibility factor. Firstly, the PDCAAS refers to overall protein digestibility, which might not reflect digestibility of individual AA (Hess *et al.*, 1998; Hess *et al.*, 2000). Thus, it would be more appropriate to use individual AA digestibilities for the PDCAAS (Darragh & Hodgkinson, 2000; Tomé *et al.*, 2002). Secondly, ileal digestibility rather than faecal digestibility should be included in the PDCAAS (Darragh & Hodgkinson, 2000; Tomé *et al.*, 2002) because it is known to be more representative of dietary protein digestibility (see Section I.3.1.1). Thirdly, estimation of digestibility in pigs rather than rats might be considered (Rowan *et al.*, 1994; Darragh & Hodgkinson, 2000), as pigs might be a better model for humans than rats (see Section I.3.2.2). Alternatively, an *in vitro* ileal AA digestibility assay, based on a

computer-controlled gastrointestinal model, to replace the rat assay has been proposed (Schaafsma, 2005), but would need further experimental validation before being widely accepted.

Another controversial matter relates to the assumption on which the PDCAAS is based, *i.e.* that the AA score determines the biological value. This implies that biological value (proportion of digestible N that has been utilized in the body) is driven only by the AA profile of the dietary protein, an assumption that has been disputed (Hegsted, 2000; Millward, 2003).

Finally, because the PDCAAS was proposed as an index to evaluate the protein quality of diets and not of a specific dietary protein, it was first decided that PDCAAS values above 1 were of no special interest. However, because the PDCAAS is widely used to evaluate individual dietary protein sources, the truncation of high PDCAAS to 1 is now seen as a limitation. Indeed, the complementary values of high-quality proteins in mixed diets, such as meat, egg, and milk, are ignored (Schaafsma, 2005), which assumes that there is no need to consider protein quality beyond the point of sufficiency (Darragh & Moughan, 1998). As argued by the 2007 FAO/WHO/UNU expert consultation, the PDCAAS must be truncated for its practical use to adjust dietary protein requirement. However, a “protein source quality index” for individual proteins with a PDCAAS higher than 1 could be established (FAO/WHO/UNU, 2007).

Additionally, the PDCAAS does not take into account the potential adverse effect of antinutritional factors sometimes present in proteins, and thus probably overestimates the nutritional quality of such proteins, as shown by comparison with the protein efficiency ratio (Sarwar, 1997; Gilani *et al.*, 2005). Conversely, the PDCAAS of proteins deficient in IAA have been reported to underestimate their nutritional value. Pea and wheat proteins amounted to 91 and 85% of the milk nutritional value based on their net postprandial utilization and to only 61 and 30% of the milk nutritional value based on their PDCAAS (Mariotti *et al.*, 2001; Bos *et al.*, 2005a). Also, other additional intrinsic values, such as the presence of conditionally indispensable AA, other AA included in alternative (non-nutritional) pathways or even bioactive peptides (Darragh *et al.*, 1998), are not included in the PDCAAS.

In summary, although the PDCAAS method is a useful method for routine determination of protein quality, revisions are still required. In particular, it does not consider the subsequent metabolism of absorbed AA and thus seems unlikely to predict with accuracy the biological value of dietary protein (Gaudichon *et al.*, 1999; Hegsted, 2000; Tomé &

Bos, 2000; Lacroix *et al.*, 2006b; Bos *et al.*, 2007; Humayun *et al.*, 2007). Net postprandial protein utilization has been proposed as a better indicator of protein quality. This method has been fully developed and applied over recent years, as discussed below. Recently, Humayun *et al.* (2007) proposed an approach based on the “indicator AA oxidation” method as a potentially useful tool for the determination of the metabolic availability of AA in human foodstuffs. However, this method needs substantial refinement before being widely applied.

## 2.2. Net postprandial protein utilization

Net protein utilization can be directly determined using the classical N balance method; however, its major limitation is in the estimation of the net protein retention as a daily gain. Protein metabolism is subject to a diurnal cycle (fed and fasting states), resulting in alternate periods of postprandial accretion and postabsorptive losses of body proteins (Tomé & Bos, 2000). Acute N deposition during the postprandial phase is critical for the deposition of dietary N in tissues (Millward & Pacy, 1995; Tomé & Bos, 2000). Dietary N utilization directly for protein synthesis can be estimated when determined in the postprandial period, and is assumed to be a good indicator of the dietary protein nutritional value (Mariotti *et al.*, 1999; Tomé & Bos, 2000). Net postprandial protein utilization was thus proposed as a more sensitive approach for assessing dietary protein quality (Gaudichon *et al.*, 1999; Tomé & Bos, 2000; Reeds *et al.*, 2000b). It can be assessed by measuring total dietary N losses usually for 8 h following ingestion of a test meal containing  $^{15}\text{N}$ -labelled dietary protein (Tomé & Bos, 2000) and using the following equation:

$$\text{Net postprandial protein utilization} = \left[ {}^{15}\text{N}_{\text{intake}} - ({}^{15}\text{N}_{\text{ileal}} + {}^{15}\text{N}_{\text{body urea}} + {}^{15}\text{N}_{\text{urine}}) \right] / {}^{15}\text{N}_{\text{intake}}$$

When combined with true ileal digestibility, the net postprandial protein utilization allows for the measure of postprandial biological value, *i.e.* the proportion of absorbed N effectively used in the anabolic pathway (Mariotti *et al.*, 1999).

Unlike the net protein utilization index, which requires long-term studies due to the adaptation period (Millward & Pacy, 1995), the net postprandial protein utilization can be determined acutely because postabsorptive losses, depending on the previous level of protein intake, are not taken into account. The net postprandial protein utilization has thus been suggested as a better criterion for protein quality evaluation (Gaudichon *et al.*, 1999; Bos *et al.*, 2002; Bos *et al.*, 2005a). In particular, it penalizes protein sources deficient in



AA, such as wheat and pea, to a lesser extent than the PDCAAS as discussed above. Additionally, it allows for a better discrimination between proteins that have undergone technological processing like spray-drying: whereas the PDCAAS differed by only 2% between spray-dried milk and microfiltered milk, the net postprandial protein utilization was 10% lower for spray-dried milk than for microfiltered milk (Lacroix *et al.*, 2006b). This suggests that the metabolic fate of dietary proteins must be taken into account when assessing nutritional quality.

### 3. Digestibility

Protein digestibility is a key component in dietary protein quality evaluation, as it indicates the extent to which dietary protein has been digested and absorbed as AA by the gastrointestinal tract, and thus provides a measure of bioavailability, *i.e.* the proportion of dietary AA that are absorbed in a chemical form that renders them potentially suitable for protein metabolism (Moughan, 2003; Fuller & Tomé, 2005; Stein *et al.*, 2007). Protein digestibility is indirectly measured from the difference between intake and gut losses. The overall protein digestibility is determined from the measure of N digestibility. Additionally, digestibility of individual AA, not always equal to N digestibility, can be determined.

#### 3.1. Terminology

##### 3.1.1. *Faecal versus ileal*

Digestibility has been determined in the past at the faecal level; however, because of a prolific microbial N metabolism within the hindgut (Low, 1980) and because AA are absorbed mainly in the small intestine (Krawielitzki *et al.*, 1990), this approach is usually recognized as being inaccurate in estimating the amount of absorbed dietary AA (Sauer & Ozimek, 1986; Darragh & Hodgkinson, 2000; Mosenthin, 2002; Moughan, 2003; Fuller & Tomé, 2005). Colonic (and caecal) microbial metabolism includes degradation of undigested AA or peptides and *de novo* synthesis of microbial AA, thus yielding a faecal AA profile that is quantitatively and qualitatively different from the pattern of undigested AA (Fuller & Tomé, 2005). Faecal digestibility coefficients are therefore likely to differ from ileal coefficients, especially for individual AA, and have been reported, in most cases, to be overestimated in pigs (Mosenthin, 2002; Moughan, 2003).

In humans, ileal digestibility and faecal digestibility have been compared in few controlled studies. N digestibility was reported to be slightly lower (1–3%) at the ileal level than at the faecal level (Rowan *et al.*, 1994; Bos *et al.*, 1999). Greater differences were observed for AA digestibilities, which were mostly lower at the ileal level than at the faecal level, with differences ranging from 2 to 17%. In summary, ileal AA and N digestibility values, especially when corrected for endogenous losses (see next section), appear to be more representative than faecal values of dietary AA/N absorbed from the intestine.

### 3.1.2. *Apparent versus true*

Apparent N digestibility is determined as the difference between the quantity of ingested N and the total N losses from the gut. A major disadvantage of apparent digestibility is the non-linear correlation between apparent N digestibility and dietary protein level, which results in non-additivity of apparent N digestibility in mixtures of protein sources (Stein *et al.*, 2007). This is due to the relative contribution of gut endogenous N to total N losses (Fan *et al.*, 1994). Endogenous N losses are composed of two components: basal or minimal losses that are inevitably lost by the body and that are independent of dietary factors, and specific losses that are influenced by dietary factors (Nyachoti *et al.*, 1997a; Darragh & Hodgkinson, 2000; Stein *et al.*, 2007). When apparent N digestibility is corrected for total (basal and specific) endogenous N losses, true digestibility, also occasionally referred to as real digestibility (de Lange *et al.*, 1990; Mariotti *et al.*, 2001), is determined. True digestibility reflects the specific fate of dietary N within the gut and corrects for any variation of the endogenous fraction related to dietary factors. It is thus a fundamental property of the protein source itself (Mosenthin, 2002) and allows the metabolic costs associated with synthesis and recycling of gut endogenous AA losses to be represented explicitly (Stein *et al.*, 2007). Unlike apparent digestibility, true digestibility gives rise to a system in which the digestible N concentrations in food are additive (Fuller & Tomé, 2005). According to Stein *et al.* (2007), a distinction should be made between “standardized digestibility” and “true digestibility”, with standardized digestibility referring to apparent digestibility corrected for basal endogenous losses only (Fuller & Tomé, 2005; Stein *et al.*, 2007).

However, in some cases, the distinction between true digestibility and standardized digestibility can be difficult to establish.

To conclude, protein digestibility when determined at the ileal level and corrected for

endogenous losses appears to be a better predictor of dietary AA bioavailability than faecal digestibility and/or apparent digestibility (Hodgkinson *et al.*, 2003; Fuller & Tomé, 2005). In some cases, digestibility might not reflect accurately bioavailability. For instance, heat treatment of protein ingredients can result in chemical modification of AA, such as lysine, which is absorbed through the intestinal membrane but cannot be utilized for protein synthesis (Moughan & Rutherfurd, 1996). Also, the recycling of intestinal dietary N, possibly in microbial AA, might contribute to differences between digestibility and bioavailability (Fuller & Reeds, 1998; Fuller & Tomé, 2005; Metges *et al.*, 2006).

## 3.2. Animal models

### 3.2.1. *The growing rat*

The growing rat has been recommended and generally accepted as a valid animal model for predicting protein digestibility in humans (FAO/WHO, 1991). Whereas the rat coefficients of apparent faecal digestibility overestimated by 14% (Bodwell *et al.*, 1980) or were poorly correlated ( $r = 0.17$ ) (Ritchey & Taper, 1981) with human coefficients, the values for true faecal digestibility were in better agreement between the species, with rat values being on average 3% higher (**Table 4**).

**Table 4.** Apparent and true faecal digestibility of nitrogen as estimated in adult humans and growing rats within controlled studies.

Protein source	n		Apparent digestibility		S <sup>1</sup>	True digestibility <sup>2</sup>		S	Reference
	Human	Rat	Human	Rat		Human	Rat		
			%			%			
Canned tuna	4	6	75,2	93,7	*	92,1	97,4	NS	} Bodwell et al., 1980
Spray dried whole egg	5	6	77,8	91,4	*	95,0	99,9	NS	
Cottage cheese	5	6	84,9	94,2	*	101,5	100,1	NS	
Peanut flour	4	6	76,3	88,9	*	93,3	96,1	NS	
Soy isolate	5	6	81,0	91,7	*	97,1	99,0	NS	
Wheat gluten	4	5	81,7	93,3	*	98,0	99,4	NS	
Mixed protein sources <sup>3</sup>						84,0	87,5	-	Forsum et al., 1981
Mixed protein sources <sup>4</sup>	8	5	87,6	89,2	NS				Bach Knudsen et al., 1994
Mixed protein sources <sup>5</sup>	7	5-6	88,4	90,8	***	93,0	96,5	***	Wisker et al., 1996

<sup>1</sup> Statistical significance: \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; NS, non significant.

<sup>2</sup> Corrected for a constant estimate of faecal endogenous nitrogen losses.

<sup>3</sup> Average digestibility over 2 mixed diets based on vegetable or vegetable/animal proteins.

<sup>4</sup> Vegetable and animal proteins. Average digestibility over 4 diets with similar protein content but different fibre content. For each diet, there was no significant difference between species.

<sup>5</sup> Vegetable and animal proteins. Average over 3 diets with similar protein content but different fibre content. For each diet, there was a significant difference ( $P < 0.05$ ) between species.

To our knowledge, controlled studies comparing human and rat ileal digestibilities have not been undertaken. Data from independent studies suggest good agreement between the species for ileal AA digestibility coefficients (Fuller & Tomé, 2005), except for glycine digestibility, which was lower by 10% in rats fed casein or soya protein isolate (Fuller & Tomé, 2005), probably due to an underestimation of the endogenous glycine flow (Skilton *et al.*, 1988; Moughan *et al.*, 1992c; Hendriks *et al.*, 2002; Rutherfurd & Moughan, 2003). However, recent data suggest that rats might be able to digest normally dietary proteins that are poorly digestible in humans, such as rapeseed protein, for which the true ileal digestibility was 84–87% in humans versus 95% in rats (Boutry *et al.*, 2008; Bos *et al.*, 2007).

The greater difference between rats and humans for apparent digestibility than for true digestibility may result from a higher contribution of endogenous N losses to total N losses in humans than in rats, although the AA compositions of their endogenous protein losses seem to be similar (**Table 5**).

The nutritional significance of rat data must be interpreted with caution when extrapolated to humans, due to nutritional, physiological and anatomical inter-species differences. A major discrepancy is the different AA requirements, as shown by the resulting AA scoring pattern (AA requirements expressed relative to the recommended protein intake, Table 3). The difference is dramatic for sulphur-containing AA, for which the rat AA score is more than twice the human AA score. In addition, adult humans have a higher maintenance requirement than growing rats (Ritchey & Taper, 1981). Nevertheless, Mitchell (1954), who compared biological values of proteins, reported a better correlation between the growing rat and adult man ( $r = 0.92$ ) than between the adult rat and adult man ( $r = 0.67$ ). There are also differences in digestive tract anatomies, especially with respect to the large caecum size relative to the overall size of the rat digestive tract (Ritchey & Taper, 1981). Furthermore, coprophagy occurring in rats might impair protein quality assessment if not prevented. Small gut mucosa protein synthesis appears to be higher in rats than in humans. The average fractional protein synthesis rate (g protein synthesized/100 g protein in the tissues) was 143% per day for rats and ranged from 22 to 50% in humans, as reviewed by Waterlow (2006a). This suggests a higher mucosal protein renewal, possibly leading to a higher degree of dietary N recycling within endogenous mucosal protein. However, when taking into consideration potential differences between the rat and the human, the rat is still a useful model for fundamental and theoretical research (Bergen, 2007).

**Table 5.** Ileal endogenous nitrogen flow and its amino acid composition (mean  $\pm$  SD) as determined in the rat, pig and human fed a protein-free diet.

	Rat <sup>1</sup>	Pig <sup>2</sup>	Human <sup>3</sup>
Number of studies	6	9	3
<i>Nitrogen flow, <math>\mu\text{g/g}</math> dry matter intake</i>	1346 $\pm$ 436	1678 $\pm$ 188	1952 $\pm$ 172
Number of studies	3	9	3
<i>Amino acid composition</i>			
<i>g/16g endogenous N</i>			
Threonine	5.9 $\pm$ 1.2	5.0 $\pm$ 0.9	6.1 $\pm$ 0.6
Valine	3.8 $\pm$ 0.3	3.9 $\pm$ 1.2	4.6 $\pm$ 0.2
Isoleucine	2.4 $\pm$ 0.1	2.9 $\pm$ 0.7	2.6 $\pm$ 0.3
Leucine	4.1 $\pm$ 0.4	4.4 $\pm$ 0.6	5.0 $\pm$ 0.5
Phenylalanine	2.7 $\pm$ 0.4	2.5 $\pm$ 0.4	3.0 $\pm$ 0.5
Tyrosine	2.5 $\pm$ 0.2	2.6 $\pm$ 1.1	2.9 $\pm$ 0.4
Lysine	3.1 $\pm$ 0.5	3.3 $\pm$ 1.0	4.8 $\pm$ 1.7
Histidine	2.2 $\pm$ 0.2	1.5 $\pm$ 0.2	2.7 $\pm$ 0.5
Serine	7.3 $\pm$ 3.2	4.5 $\pm$ 0.7	4.7 $\pm$ 0.5
Glutamic acid	13.5 $\pm$ 5.5	7.6 $\pm$ 1.8	4.5 $\pm$ 1.2
Alanine	3.4 $\pm$ 0.2	4.6 $\pm$ 1.4	3.4 $\pm$ 0.3
Proline	7.5 $\pm$ 0.3	14.4 $\pm$ 6.4	5.4 $\pm$ 1.0
Arginine	3.6 $\pm$ 0.2	3.6 $\pm$ 0.6	4.2 $\pm$ 1.5
Aspartic acid	10.4 $\pm$ 1.1	6.5 $\pm$ 0.9	7.1 $\pm$ 0.6
Glycine	8.7 $\pm$ 2.5	8.4 $\pm$ 2.3	7.6
Methionine	-	1.1 $\pm$ 0.8	0.8 $\pm$ 0.2
Cysteine	0.8	1.2 $\pm$ 0.6	2.6 $\pm$ 1.2
Tryptophan	-	0.9 $\pm$ 0.5	1.8

<sup>1</sup> Nitrogen flow (Butts *et al.*, 1992a, 1992b; Moughan *et al.*, 1992b; Donkoh *et al.*, 1995; Hendriks *et al.*, 2002; James *et al.*, 2002a). Amino acid composition (Butts *et al.*, 1992a, 1992b; Donkoh *et al.*, 1995).

<sup>2</sup> (Chung & Baker, 1992; Butts *et al.*, 1993a; Mariscal-Landin *et al.*, 1995; Leterme *et al.*, 1996a; Leterme *et al.*, 1996b; Hess *et al.*, 1998; Hess *et al.*, 2000; Hodgkinson *et al.*, 2000; Zhang *et al.*, 2002).

<sup>3</sup> (Fuller *et al.*, 1994; Rowan *et al.*, 1994; Moughan *et al.*, 2005). The values determined in Fuller *et al.* (1994) and Rowan *et al.* (1994) were calculated for an average daily dry matter intake of 410 g determined according to the data from Department for Environment (2003) and Marriott (2003).

### 3.2.2. *The growing pig*

With a physiology and a metabolism that are closer to those of the human, the growing pig has been extensively used for studying human protein metabolism (Bergen, 2007) and has been proposed as a better model for protein digestion studies (Moughan *et al.*, 1992a; Moughan *et al.*, 1994; Darragh & Hodgkinson, 2000; Moughan, 2005). Also, the pig offers the advantage of allowing continuous *in vivo* ileal collection after surgical preparations, unlike the rat, from which digesta are collected after euthanasia at a certain time post meal (Moughan *et al.*, 1994).

At the faecal level, Darragh & Moughan (1995) reported good agreement for apparent N and AA digestibility between piglets and infants fed milk formula, with N digestibility values of 97.5 and 94.5%, respectively. A similar observation was made by Forsum *et al.* (1981). To our knowledge, few studies have compared the true ileal digestibility of protein between pigs and humans. Rowan *et al.* (1994) reported true digestibility coefficients that were similar between ileostomized subjects and ileostomized pigs, except for threonine, phenylalanine, methionine and cysteine which were significantly higher (3–8% units) in humans. This is in line with data across independent studies (although not direct evidence), showing good agreement between pig and human true ileal N and AA digestibility values (for soya protein or casein), as reviewed previously (Fuller & Tomé, 2005). As reported for the growing rat, AA compositions of endogenous ileal protein losses were similar between the growing pig and the adult human (Table 5), except for proline for which the contribution was three times higher in pigs.

Although the pig is also a fast-growing animal, the AA scoring pattern is closer between the growing pig and the adult human than between the growing rat and the adult human (Table 3). Gut fractional protein synthesis rates were reported to be in the same range, with pig values of 43 to 51% per day (Simon *et al.*, 1978; Simon *et al.*, 1982) and human values of 22 to 50% per day (Waterlow, 2006a).

Overall, direct evidence to support the growing pig as an animal model for predicting ileal protein digestibility in humans is scarce.

## II. Nitrogen flows along the digestive tract

After a protein meal, N of dietary origin mixes with N present in the intestinal lumen, considered to be endogenous N as opposed to dietary N. Considerable exchange of N occurs between the intestinal lumen and the systemic pools, mainly in terms of proteins, AA and urea. Unlike dietary N, endogenous N exhibits a rather complicated pattern along the intestinal lumen as it enters at various stages, thus making it difficult to assess the contribution of each endogenous N source (Fuller & Reeds, 1998).

### 1. Endogenous nitrogen flows

#### 1.1. Oral cavity

N in the mouth originates mainly from saliva, which contains digestive enzymes ( $\alpha$ -amylase), glycoproteins (mucins), free AA, urea, uric acid and creatinine (Buraczewski, 1986). Various amounts of N secreted within saliva have been reported, with values ranging from 0.2 up to 2 g N/d (Juste, 1982; Alpers, 1987; Brunser *et al.*, 1991; Sève & Leterme, 1997). Compared with the total gut N secretion, the saliva N is relatively small (Table 6).

**Table 6.** Daily contributions of endogenous nitrogen secretions to total endogenous nitrogen entering the lumen of the digestive tract in growing pigs. Quoted in Sève & Leterme (1997); data from Leterme (1995).

Sources	g/day	% of total
Saliva	0.2 – 0.6	1 – 2.2
Gastric juice	2 – 3.3	10.5 – 12.3
Bile	1.7 – 1.9	8.9 – 7.1
Pancreas	1 – 4.6	5.2 – 17.2
Urea	6.4	33.5 – 23.9
Mucus	2	10.5 – 7.5
Epithelial enzymes + sloughed cells	5.8 – 8	30.4 – 29.8
<b>Total</b>	<b>19.1 – 26.8</b>	<b>100 – 100</b>

## 1.2. Stomach

N in gastric juice originates from digestive enzymes (pepsins), mucins, desquamated cells and, to a lesser extent, urea and ammonia (Juste, 1982; Buraczewski, 1986). Gastric juice has usually been studied together with saliva collected from pigs equipped with duodenal cannulae. The minimal quantity of endogenous N produced by saliva and gastric secretions was estimated to be 0.3–0.6 g/d in 35-kg pigs (Zebrowska *et al.*, 1983). Secretions were increased to 3.1 g N/d when 40-kg pigs received 1.7 kg/d of barley–fishmeal or barley–soya diet (Low, 1985).

## 1.3. Bile

Bile is secreted by the polygonal cells in the liver and is stored in the gall bladder, where the bile is concentrated by absorption of water and electrolytes ( $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{HCO}_3^-$ ). Bile, flowing into the duodenum through the sphincter of Oddi (Sanford, 1982), acts as a powerful emulsifying agent that helps in the digestion and absorption of fat. Neural (sympathetic and parasympathetic) and hormonal [cholecystokinin (CCK)] systems regulate the contraction of the gall bladder and thus the flow of bile into the small intestine (Sanford, 1982). Organic compounds of bile are mainly bile acids, phospholipids, cholesterol, bilirubin and mucus (Davenport, 1982). Of the N in bile, 75% comes from  $\alpha$ -amino N (95% of this originates from glycine in conjugated bile acids) and 25% comes from ammonium sulphate. Approximately one-quarter of the bile acids escape absorption in the small intestine (Davenport, 1982). The secretion of bile was reported to be 1.7–1.9 g N/d in pigs (Table 6). The amount of endogenous N from saliva, gastric juice and bile, as collected from a duodenal cannula, was reported to be 5.1–5.3 g/d in 30–35-kg pigs (Zebrowska *et al.*, 1982; Krawielitzki *et al.*, 1990), which is in line with the values reported in Table 6.

## 1.4. Pancreas

The pancreas, a gland located between the stomach and the duodenum, produces both endocrine and exocrine secretions, with the exocrine part representing, by weight, the most important part (84%) of the pancreas (Low & Zebrowska, 1989). Exocrine secretions, released into the duodenum via the sphincter of Oddi, are composed of digestive enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase, amylase, lipase, phospholipase and ribonuclease), secreted as inactive precursors, and of an alkaline fluid



with a high concentration of bicarbonates, which regulate the duodenal pH to provide an adequate medium for the pancreatic enzyme activity (Davenport, 1982). The pancreas plays a very important role in digestion; this is discussed in Section II.2.1. Pancreatic secretions are stimulated by stomach distension via a vagovagal reflex (Davenport, 1982) and by hormones secreted from the stomach (gastrin) and from the duodenal mucosa (secretin), acting mainly on the bicarbonate secretions, and CCK, acting mainly on the enzymatic secretions (Solomon, 1994). Whereas CCK seems to act by both neural and direct modes in rats, the neural mode seems to predominate in humans (Wang & Cui, 2007).

A feedback regulation of pancreatic secretions by various duodenal contents is well established (Solomon, 1994). Digestion products, mainly from protein and fat, have been reported to modulate exocrine pancreatic secretions through complex mechanisms (Swanson *et al.*, 2004). Whereas intact protein infused in the digestive tract of dogs did not stimulate pancreatic secretions, a peptic or pancreatic digest of proteins or a mixture of free AA efficiently stimulated pancreatic secretions, with phenylalanine and tryptophan, free or within peptides, being the most stimulating AA (Solomon, 1994). In particular, the caseinomacropptide has specifically been reported to stimulate exocrine pancreatic secretion in the anaesthetized rat (Pedersen *et al.*, 2000). In contrast, Green *et al.* (1983) reported that an intraduodenal infusion of whole protein induced a greater secretion of pancreatic enzymes compared with an infusion of small peptides or free AA in rats. The underlying mechanisms of the influence of intraluminal protein digestion products on pancreatic secretions mainly involve hormonal (CCK) regulations (Solomon, 1994; Zebrowska, 1999; Pedersen *et al.*, 2000).

The effect of protein level and protein sources on pancreatic secretions has not been clearly determined. Zebrowska *et al.* (1983) did not observe any difference in N from pancreatic secretions between 35-kg pigs fed diets based on casein–starch or barley–soya, with 1.9 or 2.1 g N/d, respectively. In contrast, Partridge *et al.* (1982) measured different N outputs from pancreatic secretions between 50-kg pigs fed diets based on casein–starch or barley–wheat hull–fish flour, with 1.3 or 2.3 g N/d, respectively. Li *et al.* (1998) observed different total pancreatic N outputs in pigs fed either raw or autoclaved soya flour, but observed similar AA compositions. The protein level was reported to have no effect on the volume and the protein content of pancreatic secretions (Corring *et al.*, 1984; Hee *et al.*, 1988a). However, a high dietary protein

intake stimulated the synthesis, secretion and activity of proteolytic enzymes (Hee *et al.*, 1988a).

Postprandial exocrine pancreatic secretion was reported to be higher than preprandial secretion in its volume and its protein content, but this was not affected by the amount of food intake *per se* (Botermans & Pierzynowski, 1999). The food intake pattern has been reported to affect exocrine pancreatic secretion: feeding 12 small meals to growing pigs (16–31 kg), compared with one large meal daily, tended to stimulate protein output from exocrine pancreatic secretions by 44% (Botermans *et al.*, 2000).

In summary, Juste (1982) reported values for pancreatic secretions ranging from 1.5 to 4 g N/d in 35–40-kg pigs fed a standard diet containing 15% protein, values that are similar to those reported in Table 6.

### 1.5. Small intestine

The small intestine is functionally divided into duodenum, jejunum (two-fifths of the length of the small intestine) and finally ileum, structurally indistinct from the jejunum (Sanford, 1982). The small intestine can synthesize up to 400 g protein/d in pigs (Souffrant *et al.*, 1993), which is one of the highest synthesis rate capacities in the body. The N input from the small intestine has been reported to be higher than that produced from bile, pancreas and stomach together (Juste, 1982). Quantitative and qualitative estimations of intestinal secretions are difficult because of the constant entry of N, at multiple stages, and because of constant reabsorption. Total N secreted in intestinal juice has been reported to amount to 8.9 g N/d, when determined by digesta exchange between <sup>15</sup>N-labelled 30-kg pigs (Krawielitzki *et al.*, 1990), and up to 15 g N/d, when determined in intestinal loops of 35–50-kg pigs perfused with isotonic osmotic solution (Buraczewska, 1979).

Dietary factors have been reported to influence intestinal secretions. Intestinal secretions of N compounds were markedly decreased in pigs fed a protein-free diet (Buraczewska, 1979). A stimulating effect of dietary fibre has been reported; infusion of guar gum solution (6.7 g/L) in isolated jejunal loops increased N secretion from the mucosa of the entire small intestine from 15 to 27 g/d (Low & Rainbird, 1984). Their net influence on endogenous N recovered at the terminal ileum is detailed in Section IV.

Intestinal endogenous N arises from mucins, epithelial enzymes (aminopeptidases, maltases, lactases etc.), pancreatic enzymes, desquamated cells and, to a lesser extent,

urea and some leaked plasma proteins (*e.g.* albumin, glutathione) (Fauconneau & Michel, 1970; Snook, 1973). Although not strictly endogenous, microbial proteins are usually included in the estimate of endogenous protein. Mostly synthesized in the large intestine, they are discussed in Section II.1.6. In the pig gut, infused with an N-free solution, 10% of intestinal N secretions originated from desquamated cells, whereas 50–80% of the remaining N was composed of  $\alpha$ -amino N (65% as free AA, 33% as protein). The non- $\alpha$ -amino N was essentially made up of urea (Buraczewska, 1979). Mucins and urea, which are of nutritional importance, are discussed here.

### 1.5.1. *Mucins*

Mucins are of great importance for the preservation of gut integrity. As the main component of the mucus gel layer covering the epithelium of the entire digestive tract, mucins are responsible for the unique consistency and properties of mucus, which acts as a protective barrier for the epithelial surfaces from the intestinal lumen content but also retards the free diffusion of various nutrients to the hydrolytic enzymes (disaccharidases and peptidases) of the brush border. Mucins, which can be either secreted or membrane associated (Montagne *et al.*, 2004), are synthesized within specialized mucous cells found throughout the digestive tract from the oral cavity (salivary glands) to the large intestine as well as in the pancreas and gall bladder ducts. Of the mucin production, 73% occurs in the small intestine and the rest occurs mainly in the stomach (Lien *et al.*, 1997b). Mucins, characterized by a very high molecular weight (MW; up to 2000 kDa) are glycoproteins containing by weight 50–80% of carbohydrates and 20–35% of proteins (Mantle & Allen, 1981). Carbohydrate chains, composed mainly of five different monosaccharides (N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose, sialic acids), are linked to the peptide backbones via O-glycosidic bonds with serine and threonine residues. The mucus layer is in dynamic balance between synthesis and secretion of mucins and proteolytic and physical erosion that releases mucins into the lumen (Montagne *et al.*, 2004). Luminal proteases hydrolyse mucins by cleaving peptidic bonds within the “naked” segment, an area enriched with cysteine, and hydrophobic and polar AA, thus resulting in a decrease in viscosity of the mucus layer. Heavily glycosylated domains, enriched with serine, threonine and proline, which represent up to 50% of the total AA (Neutra & Forstner, 1987), are temporarily resistant to further proteolytic hydrolysis because of their dense coat of oligosaccharide chains (Neutra & Forstner, 1987). Further peptidic degradation requires the hydrolysis of more than 50% of the oligosaccharides,

which depends on extracellular glycosidases and sulphatases of enteric microorganisms (Neutra & Forstner, 1987). Because of this high proteolytic resistance, especially in the small intestine, mucins are an important component of ileal endogenous protein losses (Montagne *et al.*, 2004). Lien *et al.* (1997b) estimated the daily ileal output of mucins to be 5.3–5.6 g/d in 55-kg pigs fed a protein-free or synthetic AA diet, which contributed to 11% of ileal endogenous protein. Threonine, proline and serine are predominant AA in mucins and reached 35, 24 and 16% of the ileal endogenous AA recovered at the terminal ileum (Lien *et al.*, 1997b). In particular, gut threonine loss makes a significant contribution to the metabolic requirement for threonine (Gaudichon *et al.*, 2002).

Dietary components have been reported to modulate mucin synthesis quantitatively and qualitatively (Montagne *et al.*, 2004). Dietary fibres were shown to increase mucin excretion at the terminal ileum (Mariscal-Landin *et al.*, 1995; Lien *et al.*, 2001; Morel *et al.*, 2003), with effects depending on the solubility and the nature of the fibres influencing mucus erosion and proteolytic degradation, respectively. The specific effect of bulk-forming (*e.g.* fibre swelling due to water absorption in the gut) was previously shown to increase mucin secretion in the small and large intestine of rats, whereas fermentable components increased mucin secretion only in the caecum (Tanabe *et al.*, 2006). Dietary protein, although less studied than fibre, has been shown to modify the recovery of mucin in endogenous protein. In calves fed diets containing 14 and 28 g crude protein/kg dry matter intake (DMI), the duodenal flow of mucin protein increased from 1.1 to 2.4 g/kg DMI, respectively, but no differences were observed for endogenous protein recovered at the terminal ileum (Montagne *et al.*, 2000). This was assumed to be due to the higher secretions of enzymes observed after an increase in the dietary protein intake, thus leading to a higher enzymatic degradation of mucins. In pigs fed a diet containing isolated soyabean protein, the hexosamine ileal excretion increased when the dietary crude protein content exceeded 55 g/kg dry matter, suggesting an increased ileal output of mucins (Mariscal-Landin *et al.*, 1995). In isolated vascularly perfused rat jejunum, enzymatic casein hydrolysate (0.5–5%) from bovine milk markedly stimulated rat intestinal mucus secretion (Claustre *et al.*, 2002). The intraluminal perfusion of  $\beta$ -casomorphin, an opioid peptide released during milk digestion, reproduced this mucin release in rat jejunum. Further data suggest that nutrient-derived opiate materials and opioid neuropeptides may participate in the regulation of intestinal mucus discharge (Trompette *et al.*, 2003). Intact protein did not have any impact on mucin secretion in isolated rat jejunum, emphasizing the importance of protein gastric digestion (Claustre *et*

*al.*, 2002). Dietary intake of threonine has been reported to influence mucin protein synthesis: when rats were restricted to a dietary intake of threonine at 30% of the requirement, the mucin protein synthesis along the entire gut was significantly reduced (Faure *et al.*, 2005). In addition, antinutritional factors, such as tannins and possibly lectins, might increase the output of intestinal mucins (Montagne *et al.*, 2004).

### 1.5.2. Urea

Urea, an end-product of protein catabolism, is produced in the liver and freely diffuses in all liquid body compartments before being excreted mainly in urine or, to a lesser extent, in sweat. It is known that urea enters the gastrointestinal tract, either by free diffusion from the circulating blood (Bergner *et al.*, 1986) or by secretion via bile and/or pancreatic juice (Bergner *et al.*, 1986; Mosenthin *et al.*, 1992). The daily endogenous secretion of urea in the gastrointestinal tract was estimated to be 6 g N/d in growing pigs [(Rérat & Buraczewska, 1986), quoted by Souffrant *et al.* (1993)]. Bergner *et al.* (1986), who infused <sup>15</sup>N-urea through the jugular vein of 34-kg pigs, reported a urea flux ranging from 1.2 to 2.4 g urea/ kg body weight/d.

Endogenous urea is hydrolysed into ammonia via intestinal bacteria that are active mainly in the large intestine (Kim *et al.*, 1998). Estimates of urea degraded by intestinal bacteria have been reported to range between 20 and 25% of the daily urea produced (Wrong *et al.*, 1981; Wrong & Vince, 1984; Fuller *et al.*, 1987; el-Khoury *et al.*, 1994). The resulting intestinal ammonia is partly reabsorbed through the intestinal wall and is then directed towards either splanchnic anabolism or reconversion to urea, thus resulting in urea recycling. The proportion of urea-N recycled was up to 18% in healthy adult humans (Long *et al.*, 1978). In addition, intestinal ammonia can be metabolized by the intestinal microflora and incorporated into microbial proteins (Torrallardona *et al.*, 1996; Petzke *et al.*, 1998; Metges *et al.*, 1999b).

Urea kinetics (production, excretion and hydrolysis) are considered to play an important role in N salvage (Jackson, 1995). The factors involved in the control of urea recycling are not clearly understood (Fuller & Reeds, 1998). Recent results from compartmental modelling showed that urea production and excretion but also urea recycling were positively correlated to the dietary protein deamination rate, *i.e.* negatively correlated to nutritional value (Juillet, 2006). Mosenthin *et al.* (1992) reported that increased plasma urea induced higher urea entry in the gut and higher urea excretion and degradation rates; however, the urea recycling rate was reduced in proportion. In summary, this would

suggest a higher urea entry in the gut, and possibly a higher faecal excretion, with low-quality protein than with high-quality protein.

In addition, the level of urease activity in the gut can be influenced by dietary components. Feeding diets containing high levels of lactose or cellulose has been shown to reduce urease activity and net ammonia production in the rat intestine (Kim *et al.*, 1998).

## 1.6. Large intestine

The large intestine, which comprised the caecum (for rats and pigs only), colon and rectum, has been reported to secrete 1.6–4.5 g endogenous N/d (Krawielitzki *et al.*, 1994; Krawielitzki *et al.*, 1996). Microbial N was reported to contribute up to 60–80% of total faecal N (Stephen & Cummings, 1980; Low & Zebrowska, 1989). However, whereas microbial activity is maximal in the large intestine, it is also present to a lesser extent in the duodenum and jejunum, and to an intermediate extent in the ileum (Bach Knudsen *et al.*, 1991). Bacteria can metabolize N from non-protein (ammonia) or protein sources from endogenous or dietary origin. Although this measure was undertaken at the ileal level, an increased dietary protein intake was shown to induce a higher intestinal flow of bacterial N, mostly due to a higher incorporation of dietary N into microbial proteins (Bartelt *et al.*, 1999). Fibres have also been reported to enhance intestinal microbial protein flow (Bartelt *et al.*, 1999; Libao-Mercado *et al.*, 2007).

Intestinal microflora may synthesize *de novo* IAA. Synthesis and utilization of microbial IAA have been demonstrated in various monogastric mammals (Niiyama *et al.*, 1979; Torrallardona *et al.*, 1996; Metges *et al.*, 1999a; Millward *et al.*, 2000b; Backes *et al.*, 2002; Torrallardona *et al.*, 2003a; Belenguer *et al.*, 2005). Their site of synthesis and absorption might not be confined to the large intestine and could occur in part in the small intestine (Torrallardona *et al.*, 2003b). Furthermore, their net contribution to the AA homeostasis of the host is still being debated (Fuller & Reeds, 1998; Baker, 2005; Metges & Petzke, 2005; Stoll & Burrin, 2006; Tomé & Bos, 2007; Blachier *et al.*, 2007). According to Torrallardona *et al.* (2003a), lysine, and more generally IAA synthesized by the gastrointestinal microflora, would significantly contribute to fulfil the metabolic IAA requirements in pigs. However, microbially derived lysine may also be seen as part of the recycling that occurs for endogenous N and IAA rather than as a net source of AA supplied in the diet (Metges *et al.*, 1999a; Metges & Petzke, 2005). An important aspect

to consider is thus the origin of N that has been incorporated into *de novo* microbial IAA. To conclude, the overall endogenous N flow (ENFL) in the gastrointestinal tract is not known with accuracy. In pigs, estimates of the total daily secretion of endogenous N into the gastrointestinal tract ranged from 11 g/d (Souffrant *et al.*, 1993) to 18 g/d (Krawielitzki *et al.*, 1990) for a daily protein intake of 24 and 40 g/d, respectively. Values of up to 26.8 g/d have also been reported (Table 6). In humans, it is considered that N flowing through the intestines (up to the terminal ileum) amounts to 11–16 g/d (FAO/WHO/UNU, 2007).

## 2. Protein digestion and absorption

### 2.1. Digestion

Digestion of endogenous and dietary proteins occurs concomitantly along the entire gastrointestinal tract, but its initiation differs, being located in the stomach for dietary protein and depending on the point of secretion/production for endogenous protein. Protein digestion is initiated in the stomach through chemical (pepsin, HCl) and mechanical (stomach contractions) mechanisms so that a mixture of peptides and free AA is released into the duodenum via the pylorus. The protease pepsin is secreted from the chief cells as pepsinogen, an inactive form that is activated at acidic pH. Pepsin is maximally active at pH values between 1.0 and 3.0 (Sanford, 1982). The gastric emptying rate influences not only the extent of gastric protein digestion but also the further steps of digestion and absorption (Mahé *et al.*, 1992), subsequently influencing the metabolic fate of dietary AA (Gaudichon *et al.*, 1994b; Dangin *et al.*, 2001). However, gastric emptying, and more generally the transit rate of food, has been reported to have no or little impact on overall protein digestibility (Huge *et al.*, 1995; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000b). The gastric emptying rate can be modulated by the quantity and the nature of ingested nutrients (Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000b), and more importantly by the energy content of the meal (Weber & Ehrlein, 1998; Fouillet *et al.*, 2001), but also by the chyme osmolarity and viscosity.

Further digestion occurs in the small intestine, and involves a series of proteolytic enzymes secreted by the pancreas into the duodenum. Ligature of the pig pancreatic duct was shown to decrease apparent N digestibility by up to 70%, illustrating the important role of the pancreas in protein digestion (Pekas *et al.*, 1964). Proteolytic enzymes

secreted by the pancreas (**Table 7**) are classified as endopeptidases, which hydrolyse peptidic bonds within the protein chain, and exopeptidases, which remove AA residues from the carboxyl end of the protein chain and which are particularly effective on hydrolysates released by endopeptidases (Davenport, 1982). Initially secreted as inactivated precursors, pancreatic enzymes are successively activated in the duodenum after initiation of the activation by the intestinal enteropeptidase (Lowe, 1994). The regulation of the pancreatic secretions has been discussed in Section II.1.4. Protein digestion is then completed by peptidases included within the membranes of brush border enterocytes. They can cleave peptidic bonds of large peptides but also of proteins such as  $\alpha$ -casein (Erickson & Kim, 1990). The most abundant peptidase is aminopeptidase, which removes the N-terminal AA from short oligopeptides. Peptidases with high hydrolytic rates for peptides containing a proline residue at their cleavage site have a complementary role to pancreatic enzymes, which have little or no ability to hydrolyse these kinds of peptides. In addition, brush border membrane activity has been shown to be higher in the ileum than in the upper intestine in humans (Erickson & Kim, 1990), which emphasizes their complementary role to pancreatic enzymes. Finally, di/tripeptidases have been reported in the cytoplasm of enterocytes (Erickson & Kim, 1990). Dietary factors may modulate the small intestinal brush border enzyme activity. For instance, dietary proteins or peptides were reported to stimulate neutral aminopeptidases to a greater extent than free AA (Poullain *et al.*, 1989).

**Table 7.** Intestinal proteolytic enzymes. From Le Floch & Sève (2000).

Enzyme	Enzyme type	Origin	Cleavage site
Trypsin	Endopeptidase	Pancreas	Arg/Lys/basic AA
Chymotrypsin A			Tyr/Phe/Trp
Chymotrypsin B			Tyr/Phe/Trp + Leu
Chymotrypsin C			Tyr/Phe/Trp + Leu + Gln/Met
Elastase I			Ala/Gly
Elastase II	Exopeptidase		Tyr/Phe/Trp
Carboxypeptidase	Exopeptidase	Intestinal brush border membrane	AA from the carboxyl end
Aminopeptidase-neutral			Arg/Leu/Met (N-terminal)
Aminopeptidase-acid			Glu/Asp (N-terminal)
Dipeptidyl aminopeptidase			Pro
Di and tripeptidase		Enterocyte cytoplasm	



## 2.2. Absorption

N enters the systemic circulation mainly as AA and di/tri-peptides but a small proportion of larger peptides or whole protein may pass directly from the intestinal lumen to the blood (Eastwood, 2003b). The absorption rate of AA has been reported to be dependent on their individual molar concentration in the gut (Rérat *et al.*, 1976; Hegarty *et al.*, 1982) but also on their form of delivery as many AA have been reported to be more efficiently absorbed in the form of peptides than as free AA (Silk *et al.*, 1975; Erickson & Kim, 1990; Rérat *et al.*, 1992; Rérat, 1995). Interestingly, when carbohydrates were added to the solution, differences in the absorption rates between molecular AA forms were lowered (Rérat, 1995).

Free AA can be absorbed via facilitated transporters ( $\text{Na}^+$ -independent), via simple diffusion through the membrane (Erickson & Kim, 1990), especially when the intraluminal AA concentration is high, and more importantly via active transporters ( $\text{Na}^+$ -dependent). Firstly, based on the AA group specificity (Rérat *et al.*, 1976), the system description appears to be more complex because of overlapping substrate specificities, as shown in **Table 8**, and also because of differences among species (Erickson & Kim, 1990).

**Table 8.** Amino acid transport systems of the intestine. Adapted from Kilberg *et al.* (1993).

<b>System</b>	<b>Membrane domain</b>	<b><math>\text{Na}^+</math>-dependent</b>	<b>Typical substrates</b>
B	Apical	Yes	Threonine, Alanine
ASC	Apical	Yes	Alanine, Glycine
IMINO	Apical	Yes	Proline, Pipecolic acid
$\beta$	Apical	Yes, with Cl	$\beta$ -Alanine
$\text{X}_{\text{AG}}^-$	Apical	Yes, with Cl	Glutamate, Aspartate
A	Apical and basolateral	Yes	MeAIB <sup>1</sup>
$\gamma^+$	Apical and basolateral	No	Arginine, Lysine
$\text{b}^{0,+}$	-	No	Lysine, Leucine
L	Apical and basolateral	No	Phenylalanine, Leucine

<sup>1</sup> MeAIB: methylamino-isobutyrate.

Additionally, an active transport system for di/tri-peptides ( $\text{H}^+$ -dependent PepT1) has been identified exclusively in the small intestinal tissues of various species (Fei *et al.*, 1994; Doring *et al.*, 1998; Daniel, 2004). There is no specificity towards AA; however, chain length, rather than the molecular volume, appears to be the limiting factor

(Eastwood, 2003b). Weak signals of another peptide transporter (PepT2) have been identified in the rabbit colon (Doring *et al.*, 1998) but this remains to be proved in humans.

Although most absorption of N has been reported to occur in the proximal intestine (Nixon & Mawer, 1970; Gausserès *et al.*, 1996; Mahé *et al.*, 1996), the distal regions of the small intestine may also play an important role, especially regarding absorption of N as di/tri-peptides (Erickson *et al.*, 1995). Indeed, the ileal expression of PepT1 was found to be twice as high as the duodenal expression (Ziegler *et al.*, 2002).

The activities of the L-AA or di/tri-peptide transporters are responsive to nutritional conditions, and especially to dietary protein (Erickson *et al.*, 1995; Ogihara *et al.*, 1999; Ferraris & Carey, 2000). Fasting or malnutrition may enhance nutrient absorption relative to mucosal mass through a variety of mechanisms, including increased transporter gene expression (Ogihara *et al.*, 1999; Ferraris & Carey, 2000). Some authors reported a modulation of the intestinal N absorption through the form of delivery of dietary AA when diets were administered for at least 3 days (Kilberg *et al.*, 1993; Shiraga *et al.*, 1999; Daniel, 2004). In contrast, Ferraris *et al.* (1988) did not observe any difference in N uptake when mice received either peptides or the corresponding free AA for 14 days.

The absorption of AA or di/tri-peptides in the large intestine is generally agreed to be minimal (Just *et al.*, 1981; Darragh *et al.*, 1994), but can not be completely excluded (Fuller & Reeds, 1998; Blachier *et al.*, 2007) as biochemical evidence has demonstrated the presence of colonic AA or di/tri-peptide transporters (Ugawa *et al.*, 2001; Ziegler *et al.*, 2002). This is also supported by the appearance of microbial AA in the systemic circulation (Niiyama *et al.*, 1979; Torrallardona *et al.*, 1996; Metges *et al.*, 1999a); however, the site of microbial AA absorption is not known with certainty (Torrallardona *et al.*, 2003b). In addition, the nutritional significance of this putative colonic AA absorption is still to be determined (Blachier *et al.*, 2007).

### **3. Ileal nitrogen losses**

N recovered at the end of the small intestine is the net result of the overall dynamics of nitrogenous compounds occurring along the digestive tract. Endogenous N has been reported to be up to 80% reabsorbed by the terminal ileum and up to 90% by the end of the digestive tract in pigs (Krawielitzki *et al.*, 1990; Souffrant *et al.*, 1993). Dietary N ingested in human diets is usually absorbed at more than 70% (FAO/WHO, 1991).

Ileal endogenous N can arise from different compounds. Basal or minimal endogenous N flow (ENFL) was reported to include 47% (Chacko & Cummings, 1988) and up to 60–80% proteic N when determined in humans and animals, respectively, given protein-free or low-protein diets for 6–10 days (Moughan & Schutttert, 1991; Butts *et al.*, 1992a). The human proteic N fraction might have been underestimated as it was determined following trichloroacetic acid precipitation which is incomplete (Moughan *et al.*, 1990; Butts *et al.*, 1991). In humans fed a rapeseed protein isolate, 79% of ileal endogenous N was proteic, as determined after ethanol precipitation (Bos *et al.*, 2007). Ileal endogenous N also arises from partially digested endogenous proteins or unabsorbed endogenous AA, which have been reported to contribute 11–27% of the basal ileal endogenous N (Moughan & Schutttert, 1991; Butts *et al.*, 1992a; Leterme *et al.*, 1996a) in animals fed a protein-free diet. Values from Butts *et al.* (1992a) and Leterme *et al.* (1996a) (20–27%) may have been overestimated as digesta autolysis was not prevented.

Non-amino N was reported to make a small contribution to basal ileal endogenous N. Obligatory ileal losses of urea amounted to 0.8–1.8% and up to 4.4% of the total ENFL in animals (Moughan & Schutttert, 1991; Butts *et al.*, 1992a) and humans (Chacko & Cummings, 1988), respectively, fed low-protein diets for more than 6 days. Ammonia N accounted for 1.1% of basal ileal endogenous N in humans (Chacko & Cummings, 1988). The AA composition of basal ENFL has been reported to be relatively constant, independent of the diet, the method used to determine endogenous flows and the level of N flows (Boisen & Moughan, 1996), but is subject to individual variation (Boisen & Moughan, 1996; Hess & Sève, 1999). The most abundant AA in basal ileal ENFL in animals and in humans appear to be the dispensable AA serine, proline, glycine, aspartic acid and glutamic acid and the IAA threonine as reported previously in pigs and in humans (Gaudichon *et al.*, 2002). The AA compositions of basal ileal ENFL and of the major proteic secretions in pigs are compared in **Table 9**. Serine, proline, threonine and, to a lesser extent, glycine, aspartic acid and glutamic acid constitute a large proportion of mucus glycoprotein (Neutra & Forstner, 1987; Lien *et al.*, 1997b), showing the high presence of mucins in ileal losses, mostly due to their resistance to enzymatic hydrolysis (Moughan & Schutttert, 1991; Stein *et al.*, 1999; Montagne *et al.*, 2000). Glycine, the most abundant AA in biliary secretions (Juste, 1982; Souffrant, 1991), escapes reabsorption as deconjugated glycine (Li & Sauer, 1994). Aspartic acid, glutamic acid and serine are present in relatively high proportions in pancreatic and intestinal secretions

(Buraczewska, 1979); however, Asche *et al.* (1989) reported that gut endogenous enzymes are hydrolysed rapidly, because little soluble protein corresponding to the MW of pancreatic enzymes was detected in ileal digesta. Taverner *et al.* (1981) commented that the high amounts of proline, glycine, glutamic acid and aspartic acid recovered in ileal digesta might result from their slow intestinal absorption.

**Table 9.** Comparison of the amino acid (AA) composition of ileal endogenous nitrogen (N) with endogenous nitrogenous secretions in the small intestine of pigs.

	Ileal	Source of N			
	endogenous N	Pancreas	Bile	Ileal bacteria	Mucins
Reference	Jansman <i>et al.</i> , 2002	Corring <i>et al.</i> , 1984	Juste, 1982	Dugan <i>et al.</i> , 1994	Lien <i>et al.</i> , 1997
	g/100 g AA	g/100 g AA	g/100 g AA	g/100 g AA	g/100 g AA
Threonine	6.1	5.2	0.3	4.8	16.4
Valine	4.9	7.2	0.3	6.5	5.9
Isoleucine	3.5	5.9	0.2	6.5	3.0
Leucine	5.4	8.3	0.4	9.9	5.7
Phenylalanine	3.8	4.4	0.2	6.6	3.5
Tyrosine	3.6	5.7	0.2	4.6	3.2
Lysine	4.3	5.1	0.3	8.0	2.8
Histidine	1.9	2.6	0.2	2.6	1.7
Serine	6.1	6.9	0.3	5.1	10.9
Glutamic acid	12.2	10.3	1.1	9.5	10.1
Alanine	5.4	5.4	-	6.4	7.4
Proline	15.8	5.0	0.3	-	12.0
Arginine	4.8	5.1	0.3	7.3	3.5
Aspartic acid	8.4	12.5	0.4	14.5	7.8
Glycine	10.3	6.2	95.0	4.8	5.5
Methionine	1.2	1.1	0.1	1.2	0.8
Cysteine	2.1	3.4	0.6	1.9	-

Basal ileal endogenous N and IAA losses (Rowan *et al.*, 1993; Fuller & Garlick, 1994; Moughan *et al.*, 2005) represent a significant proportion of the daily dietary requirement, being 11% for N and 9–19% for IAA, except for threonine for which the ileal loss amounts to some 28% of requirement (FAO/WHO/UNU, 2007). Whether endogenous N entering the colon represents an actual loss for the body is still under discussion, depending on the colonic capacity to (re)absorb AA and on the nutritional significance of this reabsorption (Blachier *et al.*, 2007), as discussed in the preceding section.

### III. Determination of ileal nitrogen flows

This section describes the methodologies available to allow collection of ileal digesta samples and to differentiate endogenous N from dietary N.

#### 1. Ileal digesta collection

##### 1.1. Marker

When a total digesta collection cannot be undertaken, non-absorbable markers have to be used to allow the sample to be extrapolated to a dietary intake or a total intestinal effluent flow). Ideally, a marker should be neither digested nor absorbed through the gastrointestinal tract; it should be inert; it should have the same dissolving properties as the substance under study and should have a homogeneous distribution within the intestinal lumen; and the method for its analysis in samples should be sensitive and specific (Modigliani *et al.*, 1973; Kozloski *et al.*, 1998). Although in fact there are no such markers, substances meeting most of criteria have been used.

Chromic oxide ( $\text{Cr}_2\text{O}_3$ ) and titanium dioxide ( $\text{TiO}_2$ ) have been widely used as dietary markers in animal digestibility studies. Jagger *et al.* (1992) demonstrated that  $\text{Cr}_2\text{O}_3$  had a lower faecal recovery (quantity collected from a total collection of faeces expressed as a proportion of that ingested, an important indicator of the marker reliability) than  $\text{TiO}_2$  (75 versus 98%) and that  $\text{TiO}_2$  induced lower standard errors for apparent ileal N digestibility than  $\text{Cr}_2\text{O}_3$ . An accurate and reproducible method for  $\text{TiO}_2$  determination was proposed by Short *et al.* (1996). In addition, carcinogenic properties of  $\text{Cr}_2\text{O}_3$  have been reported (Peddie *et al.*, 1982; Jagger *et al.*, 1992). Overall,  $\text{TiO}_2$  has been suggested as a more appropriate marker for animal digestibility studies (Jagger *et al.*, 1992). Acid-insoluble ash or celite (diatomaceous earth) has also been proposed as a reliable marker that can be used in both animals and humans (Rowan *et al.*, 1991; Kavanagh *et al.*, 2001). However, the large amount of digesta sample (1.5–2 g) required for analytical determination is a limiting factor (1993).

When digesta are collected in humans through a naso-intestinal tube, the sample data are extrapolated using an estimate of the effluent flow rate, determined according to the dilution of a non-absorbable soluble marker perfused 20 cm above the sampling site. Liquid phase markers such as phenolsulphonphthalein (phenol red) or polyethylene

glycol (PEG)-4000 have been reported to be adequate markers for this purpose (Modigliani *et al.*, 1973) and have commonly been used in human protein digestion studies (Gausserès *et al.*, 1996; Gaudichon *et al.*, 1999; Gaudichon *et al.*, 2002; Mariotti *et al.*, 2002; Bos *et al.*, 2007).

## 1.2. Ileal digesta collection in humans

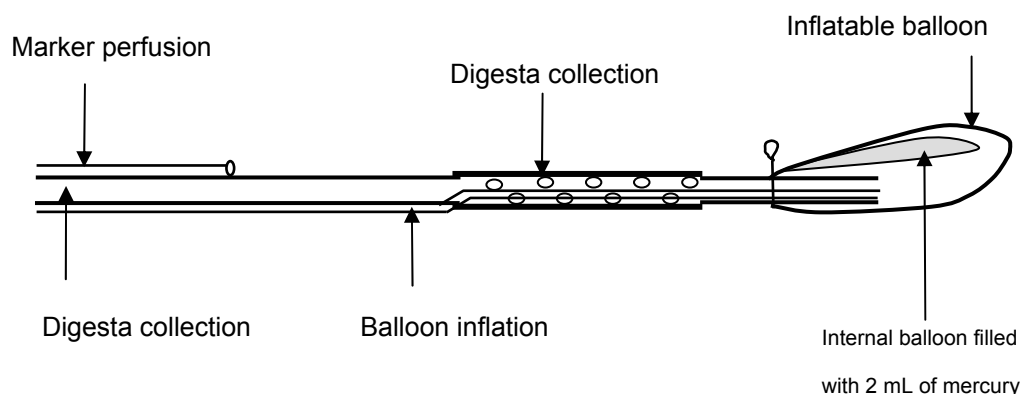
### 1.2.1. *Ileostomates*

Ileal digesta can be collected directly in subjects who have undergone an ileostomy, usually due to ulcerative colitis (Sandberg *et al.*, 1981; Chacko & Cummings, 1988; Fuller *et al.*, 1994; Rowan *et al.*, 1994). After colectomy, the distal small bowel (5–10 cm) is attached to the abdominal wall as a fistula, which allows a total and direct collection of ileal digesta. However, the validity of this approach has been questioned because ileostomates may have some alteration in their digestive function, and this may vary according to the exact disease that required ileostomy and the nature of the surgical intervention. The other limitation of this approach is in the microbial colonization of the terminal ileum that occurs after surgery. The total number of organisms recovered in ileal effluents has been reported to be up to 80 times that in normal ileal contents (Gorbach *et al.*, 1967). However, Fuller *et al.* (1994) showed that a 2-day antibiotic treatment (suppressing intestinal microbial activity) of ileostomized subjects did not modify either their N flow or their DM flow, and that AA flows were reduced at most to approximately 15%. Furthermore, colectomy induces adaptations in numerous physiological and metabolic processes (Christl & Scheppach, 1997). Data from ileostomates thus need to be interpreted with caution.

### 1.2.2. *Naso-intestinal intubation*

The intubation method (Schedl & Clifton, 1961; Modigliani *et al.*, 1973; Mahé *et al.*, 1992) allows the sampling of intestinal digesta from human volunteers with a normal digestive tract. It consists of inserting a triple-lumen polyvinyl chloride (PVC) tube (**Figure 1**) through the nose so as to progress up to the terminal ileum aided by peristaltic movements and by a terminal inflatable balloon containing mercury. Intestinal contents are continuously collected by siphoning or slight aspiration through the distal opening of the tube. The validity of this approach has been determined by the workers in France (Mariotti and Bos, personal communication), by adding a marker (*i.e.* PEG-4000) to the

meal and determining its recovery in digesta samples. To date, such results have not been published nor the method fully validated



**Figure 1.** Naso-intestinal tube used for ileal digesta collection in humans.

The effect of intubation on the transit rate of food remains unknown. Results in the literature on the effect of an intestinal tube on gastric emptying are conflicting, reporting either no effect (Longstreth *et al.*, 1975; Muller-Lissner *et al.*, 1982) or a delay in gastric emptying (Read *et al.*, 1983; Fone *et al.*, 1991; Medhus *et al.*, 1999) with a shortening of the small intestine transit time (Read *et al.*, 1983). However, the transit rate of food has been demonstrated to have no or little impact on overall protein digestibility (Huge *et al.*, 1995; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000b). It is possible that intubation influences the metabolism of absorbed AA, but this has not been determined.

A few studies have compared the intubation method with other methods available for ileal digesta collection. Langkilde *et al.* [1994, quoted by (Champ *et al.*, 2003b)] observed that there was 20% less resistant starch in ileal digesta from ileostomates than that in digesta from intubated subjects. This could be due to either an underestimation by the ileostomy method or an overestimation by the intubation method. In contrast, Noah *et al.* (1998) reported a lower quantity of resistant starch in ileal digesta from intubated pigs (tube inserted 40 cm before the ileo-caecal valve) compared with that from T-cannulated pigs. However, the digesta samples were qualitatively representative of the total digesta reaching the large intestine (Noah *et al.*, 1998).

Both methods available to collect human ileal digesta present drawbacks; however, the intubation method offers the advantage of allowing digesta collection from a healthy intact digestive tract and has thus been accepted as a valid method (Champ *et al.*, 1998; Noah *et al.*, 1998; Gaudichon *et al.*, 1999; Mariotti *et al.*, 1999; Bos *et al.*, 2007). It remains for the method to be formally validated.

### 1.3. Ileal digesta collection using animal models

#### 1.3.1. *Anaesthesia*

Ileal digesta can be collected either from a deeply anaesthetized animal (*e.g.* pig) or immediately following death (*e.g.* rat) of animals; the intestinal tract is removed and ileal digesta are usually sampled in the last 20 cm of the small intestine. These methods offer advantages of simplicity, minimal disturbance of the digestive tract pre-sampling and avoiding the need for surgery. In pigs receiving a single meal, digesta have been collected up to 9 h after feeding (Nyachoti *et al.*, 1997a). In rats fed a single meal for 3 h, digesta collection 3–4 h after the start of feeding was reported to be the optimum sampling time (Butts *et al.*, 2002). A disadvantage of the so-called “slaughter” method is the need for a dietary marker, as total digesta collection is not possible. The slaughter method may be criticized as samples represent only a short part of the feeding cycle. However, Donkoh *et al.* (1994) did not report any difference for ileal protein digestibility determined in digesta collected either punctually after pig euthanasia (9 h after the meal) or continuously in T-cannulated pigs (for 10 h after the meal).

An alternative approach with the slaughter method is that digesta can be collected from animals after being fed hourly (five to eight hourly meals, frequent feeding regimen) to ensure a more continuous flow of digesta along the digestive tract, and to allow the collection of a more representative sample (James *et al.*, 2002a; Hodgkinson *et al.*, 2003; Rutherford & Moughan, 2003). Another disadvantage of the slaughter method is that repeated measurements on the same animal, which reduces variability and the number of animals used per trial, are obviously not possible, but multiple measures along the digestive tract can be made in the same animal.

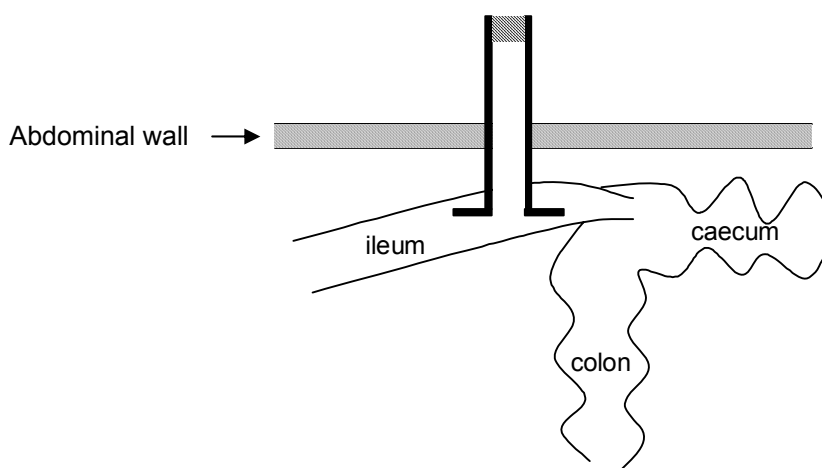
#### 1.3.2. *In vivo sampling*

##### *T-cannula*

The most straightforward cannulation technique is the implantation of a T-shaped cannula in the terminal ileum (**Figure 2**) as first described by Livingstone *et al.* (1977). This is considered to be the least invasive technique and maintains the myo-electric complexes responsible for intestinal motility. The intestinal flow is divided, with one portion diverted through the cannula and the other portion continuing along the intestine. The portion entering the cannula is assumed to be representative of the total digesta flow.



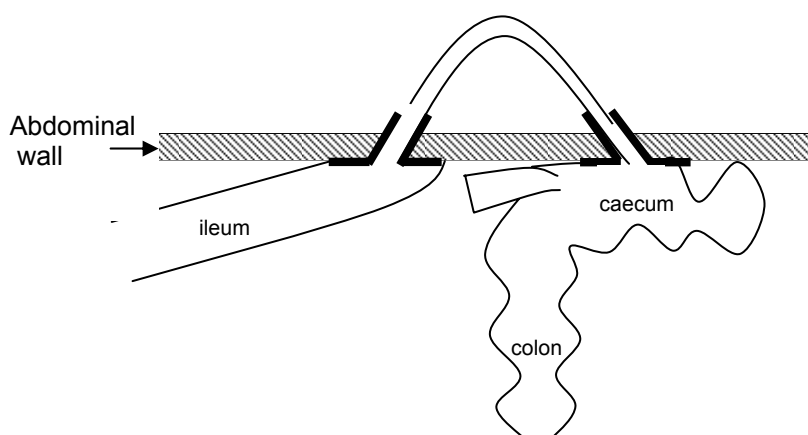
This might not always be so, especially with high-fibre diets (Hodgkinson & Moughan, 2000). As digesta collection is not total, the use of a dietary marker is required; 60–95% of the dietary marker has been reported to be recovered in T-cannulated pigs (Butts *et al.*, 1993b). As noted above, comparisons between T-cannulated pigs and slaughtered pigs resulted in similar protein digestibilities (Donkoh *et al.*, 1994). Comparisons between T-cannulated pigs and intact pigs yielded similar AA digestibilities, except for the digestibilities of methionine, proline and tryptophan (Leterme *et al.*, 1990).



**Figure 2.** T-cannula used in pigs for ileal digesta collection.

#### *Re-entrant cannula*

Another common technique is the use of a re-entrant cannula as first proposed by Cunningham (1962). To implant this cannula, the terminal ileum is transected 15–20 cm from the ileo-caecal junction (**Figure 3**).



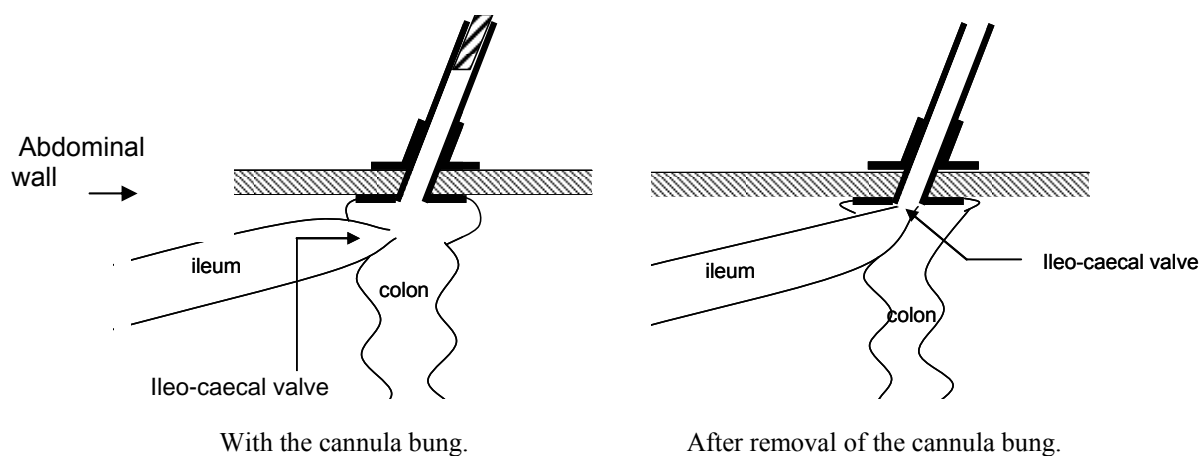
**Figure 3.** Re-entrant cannula used in pigs for ileal digesta collection.

A T-cannula is inserted into the intact ileum and a second cannula is inserted into the caecum. The cannulae are exteriorized and connected with plastic tubing, which is removed for digesta collection. Digesta can then be collected quantitatively, thus avoiding the need for a dietary marker. However, this technique has two major disadvantages: (1) intestinal motility alteration because of the complete transection of the terminal ileum; (2) frequent blockages, when the cannula is in the re-entrant position, for diets that are not finely ground.

A modification of the original re-entrant cannula has been proposed (Darcy *et al.*, 1980), such that the cannula is located immediately distal to the ileo-caecal valve. However, the surgery is traumatic and blockages still occur. Nowadays, this method is used infrequently.

#### *PVTC cannula*

Post-valve T-caecum (PVTC) cannulation (**Figure 4**) involves the removal of approximately two-thirds of the caecum, with the insertion of the cannula into the remaining caecum, opposite to the ileo-caecal valve (van Leeuwen *et al.*, 1991).



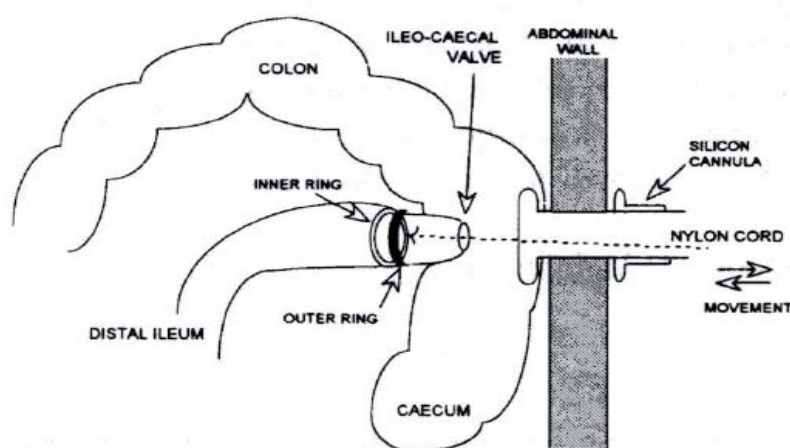
**Figure 4.** Post-valve T-caecum (PVTC) cannula used in pigs for ileal digesta collection. Adapted from van Leeuwen *et al.* (1991).

When the cannula bung is removed, the difference between the intestinal pressure and atmospheric pressure causes the ileo-caecal valve to protrude into the cannula lumen. The major advantage of this technique, above other cannulation methods, is that the small intestine is left intact. Also, if the bore of the cannula is sufficiently large, most blockages can be avoided, thus allowing the use of any diet (Hodgkinson & Moughan, 2000). Dietary marker ( $\text{Cr}_2\text{O}_3$ ) recovery has been reported to range between 72 and 106%

(Kohler *et al.*, 1990; Kohler *et al.*, 1991; Hodgkinson *et al.*, 2000), showing that a representative quantity of digesta is collected. PVTC cannulation has been reported to be without effect on pig metabolism over time (Kohler *et al.*, 1992a; Kohler *et al.*, 1992b). Normal growth was observed (Kohler *et al.*, 1992a). This was true over the entire cannulation period (12 weeks). PVTC cannulation thus appears to be suitable for digesta collection in metabolic studies and has been reported to be the current method of choice (Hodgkinson & Moughan, 2000).

#### *Steered ileal-caecal valve cannula*

A modification of the PVTC cannula is the steered ileal-caecal valve cannula (Mroz *et al.*, 1996). With this method, metal rings are placed around the terminal ileum (**Figure 5**) and a cord attached to the rings is placed through the cannula inserted in the caecum. To allow digesta collection, the cord is gently pulled, moving the terminal ileum into the mouth of the cannula. The advantage of this method over PVTC cannulation is that the caecum is not removed. However, the effect of the presence of the metal rings on the terminal ileum is not clear (Hodgkinson & Moughan, 2000).

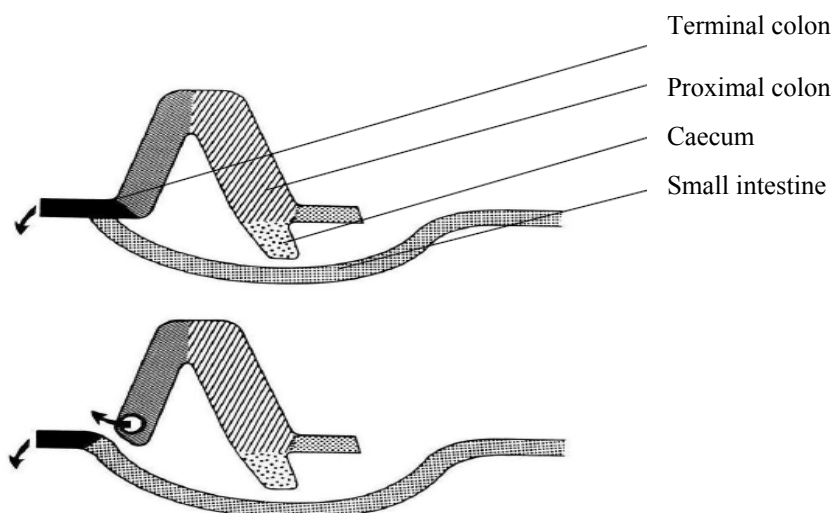


**Figure 5.** Steered ileal-caecal valve cannula used in pigs for ileal digesta collection. From Mroz *et al.* (1996).

#### *Ileo-rectal anastomosis*

Ileo-rectal anastomosis, first proposed by Fuller *et al.* (1982), consists of connecting the terminal ileum to the rectum so as to collect the ileal digesta via the anus. Initially, the large bowel was sutured at the proximal end only (**Figure 6**), thus allowing the elimination of residual faeces and gas via the anus. In order to prevent any influence of the colic microflora on the ileal AA profile, the most common practice now consists of

completely isolating the large bowel and exteriorizing it by a T-cannula so that residual colonic material is removed (Green *et al.*, 1987).



**Figure 6.** Ileo-rectal anastomosis without exteriorization of the large intestine (top figure) or with exteriorization of the large intestine by a T-cannula (bottom figure). From Sauer *et al.* (1989).

The ileo-rectal anastomosis method offers the possibility of maintaining an intact ileo-caecal valve and allows for total digesta collection, thus avoiding the need for a dietary marker. Also, there are no problems of blockages or leakages. Yin *et al.* (1993) demonstrated that this technique gave reasonable estimates of apparent ileal protein digestibility. However, the main drawbacks are the surgical complexity, the long post-surgery recovery period and the need for mineral and vitamin supplementation to compensate for the lack of the large bowel (Lahaye, 2004). Additionally, ileal chyme excretion via the anus frequently induces irritation, which is ethically subject to criticism. Kohler *et al.* (1992b) reported lower growth performance, probably due to the lower N retention observed, but this was not observed by Yin *et al.* (1993). Evidence of an increased intestinal microbial activity in anastomosed pigs (Salgado *et al.*, 2002a) and of a modified metabolism (Kohler *et al.*, 1992b; Salgado *et al.*, 2002a) has been reported, but it is not clear to what extent this influences the estimates of protein digestibility. In summary, the use of this technique seems to be limited.

## 2. Measurement of endogenous nitrogen and amino acid losses

### 2.1. Protein-free diet

When a protein-free diet is fed to animals or humans for several days, all of the N and AA recovered in ileal digesta is of endogenous origin. The use of protein-free diets is known to induce lowered ileal endogenous protein flows compared with protein-containing diets (Darragh *et al.*, 1990; Butts *et al.*, 1993a; Moughan *et al.*, 2005). This is supported by the data found throughout the literature (**Tables 10.a, 10.b** and **11**). In pigs, however, the difference between protein-free and protein-containing diets was not observed when diets contained less than 5% of peptides/proteins (Hodgkinson *et al.*, 2000; Zhang *et al.*, 2005). Feeding a protein-free diet to animals/subjects creates a non-physiological state (Low, 1980) by inducing a negative N balance, possibly leading to a decreased rate of body and possibly gut protein synthesis (Millward & Garlick, 1976). In addition, the protein-free diet might lack the protein or peptide stimulatory effect on endogenous gut protein secretions (Skilton *et al.*, 1988; Butts *et al.*, 1993a; Donkoh *et al.*, 1995). Some authors have reported higher endogenous losses of proline in pigs fed a protein-free diet (Taverner *et al.*, 1981; de Lange *et al.*, 1989b; Leterme *et al.*, 1996a; Hodgkinson & Moughan, 2000) or a diet containing a low level of N (Mariscal-Landin & De Souza, 2006). This was suggested to be due to the increased muscle protein catabolism induced by the protein-free status, releasing high amounts of glutamine to be used by the intestines, whereby its metabolism leads to proline release (de Lange *et al.*, 1989b; Mariscal-Landin & De Souza, 2006). However, other pig studies did not report any difference for proline flows (Hess *et al.*, 2000; Zhang *et al.*, 2002). In summary, it is generally agreed that the protein-free diet underestimates gut endogenous protein losses.

**Table 10. a.** Basal ileal endogenous nitrogen and lysine flows as determined in growing pigs using different methods.

Method	Dietary protein intake		Ileal endogenous flows		Reference
			nitrogen	lysine	
	g/100g diet		µg/g dry matter intake		
<b>Protein-free</b>					
	-	0	1556	286	Chung and Baker, 1992
	-	0	1834	354	Mariscal-Landin <i>et al.</i> , 1995
	-	0	1500	252	Butts <i>et al.</i> , 1993a
	-	0	1860	570	Leterme <i>et al.</i> , 1996b
	-	0	1610	407	Leterme <i>et al.</i> , 1996a
	-	0	1410	310	Hess <i>et al.</i> , 1998
	-	0	1753	-	Hodgkinson <i>et al.</i> , 2000
	-	0	1980	390	Hess <i>et al.</i> , 2000
	-	0	1600	310	Zhang <i>et al.</i> , 2002
<b>Protein-free + i.v. AA infusion</b>					
	-	0	2032	560	de Lange <i>et al.</i> , 1989b
	-	0	1840	457	Leterme <i>et al.</i> , 1996a
<b>Regression</b>					
	soyabean isolate	6-17	1428	382	Mariscal-Landin <i>et al.</i> , 1995
	soyabean meal	4-24	2640	470	Fan <i>et al.</i> , 1995
<b>Dietary AA devoid of lysine</b>					
	free AA	9	-	284	Butts <i>et al.</i> , 1993a
<b>Peptide alimentation/Ultrafiltration</b>					
	hydrolyzed casein	10	3700	448	Butts <i>et al.</i> , 1993a
	hydrolyzed casein	10	2851	456	Hodgkinson <i>et al.</i> , 2000
	hydrolyzed casein	10	1543	287	Hodgkinson <i>et al.</i> , 2003
	hydrolyzed casein	11	1817	-	Leterme <i>et al.</i> , 1996a
	hydrolyzed casein	15	2270	280	Morel <i>et al.</i> , 2003
	hydrolyzed casein	18	1490	320	Steendam <i>et al.</i> , 2004a
	hydrolyzed casein	20	3940	880	Yin <i>et al.</i> , 2004
<b>Highly digestible protein</b>					
	casein	5	3110	680	Zhang <i>et al.</i> , 2002
	casein	8	2528	221	Mariscal-Landin <i>et al.</i> , 2006
	casein	16	2273	384	Chung and Baker, 1992
	casein	16	2760	295	Mariscal-Landin <i>et al.</i> , 2006
<b>Homoarginine</b>					
	casein	9	1520	320	Libao-Mercado <i>et al.</i> , 2006
	casein	10	1179	214	Hodgkinson <i>et al.</i> , 2003
	casein	15	4728	-	Roos <i>et al.</i> , 1994
	casein	20	2304	490	Yin <i>et al.</i> , 2004
	casein	21	1160	586	Nyachoti <i>et al.</i> , 1997b
<b>Isotope dilution</b>					
<i><sup>15</sup>N-leucine infusion</i>					
	hydrolyzed casein	18	1950	-	Steendam <i>et al.</i> , 2004a
	soyabean isolate	18	2740	-	Schulze <i>et al.</i> , 1995c
	pea isolate	18	2113	-	Hess <i>et al.</i> , 2000
<i>dietary <sup>15</sup>N</i>					
	hydrolyzed casein	10	1011	-	Hodgkinson <i>et al.</i> , 2003
	casein	15	3736	-	Roos <i>et al.</i> , 1994

**Table 10.b.** Comparison of overall mean ( $\pm$  SD) of published basal ileal endogenous nitrogen and lysine flows determined in pigs using different methods<sup>1</sup>.

	<b>Nitrogen flow</b>	<b>Number of studies</b>	<b>Lysine flow</b>	<b>Number of studies</b>
	$\mu\text{g/g DMI}^2$		$\mu\text{g/g DMI}$	
Protein-free	1678 $\pm$ 63	9	360 $\pm$ 35	8
Protein-free + i.v. AA infusion	1936 $\pm$ 96	2	509 $\pm$ 52	2
Regression	2034 $\pm$ 606	2	426 $\pm$ 44	2
Dietary free AA	-		284	1
Hydrolyzed protein/ ultrafiltration	2516 $\pm$ 381	7	445 $\pm$ 93	6
Highly digestible protein	2668 $\pm$ 178	4	395 $\pm$ 101	4
Homoarginine	2178 $\pm$ 670	5	403 $\pm$ 84	4
<sup>15</sup> N-Leu infusion	2268 $\pm$ 241	3	-	
<sup>15</sup> N-meal	2374 $\pm$ 1363	2	-	

<sup>1</sup> Data from Table 10.a.<sup>2</sup> Dry matter intake**Table 11.** Mean ileal endogenous nitrogen flow as determined in adult humans using different methods.

	<b>Dietary protein intake</b>	<b>n</b>	<b>Nitrogen flow</b>		<b>Digesta</b>	<b>Reference</b>
	g		mg/d	$\mu\text{g/g DMI}^2$		
<b>Protein-free</b>						
	-	5	836	2039	ileostomy	Rowan <i>et al.</i> , 1993 <sup>3</sup>
	-	8	719	1752	ileostomy	Fuller <i>et al.</i> , 1994 <sup>4</sup>
	-	8	845	2061	ileostomy	Moughan <i>et al.</i> , 2005
<i>Overall mean</i>			800	1952		
<b>Hydrolysed protein/ultrafiltration</b>						
	27	8	1736	4233	ileostomy	Moughan <i>et al.</i> , 2005
<b>Isotope dilution/ <sup>15</sup>N dietary protein protein<sup>1</sup></b>						
milk protein	30	7	1638	3995	intubati	Gaudichon <i>et al.</i> , 2002 <sup>3</sup>
lupin-flour protein	28	9	1814	4425	intubation	Mariotti <i>et al.</i> , 2002 <sup>3</sup>
soyabean protein	30	10	1949	4753	intubation	Mariotti <i>et al.</i> , 2000b <sup>3</sup>
soyabean protein	30	6	2150	5245	intubation	Gaudichon <i>et al.</i> , 2002 <sup>3</sup>
pea globulin	30	9	2159	5265	intubation	Mariotti <i>et al.</i> , 2001 <sup>3</sup>
pea globulin and albumin	30	7	2453	5982	intubation	Mariotti <i>et al.</i> , 2001 <sup>3</sup>
<i>Overall mean</i>			2027	4944		

<sup>1</sup> Purified protein source ingested within a liquid meal.<sup>2</sup> Dry matter intake.<sup>3</sup> Data were initially reported as mg/8 h or mmol/8 h. The experimental meal was assumed to represent one-third of the daily intake, and endogenous nitrogen flows were assumed to be similar every 8-h period. The initial values were calculated for an average daily dry matter intake of 410 g (Department for Environment Food and Rural Affairs, 2003; Marriott & Buttriss, 2003).<sup>4</sup> Data were initially reported as mg/d and were converted for a dry matter intake of 410 g.

## 2.2. Free amino acids/protein devoid of one amino acid

The free AA approach consists of feeding semi-synthetic diets containing, as a sole source of N, a mixture of free L-AA devoid of certain dispensable AA, for which the endogenous flows can be determined directly. Thus, endogenous flows are determined in animals or humans who are not in a negative N balance, one of the major criticisms of the protein-free diet. However, endogenous AA flows (EAAFL) have been reported to be similar to those obtained with a protein-free diet (Skilton *et al.*, 1988; Darragh *et al.*, 1990; Butts *et al.*, 1993a), suggesting that the N balance *per se* does not affect the level of endogenous protein flows. This is supported by previous data (Table 10), where endogenous flows were obtained under simultaneous protein-free feeding and intravenous infusion of AA. Nevertheless, to date, EAAFL obtained under free AA alimentation have not been compared with those obtained with diets having the same AA composition but with AA supplied as peptide or protein forms.

Zein, a purified protein from maize, is virtually free of lysine and has been used to determine directly endogenous lysine flows in animals with a positive N balance. A simultaneous intravenous infusion of lysine is performed. Unlike the free AA approach, this method has shown significantly higher ileal endogenous lysine flows than those determined for pigs fed a protein-free diet (Butts *et al.*, 1993a).

## 2.3. Highly digestible purified proteins

Endogenous N flows can be determined using diets containing highly digestible proteins such as casein or wheat gluten (Jansman *et al.*, 2002; Stein *et al.*, 2007), thus allowing a more physiological approach than the protein-free diet. A 100% true digestibility of the ingested protein is assumed, although this might not always be exactly true and might thus lead to some degree of overestimation (Jansman *et al.*, 2002; Stein *et al.*, 2007). However, when data were compared among studies (Table 10), endogenous N and lysine flows determined with highly digestible protein-based diets were in the same range as those determined using methods such as homoarginine, isotope dilution or enzyme-hydrolysed protein/ultrafiltration. This would need to be confirmed using controlled studies but, despite some potential drawbacks, this method is straightforward and can be used in both animals and humans.



## 2.4. Enzyme-hydrolysed protein/ultrafiltration

The enzyme-hydrolysed protein/ultrafiltration method (also referred to as the ultrafiltration method) has been developed, in which dietary peptides and AA (MW < 5 kDa), assumed to be similar to those arising during digestion, are fed to animals or humans (Moughan *et al.*, 1990). This allows ENFL and EAAFL to be determined using diets containing an N source (which overcomes the protein-free diet criticism) and allows endogenous N to be discriminated from dietary N (which overcomes the highly digestible protein method criticism). This method has the advantage of determining both endogenous N and endogenous AA directly after digesta centrifugation and ultrafiltration (MW > 10 kDa). Any undigested dietary peptides are discarded in the low-MW fraction (< 10 kDa). This method might lead to some degree of underestimation as endogenous peptides and AA are also discarded (MW < 10 kDa). N from peptides and free AA (MW < 10 kDa) amounted to 11% (Moughan & Schuttert, 1991) and up to 20–27% (Butts *et al.*, 1992a; Leterme *et al.*, 1996a) of the total N collected in ileal digesta from animals fed a protein-free diet. The latter data may have been overestimated because digesta autolysis was not prevented. Questions have also been raised as to whether potentially bioactive peptides present in the hydrolysate may enhance the loss of AA from the small bowel (Jansman *et al.*, 2002; Yin *et al.*, 2004; Stein *et al.*, 2007), over and above the loss that may be found with the corresponding intact protein. Most studies (Butts *et al.*, 1991; Butts *et al.*, 1993a; Hodgkinson *et al.*, 2000; Moughan *et al.*, 2005) reported higher ENFL in animals fed dietary peptides, but this was in comparison with protein-free or synthetic AA-based diets. When casein has been compared with hydrolysed casein, conflicting results have been reported: whereas Yin *et al.* (2004) found higher ileal ENFL and EAAFL in pigs fed hydrolysed casein, Ravindran *et al.* (2004) observed similar endogenous AA flows in chickens fed hydrolysed or intact casein. However, none of these comparisons was conservative, because different casein sources and different methods (homoarginine versus enzyme-hydrolysed protein/ultrafiltration) were used between diets. Indirectly, it appears, from independent pig and human studies (Tables 10 and 11), that endogenous N and lysine flows determined using the enzyme-hydrolysed protein/ultrafiltration method are in the same range as those determined using intact casein. In contrast, Jansman *et al.* (2002) concluded in their review that the ultrafiltration method yielded higher estimates than intact protein methods based on 2 and 11 studies, respectively. Thus, controlled studies need to be conducted to compare directly the

impact of feeding hydrolysed protein and intact protein from the same source on ileal endogenous protein flows.

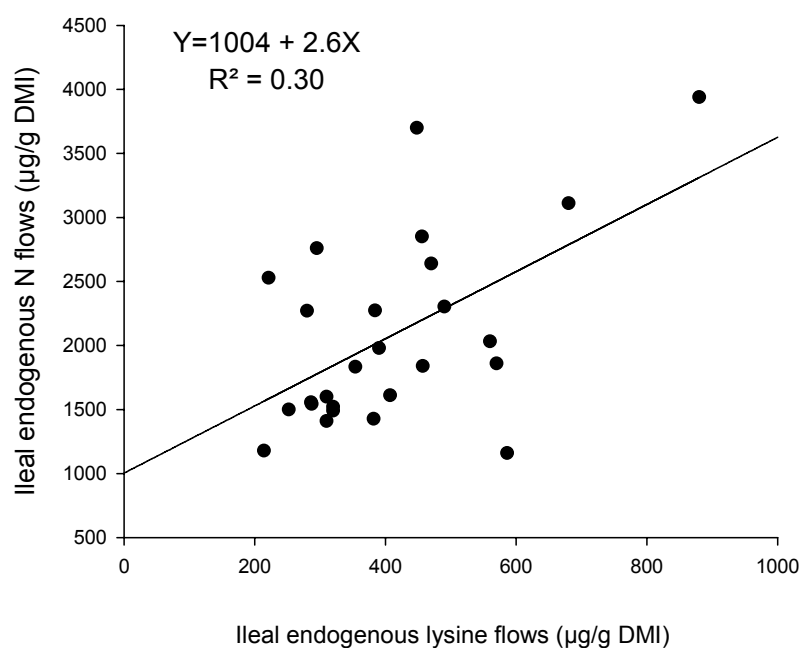
## 2.5. Regression

The linear regression method consists of feeding animals with graded levels of dietary protein (at a constant DMI), so that ileal ENFL at zero dietary protein intake can be determined by extrapolating N flows. This approach was believed to provide an estimate of ENFL under more physiological conditions than those obtained with protein-free diets (Fan *et al.*, 1995); however, several studies have since shown that the estimates are not different from those determined using a protein-free diet (Furuya & Kaji, 1989; Donkoh *et al.*, 1995). As shown by Fan *et al.* (1995), a wide range of levels of protein intake (4–24%) need to be applied so that the standard errors can be minimized. Also, mathematical extrapolation can lead to estimation errors (Hodgkinson & Moughan, 2000). Finally, it seems to be difficult to apply this method in humans because of the need for repeated measures of ileal N losses.

## 2.6. Guanidination

This method involves the transformation of dietary lysine with the agent O-methylisourea to homoarginine, a lysine derivative that does not occur naturally in proteins (Hagemeister & Erbersdobler, 1985; Rutherfurd & Moughan, 1990). Endogenous lysine can then be distinguished from undigested dietary homoarginine in ileal digesta. The homoarginine method can be applied indirectly by partially guanidinating the protein source (Hagemeister & Erbersdobler, 1985) or directly by completely guanidinating the protein source (Rutherfurd & Moughan, 1990). Both approaches rely on several assumptions that have been confirmed, as discussed earlier (Hodgkinson & Moughan, 2000). This method has the advantage of determining endogenous losses under protein feeding and can be applied to a wide variety of protein sources, thus allowing determination of basal and specific losses (Nyachoti *et al.*, 1997b; Caine *et al.*, 1998). However, the impact of the guanidination on the activity of antinutritional factors present in the protein source has not been fully tested, although it is assumed to be negligible because of the mild reaction involved with the guanidination (Hodgkinson & Moughan, 2000). Nevertheless, the major drawback is that this method allows the endogenous flow of lysine only to be determined directly; the endogenous flows of other AA and N are

determined based on assumed endogenous N or AA:lysine ratios, which seems to be invalid (**Figure 7**). Finally, a further disadvantage of this method is the accumulation of part of the ingested homoarginine in the liver, which allows the guanidinated proteins to be fed only once to animals and means the method cannot be used in humans (Hodgkinson & Moughan, 2000; Fuller & Tomé, 2005).



**Figure 7.** Relationship between ileal endogenous lysine and nitrogen flows in growing pigs. Data from 26 studies quoted in Table 10.a.

## 2.7. Isotope dilution

The use of stable isotopes allows the determination of endogenous protein flows under protein alimentation based on the determination of the dilution of the tracer in the digesta. The most common tracer is  $^{15}\text{N}$ , but  $^{13}\text{C}$  has also been used (Arentson & Zimmerman, 1995). The use of a tracer serves not only for the determination of ENFL but also to improve the understanding of AA digestion and utilization. The isotope dilution method has the advantage of being applicable to both animals and humans.

### 2.7.1. *Labelled endogenous protein*

The isotope dilution method can be used by labelling the body N pool in animals (de Lange *et al.*, 1990; Schulze *et al.*, 1995a) or in humans (Gaudichon *et al.*, 1994a; Gaudichon *et al.*, 1996). This can be achieved by prolonged administration (> 5 d) of a

labelled AA, usually  $^{15}\text{N}$ -leucine, so that other endogenous AA can be freely labelled by transamination up to a steady state.

Tracer administrations by a continuous intravenous infusion (de Lange *et al.*, 1992; Schulze *et al.*, 1995a; Lien *et al.*, 1997c) and by pulse-oral dose (Steendam *et al.*, 2004b) have been reported to give similar ENFL (Steendam *et al.*, 2004b). Ileal ENFL are determined based on isotope dilution in digesta relative to the precursor pool, generally the de-proteinized fraction of blood plasma. However, the choice of precursor pool might influence the final result, as discussed earlier (Fuller & Reeds, 1998). The tracer dilution can be determined from its isotopic enrichment in total N or, if possible, in individual AA, as non-uniform labelling of endogenous AA has been reported (de Lange *et al.*, 1992; Lien *et al.*, 1997a; Lien *et al.*, 1997c). In addition, the total N enrichment in the proteic fraction of plasma, obtained after trichloroacetic acid precipitation, might be diluted by urea and ammonia, leading to an overestimation of ileal ENFL (de Lange *et al.*, 1992; Lien *et al.*, 1997a). When the isotope dilution is estimated from a single AA, a constant AA profile of ileal endogenous protein has to be assumed. In contrast, endogenous secretions, arising from unlabelled dietary AA rapidly metabolized in the gut and subsequently re-excreted, are not included, resulting in an underestimation of ENFL (Fuller & Reeds, 1998). Despite its shortcomings, this method offers the possibility of assessing the influence of different types of diets on ENFL. Furthermore, it has provided important information regarding the quantitative contributions of endogenous secretions along the digestive tract (Krawielitzki *et al.*, 1990; Souffrant *et al.*, 1993; Krawielitzki *et al.*, 1994). In pigs, ileal ENFL determined using the  $^{15}\text{N}$ -infusion method have been reported to give higher ENFL compared with the protein-free method (de Lange *et al.*, 1990), but lower (Hodgkinson *et al.*, 2003) or similar (Schulze *et al.*, 1995a) estimates compared with the ultrafiltration method. According to some authors, this method has received major criticism and is considered to be inappropriate for determining ENFL (Nyachoti *et al.*, 1997b; Leterme *et al.*, 1998).

### 2.7.2. ***Labelled dietary protein***

Dietary protein can be labelled by applying  $^{15}\text{N}$ -labelled fertilizers or salts to growing plants (Leterme *et al.*, 1996b; Mariotti *et al.*, 1999) or by infusing  $^{15}\text{N}$ -labelled salts into the rumen of lactating cows (Mahé *et al.*, 1994a). Uniform labelling of the dietary protein is expected. It is assumed that non-labelled AA and labelled AA exert similar metabolisms and that, during the course of the experiment, no labelling of endogenous

AA from the recycling of labelled dietary AA within the splanchnic bed occurs. It is known that luminal dietary N is used for gut protein synthesis (Alpers, 1972; Stoll *et al.*, 1998b) and may be, subsequently, re-excreted, thus leading to some recycling (Roos *et al.*, 1994). Leterme *et al.* (1996b) detected, in pigs fed a  $^{15}\text{N}$ -labelled meal, some  $^{15}\text{N}$  in the blood within 10 min, in pancreatic enzymes within 50 min, in bile secretion within 90 min and in ileal mucins within 4 h. However, limiting the digesta collection to 8 h postprandially is likely to reduce the contamination of endogenous protein losses (Leterme *et al.*, 1996b). Despite possible recycling, endogenous protein flows were reported to be higher with the isotope dilution method than with the protein-free method (Hess *et al.*, 2000); however, antinutritional factors and fibres included in the  $^{15}\text{N}$ -labelled meal may have had a confounding effect. In controlled studies, the isotope dilution method was reported to induce lower ileal ENFL compared with the homoarginine method (Roos *et al.*, 1994) or the ultrafiltration method (Hodgkinson *et al.*, 2003). This was assumed to be due to the rapid incorporation of dietary  $^{15}\text{N}$  into gut endogenous protein subsequently excreted and lost at the terminal ileum. However, among different studies, the method appears to give estimates of ENFL in the same range as those determined with other protein-containing diets (Table 10). The isotope dilution method is thus considered to be a valid method for the study of endogenous protein losses (Mahé *et al.*, 1994b; Hess *et al.*, 2000; Gaudichon *et al.*, 2002). In addition, using labelled protein is of great interest as it allows both true digestibility, for which the final error might be small (Fuller & Tomé, 2005), and dietary N utilization, considered to be an accurate indicator of protein quality (see Section I.2.2), to be calculated.

In summary, all methods available to determine endogenous protein losses have advantages and disadvantages. Although the protein-free method is known to underestimate ENFL, it has the advantage of being straightforward and has less associated variability than other methods. Whereas the ultrafiltration method offers a good alternative for routine assay, it has been criticized for possible enhanced ENFL. This needs to be investigated further. Among tracer methods, the use of labelled dietary protein is technically easier and requires fewer assumptions than the  $^{15}\text{N}$  infusion method. The isotope dilution method with labelled dietary protein thus appears to be a method of choice, especially for human studies, for which methods are limited.

## IV. Dietary modulation of ileal endogenous nitrogen losses

Information regarding the influence of dietary factors on gut ENFL and EAAFL has been mainly obtained in pig studies (Boisen & Moughan, 1996; Nyachoti *et al.*, 1997a).

### 1. Dry matter intake

ENFL and EAAFL are considered to be related primarily to the physical flow of food dry matter through the digestive tract, as reviewed previously (Boisen & Moughan, 1996; Fuller & Tomé, 2005; Stein *et al.*, 2007). Ileal ENFL and EAAFL, when expressed on a daily basis, have been reported to increase linearly with the feeding level. This has been observed in pigs fed a protein-free diet (Hess & Sève, 1999; Moter & Stein, 2004) and in animals fed a 10%-hydrolysed casein diet (James *et al.*, 2002b). An increase in the feeding level from 50 to 90 g/kg BW<sup>0.75</sup> induced an increase in ileal ENFL from 1.0 to 1.9 g/d in anastomosed pigs (Hess & Sève, 1999). When pigs were fed at a level equal to one to three times the estimated energy requirement for maintenance, ileal ENFL rose from 5.6 to 7.8 g/d (Moter & Stein, 2004). Similar observations were made in pigs and rats. According to Hess *et al.* (1999) and Moter *et al.* (2004), the effect of the DMI was quantitative only as it did not affect the AA composition of the endogenous protein losses, unlike the effect observed by Stein *et al.* (1999).

As noted by Nyachoti *et al.* (1997a), the effect of DMI is closely related to the animal BW. Small pigs have been reported to have higher ileal ENFL than larger pigs (Leterme & Thewis, 2004; Mariscal-Landin & De Souza, 2006). The BW effect was limited when the pig BW was above 50–60 kg according to Leterme *et al.* (2004) or above 100 kg according to Stein *et al.* (1999). In addition, ileal ENFL have been reported to be more related to BW than to DMI *per se* at low feed intake levels (< 70 g/kg BW<sup>0.75</sup>) but to be more closely related to DMI at high feed intake levels (Mariscal-Landin *et al.*, 1995; Hess & Sève, 1999).

Until more information is available, ENFL and EAAFL are expressed on a DMI basis (Jansman *et al.*, 2002; Stein *et al.*, 2007), even though an increase in the feeding level decreased ENFL and EAAFL linearly when expressed as g/kg DMI (Stein *et al.*, 1999; Hess & Sève, 1999; Moter & Stein, 2004). For more accuracy, it has been suggested that endogenous protein losses be determined at levels of feed intake close to the voluntary feed intake of animals (Jansman *et al.*, 2002; Moter & Stein, 2004; Stein *et al.*, 2007).

However, in humans, ENFL are commonly expressed on a daily basis (Chacko & Cummings, 1988; Fuller *et al.*, 1994; Gaudichon *et al.*, 2002), thus allowing them to be related to the daily protein requirement.

## 2. Dietary protein

### 2.1. Protein quantity

The dietary inclusion of protein has been reported to increase the ileal endogenous N and AA flows, compared with a protein-free diet (Moughan & Rutherfurd, 1990; Zhang *et al.*, 2002). When graded levels of protein were fed to animals, ileal ENFL and EAAFL were reported to increase; however, different types of responses were observed. At low levels of dietary protein (50 g/kg diet), a diet based on purified guanidated soyabean protein induced a similar ileal endogenous flow of lysine, determined using the homoarginine method, to a protein-free diet (Zhang *et al.*, 2005). Libao-Mercado *et al.* (2006) reported similar ENFL for pigs fed 4 and 9% of guanidated casein. A similar observation was made in pigs fed a protein-free diet or a diet based on 50 g hydrolysed casein/kg diet, when ENFL were determined after digesta centrifugation and ultrafiltration (Hodgkinson *et al.*, 2000). In contrast, Zhang *et al.* (2002) reported higher endogenous flows in pigs fed diets containing 50 g casein/kg diet than in pigs fed a protein-free diet. It should be noted that endogenous protein flows were determined assuming 100% true digestibility for casein. A true ileal digestibility of 95% (de Vrese *et al.*, 2000; Gaudichon *et al.*, 2002) would explain the difference observed between the diets.

When the dietary protein intake was increased from 0 to 250 g/kg diet, the response of endogenous lysine flow in pigs followed a sigmoidal curve with similar lysine flow between 0 and 50 g protein/kg diet and with a plateau reached from 125 g protein/kg diet (Zhang *et al.*, 2005). A similar pattern was reported in rats fed 50–200 g zein/kg diet: the endogenous lysine flow increased sharply from 340 to 595 µg/g DMI when the dietary intake of zein was increased from 100 to 150 g/kg diet (Hodgkinson & Moughan, 2007). The increased ENFL might result from an increase in protein secretions from the pancreas (Zhang *et al.*, 2005), previously shown to be induced by an increased dietary protein intake (Partridge *et al.*, 1982; Hee *et al.*, 1988b). It is possible that a limiting secreting ability occurs when the dietary protein intake is above 100–150 g/kg diet (Zhang *et al.*, 2005). Other authors have reported a linear relationship between ENFL and

dietary protein intake. In pigs receiving diets containing 60–250 g casein/kg diet, ENFL increased from 2000 to 2800  $\mu\text{g N/g DMI}$  (Pedersen *et al.*, 2002). A similar response was observed in pigs fed 50–200 g hydrolysed casein/kg diet (Hodgkinson *et al.*, 2000).

Earlier data have shown that the N balance *per se* does not affect the ENFL when rats or pigs are fed a protein-free diet in comparison with free-AA-based diets (Table 10) or when animals fed a protein-free diet receive a simultaneous intravenous AA infusion (de Lange *et al.*, 1989b; Leterme *et al.*, 1996a). However, the free-AA-based diet has not yet been compared with a protein-based diet with similar AA composition.

## 2.2. Protein source

A limited number of controlled studies have assessed the effect of protein source *per se* on ileal endogenous protein flows. In pigs, purified gelatin versus casein or isolated soyabean protein (Moughan & Rutherfurd, 1990) or purified gelatin versus enzyme-hydrolysed casein (Moughan *et al.*, 1990) induced similar endogenous lysine flows. In contrast, human endogenous N flows were reported to be significantly higher with purified soya protein than with milk protein (Gaudichon *et al.*, 2002). Across human studies, it would appear that ileal endogenous N losses tend to be higher with purified vegetable protein (from lupin, pea, soya) than with animal protein (from milk), as reported in Table 11. However, a possible effect of residual antinutritional factors or fibres in vegetable proteins cannot be completely excluded.

## 2.3. Form of delivery of dietary amino acids

Free-AA-based diets were reported to induce similar ileal EAAFL to a protein-free diet when fed to animals (Skilton *et al.*, 1988; Darragh *et al.*, 1990; Butts *et al.*, 1993a), suggesting that the N balance *per se* does not affect the level of ileal endogenous protein flows. This is supported by data obtained in pigs under simultaneous protein-free feeding and intravenous infusion of AA (de Lange *et al.*, 1989b; Leterme *et al.*, 1996a).

The use of dietary peptides with the ultrafiltration technique has raised questions as to whether bioactive peptides potentially present in the casein hydrolysate, may enhance endogenous AA losses from the small bowel (Jansman *et al.*, 2002; Rutherfurd-Markwick & Moughan, 2005; Stein *et al.*, 2007). Milk-derived bioactive peptides have indeed been reported to modulate gastrointestinal secretions (Froetschel, 1996; Shah, 2000; Darragh, 2001; Rutherfurd-Markwick & Moughan, 2005), with some rat studies



showing an enhanced mucus secretion with bioactive peptides (Claustre *et al.*, 2002; Trompette *et al.*, 2003). However, data regarding the influence of dietary peptides *per se* on ileal endogenous protein flows are scarce. Most studies have reported an increased ileal ENFL with dietary peptides in comparison with protein-free or free-AA-based diets (Darragh *et al.*, 1990; Butts *et al.*, 1991; Butts *et al.*, 1993a; Hodgkinson *et al.*, 2000; Moughan *et al.*, 2005). Other studies comparing intact and hydrolysed casein have reported conflicting results. Whereas Yin *et al.* (2004) observed an increased ileal endogenous protein flow with hydrolysed casein in pigs, Ravindran *et al.* (2004) observed similar endogenous protein flows with hydrolysed and intact casein in chickens. Additionally, hydrolysed and intact pea proteins were shown to induce similar ileal ENFL in pigs (Hess *et al.*, 1998); however, the peptide effect might be related to the protein source. The influence of dietary peptides *per se* thus needs to be investigated further.

### **3. Antinutritional factors**

Antinutritional factors, naturally present in foods or formed during processing (*e.g.* heat, alkali treatment), may adversely affect protein digestibility (Gilani & Sepehr, 2003) and, especially when natural, may enhance gut endogenous protein losses (Nyachoti *et al.*, 1997a; Grala *et al.*, 1998a; Jansman *et al.*, 1998; Salgado *et al.*, 2002a). Antinutritional factors, commonly found in animal feedstuffs, have been extensively studied in animals. In humans, foods containing antinutritional factors (*e.g.* legumes) are commonly treated before consumption but might contain some residual activity, because of improper treatment or when a balance between inhibitor destruction and dietary protein quality has to be achieved (Lajolo & Genovese, 2002). Thus, humans are commonly exposed to antinutritional factors (Vasconcelos & Oliveira, 2004), although probably at a lower level than animals. The nutritional relevance of animal findings needs to be extrapolated with caution. In addition, different effects might be observed depending on the type of antinutritional factor, as reviewed below.

#### **3.1. Lectins**

Lectins, which refer to plant glycoproteins that are able to bind reversibly to a specific mono- or oligosaccharide (Peumans & Vandamme, 1995) or to agglutinate erythrocytes

(haemagglutinins), are found in the most common edible plant foods (*e.g.* tomato, potato, beans, pea). Usually inactivated by proper processes, they can be resistant to treatments such as dry heat (*e.g.* soya-based baked products) (Liener, 1994). In summary, animals and humans are commonly exposed to active lectins (Vasconcelos & Oliveira, 2004). Lectins, known to be resistant to digestive enzymes (Vasconcelos & Oliveira, 2004; Le Gall *et al.*, 2005), have been reported to enhance ileal ENFL (Huisman *et al.*, 1992; Schulze *et al.*, 1995b; de Lange *et al.*, 2000). Depending on the level and type of lectins, different effects were observed (Grant *et al.*, 1983; Sasaki *et al.*, 2002; George *et al.*, 2007). Yin *et al.* (2004) did not observe any effect on ileal ENFL in pigs receiving a 266 mg/kg diet of purified jack bean lectin. In contrast, Schulze *et al.* (1995b) reported a significant increase in ENFL, by 30 and 47% in pigs receiving 160 and 960 mg/kg diets of purified soyabean lectins, respectively. Similarly, in piglets, Huisman *et al.* (1992) reported ileal endogenous crude protein losses three times higher with a 8300 mg lectin/kg diet compared with a 2100 mg lectin/kg diet, although different lectins were compared [toasted common beans (*Phaseolus vulgaris*) and peas]. The stimulative effect of lectins on endogenous protein losses is related to their binding to glycoprotein receptors on the epithelial cells lining the intestinal mucosa, thus inducing degradation, and probably faster renewal, of the brush border cells (Vasconcelos & Oliveira, 2004; George *et al.*, 2007). In addition, a stimulative effect on the secretion of pancreatic digestive enzymes, possibly partially mediated through CCK, has been reported (Lajolo & Genovese, 2002). Lectins are also known to interfere directly with the digestive and absorptive processes (Liener, 1994; Vasconcelos & Oliveira, 2004).

### 3.2. Tannins

Tannins, which are plant polyphenols, can form stable complexes with proteins that are present in the intestinal lumen, and thus limit the activity of proteolytic enzymes (Mangan, 1988; Jansman *et al.*, 1994a; Nyachoti *et al.*, 1997a). Condensed tannins, unlike hydrolysable tannins, are the most widespread tannins (Mangan, 1988). Tannins from winged bean, chickpea or blackgram were shown to be greatly reduced by autoclaving (Jood *et al.*, 1987; Kadam *et al.*, 1987) but not by dry-heat treatment (Kadam *et al.*, 1987). Ileal ENFL for pigs fed a diet containing 0.5 g catechin equivalents/kg diet (from quebracho extract) were significantly increased, by 230%. However, a lower impact was reported for a diet containing 0.7 g catechin equivalents/kg diet from

condensed tannins, which increased ileal ENFL in piglets by 43% (Jansman *et al.*, 1995). When the level of condensed tannin in the diet was increased by approximately 0.2%, there was no influence on ileal ENFL in pigs (Mosenthin *et al.*, 1993). The effect of tannins on ileal endogenous protein losses is likely due to the formation of a stable complex with endogenous protein, thus reducing the extent of their digestion and subsequently of their reabsorption, as reported above; in addition, tannins have been reported to stimulate the secretion of the proline-rich salivary protein (Jansman *et al.*, 1994b).

### 3.3. Enzyme inhibitors

Protease inhibitors, found in plants, animals and microorganisms, can form stable complexes with proteases, thus inhibiting protease activity (Lajolo & Genovese, 2002). From a nutritional aspect, trypsin and chymotrypsin inhibitors from plant foodstuffs are the most important. Two groups can be distinguished: Kunitz inhibitors, which act specifically against trypsin (found mostly in soyabeans and common beans) and Bowman-Birk inhibitors, which inhibit trypsin and chymotrypsin simultaneously at independent binding sites (found mostly in soyabeans) (Lajolo & Genovese, 2002). Protease inhibitors can be destroyed by heat treatment; however, most processed soya products for human consumption have been reported to generally retain 5–20% of their initial trypsin inhibitor activity (TIA) (Liener, 1994). A dose-dependent effect of purified trypsin inhibitor on ileal endogenous N has been reported. Grala *et al.* (1998b) observed an increase in ileal ENFL by 20 and 75% when young pigs were fed diets containing either a low (162 mg TIA/100 g diet) or a high (428 mg TIA/100 g diet) level of trypsin inhibitors compared with the control level (45 mg TIA/100 g diet). In humans, an intake of 247 mg TIA/100 g diet from pea proteins did not trigger any additional ileal endogenous N losses, and slightly limited the protein digestibility (Mariotti *et al.*, 2001). However, humans were not adapted to the test diet, unlike in previous pig trials where the trypsin inhibitor was reported to enhance ileal ENFL significantly (Barth *et al.*, 1993; Nyachoti *et al.*, 2000; Salgado *et al.*, 2002b). Trypsin inhibitor has been reported to enhance pancreatic secretions in humans (Liener *et al.*, 1988; Reseland *et al.*, 1996) and in rats (Liener, 1994), probably through a CCK mediation (Liener, 1994). Enhanced ileal losses of specific endogenous serine-protease proteins sharing similar N-terminal sequences with trypsin and chymotrypsin were observed in weaned piglets receiving

trypsin inhibitors in their diet (Salgado *et al.*, 2002b). It should be noted that other antinutritional factors were included in the diet. Nyachoti *et al.* (2000) reported a higher fractional protein synthesis rate in the pancreas but not in the intestines in pigs fed diets containing trypsin inhibitors among other antinutritional factors (barley–canola-based diet), compared with pigs fed a casein-based diet.

#### 4. Dietary fibre

Dietary fibre is defined as the structural and non-structural plant polysaccharides and lignins that are not digested by mammalian enzymes (Souffrant, 2001). From chemical analysis, dietary fibre can be classified as crude fibre, neutral detergent fibre, acid detergent fibre and non-starch polysaccharides. However, based on physiological considerations, questions have been raised as to whether the definition of dietary fibres should be extended to resistant starches and non-digestible oligosaccharides (Champ *et al.*, 2003a).

Dietary fibre intake has been reported to increase ileal ENFL (de Lange *et al.*, 1989a; Schulze *et al.*, 1994; Mariscal-Landin *et al.*, 1995; Schulze *et al.*, 1995c; Leterme *et al.*, 1996c; Morel *et al.*, 2003; Libao-Mercado *et al.*, 2006). Mariscal-Landin *et al.* (1995) observed that the excretion reached a plateau at high dietary crude fibre intakes, whereas Schulze *et al.* (1994) reported a linear increase in ileal ENFL by approximately 50% when the intake of purified neutral detergent fibres was increased from 0 to 180 g/kg diet. The difference observed might result from the different fibres used between studies.

The effects of dietary fibre on endogenous protein losses, such as on digestion, depend on their source and nature and relate to their physico-chemical properties, mainly their solubility, viscosity and water-holding capacity (Souffrant, 2001; Wenk, 2001). A greater effect of purified pectin than of purified cellulose on ileal ENFL in pigs was reported by de Lange *et al.* (1989a), possibly because of the higher digesta viscosity induced by pectin. Ikegami *et al.* (1990) showed that the viscous property of fibre (such as for pectin, guar gum etc.) was a major factor affecting gastrointestinal function, inducing increased pancreatic biliary secretions and possibly hindering adequate interaction between endogenous protein and digestive enzymes, thus reducing the recycling of endogenous N (Nyachoti *et al.*, 2000). Piel *et al.* (2005) showed that an increase in the viscosity of the intestinal contents in piglets fed carboxymethylcellulose increased ileal mucin output and the numbers and maturation of goblet cells in ileal villi. Similarly, the increased mucin

flows observed in pigs fed non-starch polysaccharides (Morel *et al.*, 2003) were assumed to be due to the water-holding capacity of the non-starch polysaccharides. However, Bartelt *et al.* (2002) reported that the digesta viscosity *per se* did not cause increased ileal ENFL in pigs receiving carboxymethylcellulose or xylanase but that other properties such as digesta passage rate or bacterial activity were probably responsible for the increased ENFL. Abrasiveness and increased proteolytic activities have also been reported to be responsible for the increased mucin secretions induced by fibres (Montagne *et al.*, 2004). Leterme *et al.* (1996c) showed that the neutral detergent fibre content from different fibre sources was not indicative of their effect on ileal ENFL but that the increased ENFL were associated with the water-holding capacity of the fibres, as observed by Morel *et al.* (2003). This suggests the importance of the chemical properties of dietary fibres. The representativeness of synthetic fibres compared with fibres inherent in foodstuffs is thus questionable (Nyachoti *et al.*, 1997a).

## 5. Dietary electrolyte balance

The dietary electrolyte balance (DEB) refers to the dietary cation/anion balance and is determined as follows:  $DEB = Na^+ + K^+ - Cl^-$ , where the ions are expressed as milliequivalents (mEq) (Mongin, 1981). Although other ions, potentially metabolizable, could influence the DEB, they are not included in the common DEB because of their extensive analytical preparation (Patience, 1990). Previous studies have suggested an influence of the DEB on N digestibility. In growing pigs fed a corn–soyabean meal diet, Haydon *et al.* (1990) demonstrated that an increased DEB from –50 to 400 mEq/kg linearly increased the apparent ileal N digestibility by 10%. Similarly, an increase in DEB from –100 to 200 mEq/kg diet was shown to increase the apparent ileal N digestibility of a corn–soyabean meal diet by 4% (Dersjant-Li *et al.*, 2001b). This was observed only in a diet containing 10% non-starch polysaccharide; the opposite effect was observed in a diet containing 15% non-starch polysaccharide (Dersjant-Li *et al.*, 2001b). When the DEB ranged from 60 to 300 mEq/kg, there was no effect on ileal and faecal N digestibility in piglets fed a casein-based diet (Officer *et al.*, 1997). For the apparent faecal digestibility, a significant increase of 4% was observed when the DEB was increased from –100 to 500 mEq/kg diet (Dersjant-Li *et al.*, 2001a); however, there was no difference when the DEB ranged between –100 and 200 mEq/kg diet. Similarly,

an increase in DEB from 130 to 230 mEq/kg diet had no impact on the apparent faecal N digestibility in growing pigs fed a corn–soyabean meal diet (Wondra *et al.*, 1995). Together, these data suggest that an increase in the DEB, above a certain range, possibly enhances N digestibility. This can result either from a higher degree of absorption of dietary N or from lower ENFL. However, the underlying cause has not been assessed.

## V. Postprandial metabolic fate of dietary nitrogen

After ingestion of a protein meal (postprandial or absorptive phase), the intestines release dietary AA to other tissues, resulting in a net whole-body protein deposition that replenishes tissue proteins depleted during the fasting state. This is of great importance in maintaining long-term homeostasis of the free AA pool. In contrast, during the postabsorptive (fasting) state, muscles release endogenous AA to the other tissues, especially to the splanchnic area and the kidneys (Brosnan, 2003). The net protein deposition is the result of co-ordinated changes in the rates of protein synthesis, protein breakdown and AA oxidation (Waterlow, 2006b), which are influenced by both the nutritional/physiological status and the specific meal composition. The synergistic effects of dietary AA (Wolfe, 2002) and hormones, primarily insulin (Tessari, 1994; de Feo, 1996), have been reported to be largely involved in the regulation of postprandial protein metabolism.

Methods based on the dilution of a tracer, usually L-[1-<sup>13</sup>C]-leucine, in the precursor pool of protein synthesis (free AA pool), have allowed whole-body protein metabolism to be assessed. Initially used under steady-state conditions, implying the need for continuous feeding, the development of a non-steady-state approach (Boirie *et al.*, 1996) has allowed postprandial protein (leucine) metabolism to be assessed after ingestion of a single meal, which is more representative of human nutritional behaviour. A combination of the tracer method and the balance method (*e.g.* arteriovenous or net splanchnic organ and portal blood) has provided information regarding the relative contribution of various tissues and proteins to whole-body changes (Fouillet *et al.*, 2002a; Stoll & Burrin, 2006). These invasive techniques are mostly applicable in animal models, such as pigs, which have largely contributed to our understanding of protein metabolism (Bergen, 2007). Mathematical compartmental modelling has allowed the fate of regional dietary N to be predicted from accessible data collected in humans (Fouillet *et al.*, 2000; Fouillet *et al.*, 2002a). This section commences with the relative contribution of the splanchnic and

peripheral tissues to whole-body protein metabolism during the postprandial phase in healthy adults and then discusses the influence of dietary factors on postprandial protein metabolism.

## 1. Regional metabolism and availability of dietary amino acids

### 1.1. Splanchnic tissues

The splanchnic area, *i.e.* the portal-drained viscera (PDV; intestines, spleen, stomach, pancreas) and the liver, plays a significant, and co-operative, role in whole-body protein metabolism (Brosnan, 2003) with a major contribution to total protein accretion (Mariotti *et al.*, 2000a; Le Floch & Sève, 2000; van der Schoor *et al.*, 2002; Stoll & Burrin, 2006). This is related to the high protein turnover of the gut despite its low protein content (Fouillet *et al.*, 2002a). As the first tissues to be exposed to dietary nutrients, the requirements of the splanchnic tissues are met first, thus largely influencing dietary AA availability for the peripheral tissues (Yu *et al.*, 1990; Wu, 1998; Stoll *et al.*, 1998b; van der Schoor *et al.*, 2002; Bos *et al.*, 2005b; Stoll & Burrin, 2006).

In humans, the splanchnic retention of dietary N (from casein or soyabean) was reported to range from 20 to 35%, based on predicted values (Fouillet *et al.*, 2000) or on <sup>13</sup>C-leucine kinetics (Metges *et al.*, 2000; Luiking *et al.*, 2005). This is similar to values found in piglets, for which the total splanchnic retention of dietary N (from milk protein) was 20% (Bos *et al.*, 2005b). Information regarding the contribution of individual tissues in humans is scarce, although invasive studies in animals have provided important information, demonstrating that half of the dietary N retained in the splanchnic area is sequestered in the intestines in pigs (Bos *et al.*, 2005b), whereas this value is closer to 30% in growing rats (Morens *et al.*, 2001).

#### 1.1.1. *Portal-drained viscera tissues*

The intestines, because of a high protein synthetic activity for rapid cell renewal, enzyme production or secretion of mucus, modify the AA availability for the rest of the body and represent a high proportion of total body protein synthesis (Le Floch & Sève, 2000). Once absorbed within the enterocytes, dietary AA, as well as endogenous AA, either are transported out of the cell into the portal blood or are metabolized *in situ* through various pathways. This includes incorporation into endogenous protein: 7–10% of dietary N has

been reported to be incorporated into mucosal proteins in pigs (van der Meulen & Jansman, 1997; Stoll *et al.*, 1998b), the quantity likely to be underestimated because of mucosal protein secretions into the lumen (Stoll *et al.*, 1998b). In addition, AA entering the enterocytes can be subject to transamination (except for lysine and threonine), used for other biosynthetic purposes or completely oxidized to CO<sub>2</sub>. IAA that are irreversibly metabolized or completely oxidized to CO<sub>2</sub> are nutritionally lost to the animal (Stoll & Burrin, 2006).

During the postprandial phase, the PDV tissues receive AA both of direct dietary origin, released from the intestinal lumen, the portal blood or the arterial blood (second-pass), and of endogenous origin, mainly from arterial circulation. In piglets, the fractional use (uptake/intake) of dietary AA has been reported to be much greater (20–95%) than that of arterial AA (5–15%) even though the quantitative input of arterial AA into the PDV tissues was found to be from 3- to 5-fold higher than that for the dietary AA (Stoll *et al.*, 1998b; Stoll & Burrin, 2006). Interestingly, there is a rapid release into the portal blood of one-third of the early first-pass uptake (0–4 h after the meal) of a dietary AA such as lysine during the late postprandial phase (4–8 h after the meal) (Bos *et al.*, 2003b). Also, AA absorbed from the intestinal lumen can be of endogenous origin when they arise from hydrolysed gut endogenous protein previously secreted into the gut. This was reported to contribute to the overall systemic dietary AA availability: over 24 h, 17% of the dietary protein had reappeared in the portal vein of piglets by way of recycling of intestinal secretions (van der Schoor *et al.*, 2002). This emphasizes the nutritional importance of N recycling within the splanchnic bed. It should be noted that, when the gut was supplied only with endogenous AA (postabsorptive state), the fractional protein synthesis rate of the duodenal mucosa was reported to be similar to that determined when the gut receives dietary AA (Bouteloup-Demange *et al.*, 1998).

The measure of the net portal AA balance (based on the difference between AA flows in the portal vein and in the carotid artery) in pigs has provided important information regarding the first-pass extraction of individual AA within the PDV tissues. Despite the different methods used in the studies, there is relatively good agreement among the determined net balances, as reported in **Table 12**. The biological explanation of the different extractions among AA is only partially understood at present (Stoll *et al.*, 2006). It appears that dietary glutamine, glutamate and aspartate are virtually totally catabolized within the PDV (net portal balance close to zero or negative). It is known that glutamine,



glutamate and aspartate provide the majority of the intestinal oxidative energy (Le Floch & Sève, 2000; Reeds *et al.*, 2000a; Stoll & Burrin, 2006). In contrast, it appears that a net synthesis of arginine, alanine and, in some cases, tyrosine and proline occurs in the intestinal tissues (net portal balance > 100% of the dietary intake). It should be noted that, whereas experimental data in adult rats suggested that arginine is synthesized essentially in the kidney, this result indicates a possible synthesis in intestinal mucosa cells of piglets (Stoll & Burrin, 2006). Finally, most IAA seem to be used to a significant extent within the splanchnic bed (net portal balances < 100%).

**Table 12.** Estimates of portal amino acid balance in young pigs fed liquid-milk replacer<sup>1</sup>.

	Single oral bolus (8 h) <sup>2</sup>	Gastric hourly bolus (6 h) <sup>3</sup>	Continuous duodenal (24 h) <sup>4</sup>
	% of dietary intake		
Threonine	62	38	33
Valine	72	61	51
Isoleucine	78	70	30
Leucine	74	60	66
Phenylalanine	61	61	49
Tyrosine	96	167	168
Lysine	79	54	64
Histidine	70	-	-
Serine	84	58	51
Glutamate	29	7	10
Glutamine	-29	-8	-18
Alanine	190	205	112
Proline	88	62	43
Arginine	149	138	155
Aspartate	24	4	6
Glycine	69	52	89
Methionine	70	48	75
Cysteine	17	-	-

<sup>1</sup> The net portal balance is determined from the product of the portal blood flow rate and of the difference in AA concentrations in the portal vein and carotid artery.

<sup>2</sup> The cumulative portal balance was measured for 8 h (Bos *et al.*, 2003b).

<sup>3</sup> Pigs were fed orally via hourly boluses for 6 h, and the average portal balance was measured between 4 and 6 h (Stoll *et al.*, 1998b).

<sup>4</sup> Pigs were fed continuously via intraduodenal infusion for 12 h and then were fasted for 12 h; the cumulative portal balance was measured for a 24-h period (van der Schoor *et al.*, 2002).

Catabolism was reported to dominate the first-pass extraction of IAA in the intestines, especially in the mucosa; out of the dietary IAA extracted by the intestines (*i.e.* about

one-third of the dietary intake), 50–75% was catabolized (Stoll *et al.*, 1998b). However, N from catabolized AA can be reused for the synthesis of dispensable AA. An example would be the synthesis of alanine, highly released by the intestines (Table 12), and thus transporting a high proportion of the ingested dietary N outside of the PDV (Stoll *et al.*, 1998b; Bos *et al.*, 2005b). Threonine appears to be the most utilized IAA within the PDV, as its net balance suggests a PDV first-pass extraction of 38–67% (Table 12), and is thus the first limiting IAA for extra-intestinal protein deposition (Schaart *et al.*, 2005). Interestingly, a previous study reported that only 10% of the dietary threonine was recovered in constitutive mucosal proteins (Stoll *et al.*, 1998b). It has been speculated that the majority of dietary threonine utilized by the gut is incorporated into secreted mucin proteins (Stoll & Burrin, 2006).

The importance of intestinal tissue metabolism within the splanchnic area is well recognized (Stoll *et al.*, 1998a; Stoll *et al.*, 1998b; Stoll *et al.*, 1999; Bos *et al.*, 2005b). Of the total AA extracted in the splanchnic area, 75–85% has been reported to be sequestered in the intestinal tissues (Stoll *et al.*, 1998a; Stoll *et al.*, 1998b; Stoll *et al.*, 1999). However, these values might have been somewhat overestimated (van Goudoever *et al.*, 2000). After ingestion of a protein-based meal, especially when the AA influx is rapid, the gut may temporarily modulate its retention of dietary AA into a “labile protein pool” to prevent an immediate release of the dietary AA into the portal vein and their possible degradation in the liver to urea (Mariotti *et al.*, 2000a; Luiking *et al.*, 2005).

### 1.1.2. **Hepatic metabolism**

As for the PDV tissues, the liver is known to play an important role in AA metabolism (Stoll & Burrin, 2006; van de Poll *et al.*, 2007). The fractional synthesis rate of total liver proteins (stationary and exported) has been reported to be approximately 25% of the liver protein content daily in humans (Barle *et al.*, 1997). After ingestion of a protein-containing meal, the liver receives AA from the portal circulation (mostly dietary AA) and from arterial circulation (both endogenous AA and dietary AA). Dietary AA have been reported to be the preferred source of AA for hepatic protein synthesis (Cayol *et al.*, 1996; Stoll *et al.*, 1998a; Stoll *et al.*, 1999). Also, the AA content of portal blood, which depends on intestinal uptake, has been shown to influence AA hepatic extraction (Bloomgarden *et al.*, 1981). Nutritionally significant quantities of dietary IAA have been reported to be consumed in the first-pass extraction by the liver for protein synthesis

(Rérat *et al.*, 1992; Capaldo *et al.*, 1999). However, branched-chain AA (BCAA) have been shown to be spared by the liver for their further use in peripheral tissues (Rérat *et al.*, 1992; Capaldo *et al.*, 1999).

A major role of the liver is its capacity to synthesize urea. The liver has been shown to play an important role in AA homeostasis by preventing hyperaminoacidaemia (Bos *et al.*, 2003a; Luiking *et al.*, 2005; Lacroix *et al.*, 2006a; van de Poll *et al.*, 2007). A high influx of dietary AA into the liver appears to elicit a higher ureagenesis (Bos *et al.*, 2003a; Luiking *et al.*, 2005; Lacroix *et al.*, 2006a) but also a greater incorporation into exported liver protein (Bos *et al.*, 2003a; Lacroix *et al.*, 2006a), probably albumin (Fouillet *et al.*, 2002a). The increase in albumin synthesis would prevent irreversible oxidative losses of a significant fraction of ingested AA and serve as a vehicle to capture excess dietary AA and to transport them to peripheral tissues to sustain local protein synthesis (de Feo *et al.*, 1992), although the concept of a temporary storage of absorbed AA remains controversial.

However, a high and fast influx of AA into the liver has been shown to induce a less efficient use of AA (leucine) for the constitutive liver proteins compared with a slower AA influx (Daenzer *et al.*, 2001). In addition, the liver has been reported to play a major role in N salvage. A shift of liver N metabolism from urea toward glutamine production was reported when animals were fed diets containing 11% casein, compared with rats fed diets containing 22% casein (Remesy *et al.*, 1997). Finally, the liver has an important activity of neoglucogenesis. In the dog, it was reported that one-third of the glycogen synthesized after ingestion of a protein-containing mixed meal was derived from neoglucogenic AA (Moore *et al.*, 1994).

In animal studies, liver protein synthesis has been estimated to represent 40–70% of the total anabolic use of dietary N in the splanchnic area, with a higher synthesis of constitutive proteins (60–70% of the total liver protein synthesized) than of exported liver proteins (Morens *et al.*, 2000; Morens *et al.*, 2001). Among exported liver proteins, the albumin, but not fibrinogen, synthesis rate has been reported to be stimulated by protein ingestion (Fouillet *et al.*, 2002a).

## 1.2. Peripheral tissues

After ingestion of a protein-containing meal, it has been established in humans that around one-third of the ingested N is released from the splanchnic area and is made

available for the peripheral tissues (muscles, skin and kidneys) (Capaldo *et al.*, 1999; Fouillet *et al.*, 2000; Fouillet *et al.*, 2003). This was confirmed in rat studies, in which the proportion of ingested N taken up by peripheral tissues 8 h after a meal ranged from 20 to 50% depending on the level of dietary protein intake (Morens *et al.*, 2000; Morens *et al.*, 2001). In pigs, this proportion was estimated to be 42%, with an anabolic use of 31% of the ingested N (Bos *et al.*, 2005b). Modelling data showed that 15–25% of the ingested N was incorporated into peripheral proteins, thus highlighting the dominance of the anabolic pathway in the peripheral area (Fouillet *et al.*, 2001; Fouillet *et al.*, 2003).

After ingestion of a protein-containing meal, BCAA are quantitatively the most available AA for peripheral tissues, and account for more than half of total splanchnic AA output (Wahren *et al.*, 1976; Aoki *et al.*, 1976). More recently, it has been reported that 38% of the dietary BCAA are released towards the peripheral tissues, compared with 29% of the other AA, and that BCAA, rather than non-BCAA, are preferentially taken up by peripheral tissues (leg) (Capaldo *et al.*, 1999). The proportion of BCAA taken-up by the exercising muscle is likely to be enhanced, as physical activity has been reported to stimulate protein synthesis in muscle (Rennie, 2007), and this might have consequences on postprandial protein utilisation. During the postprandial period, muscles not only receive AA but also release neoglucogenic AA, such as glutamine and alanine, towards the peripheral tissues (Matthews, 2005). This emphasizes the importance of interorgan AA exchange (Matthews, 2005).

The peripheral area includes a heterogeneous group of tissues in terms of protein synthesis rate. Although muscles have a high protein content, they exhibit a low protein turnover, with a fractional synthesis rate of < 2% per day (Fouillet *et al.*, 2002a). In animals, the fractional synthesis rate of the skin has been reported to range between 4 and 26% per day, a value that is consistently higher than that measured in skeletal muscle, by a factor of 2 or 3 (Wykes *et al.*, 1996; Zhang *et al.*, 1998; Volpi *et al.*, 2000; Davis *et al.*, 2002). Finally, the fractional synthesis rate of the human kidney has been estimated at 40% per day (Tessari *et al.*, 1996b) despite lower protein content. Contradictory results have been found regarding the modulation of protein synthesis in muscles after the ingestion of a protein-containing mixed meal, with some studies indicating a stimulation (Tessari *et al.*, 1996a) and other studies reporting no effect (McNurlan *et al.*, 1993). The skin seems to be unaffected by the nutritional status (fasting or fed state) (Zhang *et al.*, 1998) and appears to have a high degree of AA recycling (Zhang *et al.*, 1998). The nutritional modulation of the kidneys remains to be studied.

## 2. Dietary modulation of postprandial metabolism of dietary nitrogen

The postprandial metabolism of dietary N is modulated through complex factors that are not yet fully understood. Dietary factors play a major role in the regulation of AA metabolism, largely through the modulation of hyperaminoacidaemia and hyperinsulinaemia (Liu & Barrett, 2002).

### 2.1. Dietary protein

#### 2.1.1. *Dietary protein quantity*

A trend towards an increased body N balance with an increased level of dietary protein intake above the protein requirement has been demonstrated (Motil *et al.*, 1981; Pacy *et al.*, 1994; Price *et al.*, 1994). This has been assessed mainly after chronic adaptation, inducing a series of adaptive processes. Garlick *et al.* (1999) suggested that part of the N retained might result from the modulation of the body urea pool and, to a lesser extent, from an extension of the body free AA pool; however, it is not clear whether there is an increase in protein across tissues. These hypotheses were confirmed in adapted rats (Morens *et al.*, 2001), in which a 50% protein diet increased dietary N transferred to urea in comparison with a 14% protein diet (2.2 and 1.3 mmol N/100 g BW, respectively). Dietary N incorporation into muscle protein was reduced in rats fed the high-protein diet, whereas more dietary N was accumulated in the free N pool (Morens *et al.*, 2001). This emphasizes the important role played by splanchnic catabolism in adaptation to a high-protein diet, in contrast to muscle tissue. The higher transfer to urea was related to a marked increase in AA oxidation rate in response to hyperaminoacidaemia (Motil *et al.*, 1981; Pacy *et al.*, 1994; Price *et al.*, 1994), highlighting a decrease in the yield of protein utilization with a high level of protein intake (Price *et al.*, 1994; Morens *et al.*, 2003). In terms of protein dynamics, the key factor determining the rate of protein deposition was shown to be related to the extent of inhibition of protein breakdown rather than the modulation of protein synthesis (Price *et al.*, 1994; Garlick *et al.*, 1999; Waterlow, 2006b). In addition, increased protein intake was reported to enhance the amplitude of the diurnal cycling of body protein N, with more pronounced fasting losses and higher postprandial repletion (Price *et al.*, 1994).

### 2.1.2. *Dietary protein source and amino acid composition*

A possible influence of the protein source on postprandial protein metabolism has been reported, as illustrated by the lower net postprandial protein utilization for vegetable (rapeseed, soya, wheat, pea, lupin) compared with animal (milk) proteins (Mariotti *et al.*, 2001; Gaudichon *et al.*, 2002; Mariotti *et al.*, 2002; Bos *et al.*, 2003a; Bos *et al.*, 2005b; Bos *et al.*, 2007). The lower efficiency of soya protein to achieve a postprandial protein gain has been shown to be related to a higher deamination rate and greater liver protein synthesis than those observed for casein, a difference that is in part attributable to kinetic factors (Deutz *et al.*, 1998; Bos *et al.*, 2003a; Luiking *et al.*, 2005). Soya protein was thus predicted to induce similar splanchnic retention but a lowered peripheral dietary N uptake (Fouillet *et al.*, 2002b), a prediction that was further demonstrated in exercising subjects (Hartmann & Meisel, 2007). Although the digestion/absorption kinetics may play an important role in the differences observed between casein and soya (see Section V.2.1.3), an impact of the different AA compositions cannot be ruled out (Bos *et al.*, 2003a; Luiking *et al.*, 2005).

Dietary AA, besides acting as a substrate for protein synthesis, have been reported to act as a regulation signal for muscle protein synthesis, with differential impact depending on the AA (Rennie, 2007). A perfusion of AA deficient in isoleucine in human subjects with hyperinsulinaemia induced leucine oxidation, thus reducing leucine utilization for protein synthesis; in contrast, when the perfusion was deficient in threonine, this was not observed (Lecavalier *et al.*, 1991). The importance of BCAA, and especially leucine, in promoting muscle protein anabolism has long been recognized (Buse & Reid, 1975; Sherwin, 1978). It is not clear whether the anabolic effect of leucine results from a reduction in muscle protein breakdown and/or an enhancement of muscle protein synthesis (Matthews, 2005). Whereas, in rats, the anabolic drive of leucine was reported to be due to a stimulation of muscle protein synthesis (Kimball & Jefferson, 2004; Crozier *et al.*, 2005), in humans, Matthews (2005) reported that this was mostly due to an inhibition of protein breakdown. Koopman *et al.* (2005; 2006) showed in humans that addition of leucine to a drink containing carbohydrate and protein induced an increased muscle fractional synthesis rate and a lower oxidation rate. The latter result is probably due to a synergistic effect of leucine and carbohydrates. Indeed, carbohydrates are known to induce the secretion of insulin, a hormone that is reported to stimulate the anabolic pathway (Millward, 1990; de Feo, 1996). Leucine has also been reported to have an insulinotropic effect (van Loon *et al.*, 2000; Koopman *et al.*, 2005). Also, leucine

anabolic drive may involve the regulation of the signal transduction pathways that regulate mRNA translation (Kimball & Jefferson, 2004; Crozier *et al.*, 2005).

Finally, a balanced dietary AA input is an important factor in sustaining basal rates of protein synthesis (Nissen & Haymond, 1986; Frexes-Steed *et al.*, 1992).

### 2.1.3. *Dietary protein digestion and absorption rates*

Protein digestion and absorption kinetics have been demonstrated to play a major role in the fate of absorbed dietary N and possibly in its efficiency of utilization (Boirie *et al.*, 1997b; Daenzer *et al.*, 2001; Dangin *et al.*, 2001; Bos *et al.*, 2003a; Lacroix *et al.*, 2006a; Wilkinson *et al.*, 2007). The concept of “slow” (slowly digested and absorbed) and “fast” (rapidly digested and absorbed) proteins has been developed after its first introduction by Boirie *et al.* (1997a) based on tracer techniques in the non-steady state. Fast proteins were demonstrated to induce higher protein synthesis rates, but also a higher rate of oxidation and a lower inhibition of protein breakdown, based on data obtained from whole-body <sup>13</sup>C-leucine kinetics in young adults. As a result, a lower net postprandial protein (leucine) balance was observed with fast protein compared with slow protein (Boirie *et al.*, 1997a; Dangin *et al.*, 2001), which is in line with rat or human studies following the postprandial metabolic fate of a <sup>15</sup>N- or <sup>13</sup>C-labelled dietary protein source (Daenzer *et al.*, 2001; Bos *et al.*, 2003a; Lacroix *et al.*, 2006a).

The different behaviour between proteins was assumed to be modulated via the aminoacidaemia, reported to be fast, high and transient with fast protein and lower but more prolonged with slow protein (Boirie *et al.*, 1997b; Daenzer *et al.*, 2001; Dangin *et al.*, 2001; Bos *et al.*, 2003a; Lacroix *et al.*, 2006a). Aminoacidaemia was in turn assumed to be essentially driven by the digestion/absorption kinetics. The latter may be modulated by the emptying rate of the stomach (Mariotti *et al.*, 2000b). Mahé *et al.* (1992; 1996) reported a slow gastric emptying rate for casein (used as a model of slow protein in most studies) because of clotting in the stomach at acidic pH. In contrast, Calbet *et al.* (2004) measured similar gastric emptying rates between casein and hydrolysed casein or whey protein (fast protein). However, the gastric emptying measure may not accurately account for the intestinal transit time, which may be independently affected by dietary factors (Read *et al.*, 1982; Fouillet *et al.*, 2002b). Indeed, opioid peptides ( $\beta$ -casomorphins) released during casein digestion may slow down the intestinal transit time of casein (Daniel *et al.*, 1990; Allescher *et al.*, 2000; Patten *et al.*, 2001). Conversely, the hydrolysis of casein prior to its ingestion has been shown to suppress this opioid activity

(Mihatsch *et al.*, 2005).

For some of the studies in which slow and fast proteins were compared (Boirie *et al.*, 1997b; Bos *et al.*, 2003a; Lacroix *et al.*, 2006a), it cannot be excluded that the dietary AA pattern, which differed between slow and fast proteins, contributed to the differences observed (see Section V.2.1.2). Moreover, non-protein nutrients, which likely affect postprandial AA metabolism (see Section V.2.2), were added to the test meal in the studies of Bos *et al.* (2003) and Daenzer *et al.* (2001) only.

It is interesting to note that, in steady-state studies, in which human subjects received their meals either through a continuous enteral infusion (Collin-Vidal *et al.*, 1994; Luiking *et al.*, 2005) or orally every hour (Metges *et al.*, 2000), a higher oxidation and a lower net leucine (protein) balance were observed for proteins such as hydrolysed casein, free AA and soya protein (previously reported as a fast protein) compared with casein (previously reported as slow protein) (Collin-Vidal *et al.*, 1994; Metges *et al.*, 2000; Luiking *et al.*, 2005). Even though a steady state was aimed for, different plasma leucine appearances were still observed either at the start of the experiment (Collin-Vidal *et al.*, 1994; Luiking *et al.*, 2005) or after each hourly meal (Metges *et al.*, 2000).

Insulin, known to drive dietary AA towards anabolic pathways (Tessari, 1994; de Feo, 1996), was reported to have similar plasma concentrations in humans given either slow protein (casein) or fast protein (whey or soya) (Boirie *et al.*, 1997a; Bos *et al.*, 2003a). Dangin *et al.* (2001) observed a moderate increase in insulin after ingestion of fast protein (whey protein or free AA) but not after ingestion of slow protein (repeated meal of whey protein or casein). In summary, it is likely that a complex interaction of factors, namely digestion/absorption kinetics, dietary AA profile and meal composition, contributed to modulations of the metabolic fate of absorbed AA.

Few data are available regarding the influence of dietary AA kinetics on regional metabolism. Modelling data showed similar total splanchnic retention for casein and soya protein, but a reduced peripheral dietary N uptake for soya protein compared with casein (Fouillet *et al.*, 2002b), mostly due to the higher oxidation induced by soya protein (Bos *et al.*, 2003a). Indeed, the massive influx of dietary AA into the splanchnic area appears to elicit a higher metabolic activity in the liver, with increased dietary AA catabolism and incorporation into exported liver protein, so as to prevent hyperaminoacidaemia (Bos *et al.*, 2003a; Lacroix *et al.*, 2006a).

A better postprandial protein accretion has been observed after acute ingestion of slow protein rather than fast protein (Boirie *et al.*, 1997b; Daenzer *et al.*, 2001; Dangin *et al.*,



2001; Bos *et al.*, 2003a; Lacroix *et al.*, 2006a). In contrast, long-term N balance studies have reported a similar or lower N retention for intact casein (slow protein) compared with hydrolysed casein (fast protein), as reported in **Table 13**.

**Table 13.** Effect of the delivery form of dietary amino acids<sup>1</sup> [intact protein (IP), hydrolysed protein (HP), corresponding free mixture (AA)] on the total nitrogen (N) balance.

Reference	Species	Digestive tract condition	Protein source	Diet duration	N balance <sup>2</sup>		
					IP	HP	AA
Forsum <i>et al.</i> , 1978	rat	normal	casein	9 days	+		+
		normal/ gastroectomized/ hepatoectomized					
Yamamoto <i>et al.</i> , 1985	rat	gastroectomized/ hepatoectomized	casein	21 days	+	+	+
Sales <i>et al.</i> , 1995	rat	gut resection	casein	12 days	+	+	+
Boza <i>et al.</i> , 1995	rat	normal	casein	2 days <sup>3</sup>	+	++	
Boza <i>et al.</i> , 1995	rat	normal	whey	2 days <sup>3</sup>	+	++	
Poullain <i>et al.</i> , 1989	rat	normal	whey	4 days <sup>3</sup>	+	++	+
Moriarty <i>et al.</i> , 1985	human	normal	lactalbumin	14 days	+	+	+

<sup>1</sup>The protein source was ingested within a complete mixed meal.

<sup>2</sup>The N balances that do not share the same symbol within a line are different, with ++ indicating a higher N balance than +.

<sup>3</sup>After 3 days of starvation. Results for Poullain *et al.* (1989) are for rats after 0 or 3 days starvation

Similarly, a better N retention was reported for a diet based on hydrolysed milk protein compared with a diet based on the corresponding free AA mixture (Monchi & Rérat, 1993; Rérat, 1995). Whether the differences observed are due to different methods used to estimate protein retention (postprandial versus daily retention) or to other influencing factors (dietary AA profile, meal composition) remains unknown.

More investigations are required to determine the impact of the digestion/absorption kinetics *per se* on the postprandial metabolic fate of dietary N, especially in a representative context, *i.e.* ingestion of protein within a mixed meal.

## 2.2. Non-protein nutrients

The dietary energy intake is known to be an important factor for the effective utilization of dietary protein (FAO/WHO/UNU, 2007). The addition of carbohydrate or fat to a

protein meal has been reported to exert an N-sparing effect (Fuller & Crofts, 1977; Millward & Jackson, 2004); however, it is not clear whether the relative impacts of both types of nutrients are similar (Tessari *et al.*, 1996a; Gaudichon *et al.*, 1999; Millward & Jackson, 2004).

### 2.2.1. **Carbohydrate**

The addition of carbohydrates to a protein meal is known to enhance dietary protein efficiency (Deutz *et al.*, 1995; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000b; Fouillet *et al.*, 2002a). The addition of sucrose (100 g) to a meal containing milk protein (30 g) increased the net postprandial protein utilization of milk protein by 5% (Gaudichon *et al.*, 1999), which was related to a reduction in the deamination of dietary N (Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000b). This induced a different regional distribution, with the dietary N retention being increased in the splanchnic tissues (by 17% of ingested N from milk protein) and decreased in the peripheral tissues (by 8% of ingested N from milk protein) as predicted by mathematical modelling (Fouillet *et al.*, 2002a). A similar distribution was predicted when soya and sucrose were ingested by humans (Fouillet *et al.*, 2002a). Several mechanisms have been reported to be involved in this modulation. Firstly, the addition of carbohydrate in the meal stimulates the secretion of insulin (Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000b), a hormone known to stimulate protein anabolism (Millward, 1990; de Feo, 1996). The exact insulin effect has long been debated as being attributable to an inhibition of protein breakdown and/or a stimulation of protein synthesis (Millward, 1990; de Feo, 1996; Matthews, 2005; Rennie, 2007). However, it would appear that the potential stimulatory effect of insulin on muscle protein synthesis depends on an adequate availability of intramuscular AA (Wolfe, 2000; Fujita *et al.*, 2006).

The increased protein efficiency when protein and carbohydrates are ingested together might result from the presence of an exogenous source of glucose, thus sparing the use of dietary AA for hepatic gluconeogenesis (Capaldo *et al.*, 1999).

Furthermore, the modulation of protein digestion/absorption kinetics by carbohydrates may play a role in the enhancement of dietary protein efficiency. Mariotti *et al.* (2000b) demonstrated that the addition of sucrose (100 g) to a protein meal (30 g) delayed the half-time of its gastric emptying by 108 min. This is likely to explain the more prolonged appearance of dietary AA into the systemic circulation (Gaudichon *et al.*, 1999; Deutz *et al.*, 1995), and the subsequent increased retention of dietary N in the splanchnic area

(Deutz *et al.*, 1995; Fouillet *et al.*, 2002a) that was observed when dietary proteins were ingested with carbohydrates rather than alone. It appears that dietary N when ingested with carbohydrates has a similar behaviour to that observed for slow protein (see Section V.2.1.3) and stresses the importance of the digestion kinetics on the postprandial metabolic fate of dietary N. In addition, the rate of appearance of glucose in the portal vein was reported to influence the net portal flux of AA, which suggests a possible different influence of the carbohydrate depending on its rate of digestion/absorption (van der Meulen *et al.*, 1997).

### 2.2.2. ***Fat***

The influence of fat *per se* on dietary protein utilization has been studied by Gaudichon *et al.* (1999), who reported no influence on plasma dietary AA appearance, deamination kinetics and protein retention. Most studies investigated the influence of fat on protein metabolism in relation to the dietary carbohydrate intake. When healthy humans received a high-fat mixed diet (energy ratio fat:carbohydrate 2:1), the N balance was higher than that observed in humans fed a high-carbohydrate diet (energy ratio fat:carbohydrate 1:1) (McCargar *et al.*, 1989). Similarly, growing rats fed a high-fat diet (fat:carbohydrate 1:1) exerted lower urinary N output than those fed a high-carbohydrate diet (fat:carbohydrate 0.5:1) (Hartsook *et al.*, 1973). Millward (2004) reported that a fat–glucose regimen was previously demonstrated to have a higher N-sparing effect than the addition of glucose when fed to subjects under long-term total parenteral nutrition. Similarly, the re-nutrition of starved chicks with a complete meal induced a higher fractional protein synthesis rate in the liver than that observed with a meal containing protein only or supplemented with one of the macronutrients (Yaman *et al.*, 2000). The N-sparing effect of the high-fat diet was not explained by hormone levels but might be substrate mediated (McCargar *et al.*, 1989). In summary, it appears that there is a synergistic effect between carbohydrates and fat on dietary protein utilization; however, this needs to be investigated further.

### 2.2.3. ***Dietary electrolyte balance***

Few studies have assessed the impact of the DEB on postprandial N metabolism. An increased DEB from –50 to 400 mEq/kg diet resulted in growing pigs in an increased daily urinary N excretion, thus increasing the net protein retention by 7% (Haydon & West, 1990). However, when the DEB ranged from –20 to 163 mEq/kg diet (Patience &

Chaplin, 1997) or from 64 to 302 mEq/kg diet (Officer *et al.*, 1997), the net protein retention in pigs was not affected.

## **Conclusions and justification of the experimental work**

Evaluation of dietary protein quality requires the determination of an index that is representative and that can be routinely determined. Whether the PDCAAS is suitable as such an index is still open to debate. The net postprandial protein utilization, taking into account the subsequent metabolism of the absorbed AA, has recently been demonstrated to be an accurate indicator of protein quality. Whatever index is determined, protein digestibility, and especially true ileal AA digestibility, is a key factor in protein quality evaluation. Although human data are obviously the most valuable, animal models are needed for routine digestibility assays. Whereas the growing rat has been extensively used and accepted as an animal model, the growing pig, which has more similarities (physiology of the digestive system, no coprophagy, meal eating-pattern) to the human, has been proposed as a potentially better animal model for digestibility studies. However, this has been assessed in a limited number of studies.

The collection of ileal digesta has required the development of techniques allowing access to the terminal ileum. In humans, ileal digesta can be collected in ileostomized subjects; however, this approach has been criticized (prolific ileal microflora, potential alteration in digestive function). In contrast, the intubation method allows for sampling of ileal digesta from a normal entire digestive tract with conscious subjects. Despite its wide application and its recognition as a valid method, its validity has not been clearly demonstrated because of the technical difficulties implied. In animals, digesta collection under deep anaesthesia is a straightforward method. Nevertheless, the use of cannulated pigs offers advantages other than technical simplicity (continuous digesta collection, larger sample size, etc.) In particular, the PVTC cannula, which, unlike other cannulae, does not require the transection of the small intestine, is assumed to allow a virtually complete digesta collection and has only minor effects on pig metabolism.

The determination of true digestibility, as opposed to apparent digestibility, requires the measurement of ileal endogenous N losses. The straightforward and non-ambiguous method of protein-free feeding has been widely used and can still be considered to be a

valid method when used for relative comparisons. However, this method is known to underestimate endogenous N losses. Methods based on protein-containing diets have thus been developed, using either labelled protein or mathematical methods. The enzyme-hydrolysed protein/ultrafiltration technique was developed as an alternative approach, whereby the gut is directly supplied with AA and peptides, usually derived from casein, that are assumed to be similar to those arising during digestion in the gut. This method, which allows a “direct” simultaneous determination of endogenous flows for all AA and N, is of practical interest for the development of routine digestion assays. Questions have been raised as to whether potentially bioactive peptides present in the casein hydrolysate may enhance the loss of AA from the small bowel (Jansman *et al.*, 2002; Stein *et al.*, 2007), over and above the loss that may be found with the corresponding intact protein. Dietary factors, such as antinutritional factors or fibres, may also enhance endogenous protein losses. They have been extensively studied in animals, but the extrapolation of these results to humans has to be done with caution as animal feedstuffs generally contain higher levels of antinutritional factors and fibres than human foodstuffs. More studies testing physiological concentrations of AA should be undertaken.

Following intestinal absorption, dietary AA are delivered to the peripheral tissues after their first-pass metabolism in the splanchnic tissues. The latter play a key role in the regulation of AA availability for peripheral tissues. Exogenous factors, in particular dietary factors, are known to modulate the metabolic fate of dietary N. There has been increased interest in the influence of the form of delivery of dietary AA (free, peptide-bound or protein-bound) on their metabolic fate. The concept of slow and fast digested proteins has been put forward, with the paradigm that slow proteins sustain a better N utilization than fast proteins. However, this concept has been based on studies whereby dietary proteins or peptides were ingested alone, and furthermore the net protein retention was not directly assessed. As non-protein nutrients have been reported to influence postprandial protein metabolism, it is important to compare the metabolic events following ingestion of slow and fast protein within a mixed meal. The assessment of the nutritional value of hydrolysed proteins versus free AA is of practical importance as they are used within enteral formulas for patients with compromised digestive capacities (Grimble *et al.*, 1989) or for infants suffering from milk allergy (Hernell & Lonnerdal, 2003).

Based on this background, the major objectives of the experimental work described in this dissertation were as follows:

- To investigate in a practical context the influence of antinutritional factors on ileal endogenous protein flows.
- To determine the influence of a casein hydrolysate on ileal endogenous protein flows compared with that of the parent intact casein or of the corresponding free AA mixture.
- To verify the validity of the intubation method used for sampling intestinal digesta from conscious human subjects.
- To determine the influence of the form of delivery of dietary AA on their postprandial metabolic fate.
- To assess the validity of the growing pig as an animal model for predicting ileal protein digestibility in the adult human.

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## CHAPTER II

### Commercial *Phaseolus vulgaris* extract (Starch Stopper) increases ileal endogenous amino acid and crude protein losses in the growing rat

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The objective of the present study was to investigate the influence of antinutritional factors on gut endogenous protein losses (flows) within a practical context. To this end, growing rats were fed protein-free diets to which different levels of a natural source of antinutritional factors, a crude extract of raw white kidney bean (*Phaseolus vulgaris*) consumed as a “starch stopper” to assist in body weight loss in humans, were added.

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## ABSTRACT

The effect of a commercial *Phaseolus Vulgaris* extract (PVE, Starch Stopper) on ileal and faecal endogenous protein losses was studied. Growing rats were fed for 14 days a protein-free diet containing PVE at nutritional concentrations: 0% (PF1), 0.4% (PF2) or 1.1% PVE (PF3), or 1.1% autoclaved PVE (PF4). An indigestible marker (TiO<sub>2</sub>) was included in each diet. Ileal endogenous amino acid (AA) losses were significantly higher ( $P < 0.05$ ) in PF3 (20% higher than in PF1), except for proline, glycine, alanine and histidine. Endogenous ileal nitrogen (N) losses were 22% higher in PF3 than in PF1. Endogenous faecal AA and N losses were all significantly higher ( $P < 0.05$ ) in PF3. Starch digestibility (~100%), food intake (single daily meal, d10-23) and body weight loss were not significantly different among the groups. PVE, at 1.1% of the diet, not only was ineffective in reducing starch digestibility but also led to increased ileal endogenous N losses, possibly due to the antinutritional factors (trypsin inhibitor, lectin) present in the PVE.

## INTRODUCTION

Beans contain several antinutritional factors (ANF), such as enzyme (alpha-amylase, trypsin, chymotrypsin) inhibitors, lectins, phytic acid, flatulence factors, saponins and toxic factors (Bowman, 1945). The  $\alpha$ -amylase inhibitor ( $\alpha$ AI), *phaseolamin*, has been isolated from kidney beans (*Phaseolus vulgaris*) and characterized by Marshall and Lauda (1975). It specifically inhibits animal  $\alpha$ -amylases, especially human saliva amylase and human and porcine pancreatic amylases, but has no activity towards plant, bacterial or fungal enzymes (Marshall & Lauda, 1975).

The  $\alpha$ AI activity of kidney bean extracts has been exploited to produce products (commonly referred to as “starch blocker” or “starch stopper” products) that claim to reduce starch digestion and absorption in humans and thus assist in body weight loss. As a crude extract of kidney beans, starch stopper products contain not only  $\alpha$ AI but also other antinutritional factors, found in variable concentrations (Liener *et al.*, 1984). The major ANF in beans that can cause adverse physiological responses are trypsin–chymotrypsin inhibitors and lectins (Pusztai *et al.*, 1982). Lectins are sugar-binding proteins that bind to the epithelial cell lining of the intestinal mucosa, causing damage to the microvilli and intestinal malabsorption. Trypsin–chymotrypsin inhibitors,



part of the Bowman-Birk type inhibitors, not only inhibit intestinal protein digestion but can also lead to hypertrophy and hyperplasia of the pancreas and hypersecretion of digestive enzymes in rodents (Lajolo & Genovese, 2002). Lectins and trypsin inhibitors, moreover, may increase overall gut endogenous protein losses in simple-stomached animals (Grala *et al.*, 1998; Jansman *et al.*, 1998). Costa de Oliveira *et al.* (1986) reported that endogenous protein losses in rat faeces were higher when rats were fed a diet containing 10% of raw *Phaseolus vulgaris* than when they were fed a casein-based diet. There is, however, little information on this aspect of the effects of *Phaseolus vulgaris* when ingested as a commercial extract, known as “starch stopper”. The present study thus aimed to determine the effect of a commercial *Phaseolus vulgaris* extract (PVE), at nutritional concentrations, on ileal and faecal endogenous losses of amino acids (AA) in the growing rat. Protein-free diets were used to determine endogenous protein and AA flows (losses) at the terminal ileum and in the faeces of the growing rat. PVE was added to a protein-free diet at concentrations equivalent to what a human subject would consume, according to the manufacturer’s recommendations. Any undigested protein originating from the PVE was assumed to be a negligible proportion of the protein losses at the terminal ileum and in the faeces. Two controls were included: a standard protein-free diet and a protein-free diet to which autoclaved PVE (aPVE) was added.

## MATERIALS AND METHODS

### *Phaseolus vulgaris* extract and analysis of ANF

The commercial *Phaseolus vulgaris* extract (PVE, “starch stopper”) was purchased from a pharmacy (Palmerston North, New Zealand) as capsules containing dry powdered (500 mg/capsule) white kidney bean extract. According to the information supplied by the company, 1 g of PVE was extracted with water from 12 g dry beans. PVE was removed from the capsules prior to use and was added to the diets either “as is” or after inactivation of the ANF (aPVE).

To inactivate the ANF, PVE was soaked for 15 h in deionised water, as advocated by Carvalho and Sgarbieri (1997). The wet flour was autoclaved at 121°C for 15 min, as described in previous studies (Kadam *et al.*, 1987), and freeze-dried. The major ANF known to be present in beans ( $\alpha$ AI, lectin and trypsin inhibitor) were determined in both

PVE and aPVE. Amylase and  $\alpha$ AI activities were determined by an adaptation of Bernfeld's method (1955), whereby  $\alpha$ AI was extracted from PVE in a 0.02 M sodium phosphate buffer (pH 6.9) containing NaCl (9 g/L). After 1 h vigorous shaking and centrifugation (15 min, 14000g, 4°C), the supernatant was collected [adapted from (Liener *et al.*, 1984) and (Grant *et al.*, 1995)], mixed with 1  $\mu$ g porcine  $\alpha$ -amylase (1:1, v/v, Sigma Chemical Co., St. Louis, MO) and pre-incubated (1 h, 37°C, pH 6.9). When 50% of porcine  $\alpha$ -amylase was inhibited, the  $\alpha$ AI units were determined and expressed as the number of amylase units inhibited per g starch stopper dry matter (DM) (Liener *et al.*, 1984). One amylase unit was defined as the amount of enzyme that released 1  $\mu$ mol of maltose per minute (Bernfeld, 1955). Appropriate blanks were included.

Lectin concentration was determined by the method of Burbano *et al.* (1999). A competitive indirect ELISA for quantification of the *Phaseolus vulgaris* Lectin (PHA) using anti-PHA IgG antibody (Sigma Chemical Co., St. Louis, MO) was performed.

Quantitative trypsin inhibitor measurements were performed and trypsin inhibitor units (TIU) defined as previously described (Muzquiz *et al.*, 2004).

Total dietary fibre was determined on PVE and aPVE using the kit Megazyme Total Dietary Fibre (Megazyme International Ireland Ltd., Wicklow, Ireland), which follows the method of Prosky *et al.* (1988).

The protein profiles of PVE and aPVE were assessed by electrophoresis. Soluble protein was extracted by stirring the dried sample in borate buffer (100 mmol/L H<sub>3</sub>BO<sub>3</sub>, 150 mmol/L NaCl, pH 8.0) for 1.5 h and centrifuging at 12000 g for 10 min at room temperature. The supernatants were collected and stored at -20°C until electrophoresis analysis. Soluble protein was measured using the bicinchoninic acid protein (BCA) assay (Pierce Chemical Co., Rockford, IL), based on a colorimetric reaction. Electrophoresis was carried out using a mini-gel apparatus (Mini-Protean system, Bio-Rad, Richmond, CA) in a 125 g/L acrylamide separating gel and a 45 g/L acrylamide stacking gel, according to the method of Laemmli (1970). The samples were dissolved in 1 mol/L Tris-HCl buffer (pH 6.8) containing 2.7 mol/L glycerol, 0.139 mol/L sodium dodecyl sulphate (SDS) and 0.4 mmol/L mercaptoethanol and heated to 100°C for 3 min, for reduction of disulfide bonds. The samples were loaded onto the gel, with equivalent amounts of soluble proteins (200  $\mu$ g) deposited in each well. Molecular mass standards obtained from Bio-Rad Laboratories (Hercules, CA) were also loaded in a separate well. Electrophoresis was performed in 62.5 mmol/L Tris-HCl buffer with 3.4 mmol/L SDS for 1.15 h. The electric field conditions were 170 V and 40 mA and were

set in a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA). The protein bands were fixed and stained using a solution of Coomassie Brilliant blue R-250.

### *Animals and housing*

Sixty-four Sprague–Dawley male rats (170–175g body weight, 44 days of age) were obtained from the Small Animal Production Unit, Massey University (Palmerston North, New Zealand). Ethics approval was received from the Massey University Animal Ethics Committee (protocol 03/135). The animals were housed individually in raised stainless steel cages with wire mesh floors above individual trays collecting faeces and urine. The room was maintained at  $20 \pm 2^\circ\text{C}$  with a 12 h light/dark cycle. Food was given during the light cycle. Water was continuously available.

### *Diets*

Five diets were prepared including a preliminary diet, given to all the rats, and four experimental diets, each given to 16 rats. The experimental diets, based on an essentially protein-free diet, consisted of a control (no PVE, diet PF1), two diets to which PVE was added at physiologically meaningful concentrations (0.4% PVE, diet PF2; 1.1% PVE, diet PF3), and a second control which contained 1.1% of aPVE (diet PF4). The experimental diets, PF1, PF2 and PF3, contained 0.06, 0.07 and 0.09 g N/100 g diet, which correspond to 0.36, 0.45 and 0.59 g crude protein/100 g diet, respectively (conversion factor: 6.25). PF4 contained the same concentration of crude protein as PF3.

PVE was included in the diet of the growing rat so as to provide an amount of PVE, on a food intake basis, equivalent to that ingested by humans taking the commercial starch stopper product. On the basis of the manufacturer's recommendations, a human would consume some 3 g PVE per day. Assuming a daily food intake for the adult human of 436 g DM (Marriott & Buttriss, 2003) and a daily food intake for the growing rat of 11 g DM (Butts *et al.*, 1992) (same breed, similar body weight and diet), the equivalent dose of PVE for the rat was calculated to be 75 mg/d. Lower and higher test doses (45 and 120 mg/d/rat) were chosen, equivalent to 0.4 and 1.1% PVE added to the air-dry diet. Titanium dioxide was added to each diet as an indigestible marker. Dietary ingredient compositions are given in **Table 1** and determined AA and nitrogen (N) compositions of PF1, PVE and aPVE in **Table 2**.

**Table 1.** Ingredient compositions of the preliminary and experimental diets.

Ingredient	Diet <sup>1</sup>				
	Preliminary	PF1	PF2	PF3	PF4
	<i>g/kg air dry weight</i>				
Cornflour <sup>2</sup>	690	747	743	736	736
Cellulose <sup>3</sup>	10	50	50	50	50
Vitamin mix <sup>4</sup>	50	50	50	50	50
Salt mix <sup>5</sup>	50	50	50	50	50
Soya oil	80	100	100	100	100
Lactic casein <sup>6</sup>	120	-	-	-	-
Titanium dioxide	-	3	3	3	3
PVE <sup>7</sup>	-	-	4	11	-
aPVE <sup>8</sup>	-	-	-	-	11

<sup>1</sup> The preliminary diet was fed to the rats during the first 10 days. The other diets were based on an essentially protein-free diet with no commercial *Phaseolus Vulgaris* extract, PVE (PF1) or 0.4% PVE (PF2), 1.1% PVE(PF3) and 1.1% inactivated PVE (PF4).

<sup>2</sup> Goodman Fielder Industries Limited, Summerhill, NSW, Australia.

<sup>3</sup> Avicel PH101. Commercial Minerals Ltd., Auckland, New Zealand.

<sup>4</sup> Crop & Food Research, Palmerston North, New Zealand. The mixture supplied: (mg/kg diet) retinol acetate 5.0, DL- $\alpha$ -tocopheryl acetate 200, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20, folic acid 2.0, nicotinic acid 20, D-biotin 1.0, myo-inositol 200, choline chloride 1500; ( $\mu$ g/kg diet) ergocalciferol 25, cyanocobalamin 50.

<sup>5</sup> Crop & Food Research, Palmerston North, New Zealand. The mixture supplied: (g/kg diet) Ca 6.29, Cl 7.79, Mg 1.06, P 4.86, K 5.24, Na 1.97; (mg/kg diet) Cr 1.97, Cu 10.7, Fe 424, Mn 78.0, Zn 48.2; ( $\mu$ g/kg diet) Co 29.0, I 151, Mo 152, Se 151.

<sup>4,5</sup> The mixtures were formulated to meet the vitamin and mineral requirements of the growing rat as described by the Agricultural Research Council (National Research Council, 1995).

<sup>6</sup> Lactic casein 30 mesh. Alacid, New Zealand Milk Products, Wellington, New Zealand.

<sup>7</sup> *Phaseolus Vulgaris* extract as dry powder taken from capsules of proprietary starch stopper product (50 Caps/packet). Each capsule contained 500mg of a water extract of *Phaseolus vulgaris*, equivalent to 6 g of raw *Phaseolus vulgaris*.<sup>8</sup> Autoclaved PVE. PVE was soaked, autoclaved, and freeze-dried.

**Table 2.** Determined amino acid and total nitrogen contents of the essentially protein-free diet (PF1) and of the crude *Phaseolus vulgaris* extract (PVE) and autoclaved *Phaseolus vulgaris* extract (aPVE).

	Diet PF1	PVE	aPVE
		%	
Aspartic acid	0.008	1.9	1.9
Threonine	0.003	0.6	0.6
Serine	0.005	0.7	0.7
Glutamic acid	0.012	2.7	2.3
Proline	0.000	0.8	0.6
Glycine	0.005	0.6	0.6
Alanine	0.004	0.6	0.6
Valine	0.007	0.6	0.7
Isoleucine	0.003	0.5	0.6
Leucine	0.006	1.0	1.1
Tyrosine	0.001	0.6	0.5
Phenylalanine	0.001	0.5	0.8
Histidine	0.010	0.5	0.4
Lysine	0.003	0.9	0.9
Arginine	0.000	0.9	0.8
Nitrogen	0.058	3.1	2.7

### ***Experimental design***

The rats were acclimatized to the cages and to the feeding regimen over a 10-day period (days 0–9) and for the entire study received a single meal daily (0900 to 1200 h). During the acclimatization period the rats were fed the preliminary diet. The rats were then randomly and equally allocated to the four experimental diets for a 14-day experimental period (days 10–23).

The rats were weighed on days 9 and 22. Food intake was recorded daily. Faeces were collected on absorbant paper. Collection was made three times a day on days 20, 21, and 22. Faeces were cleaned of any spilled food and were immediately frozen at -20°C.

On day 23, 3h ± 15 min after the start of feeding, the rats were asphyxiated with carbon dioxide gas and decapitated (immediately ceasing all neural stimulation to the gut). The abdomen was opened by an incision along the mid-ventral line, and the skin and musculature were folded back to expose the viscera. The final 20 cm of the ileum were dissected from the body, rinsed with deionised 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 10ml of deionised water from a plastic syringe and the pH was adjusted to 3.0 by the addition of 6M HCl to prevent bacterial activity. The samples were immediately frozen at  $-20^{\circ}\text{C}$ .

### ***Chemical analysis***

Faeces, digesta and diet samples were freeze-dried and ground. Faeces were pooled for each rat over the 3 days of collection and then pooled from two randomly selected rats within the same diet. Ileal digesta were pooled using the same pair of rats as for the faeces. Furthermore, composite digesta samples were obtained by pooling equal quantities of ileal digesta across rats receiving the same diet. Samples were stored in a desiccator.

The starch content was measured in the faeces, in the composite digesta samples, and in the diets, using the kit Megazyme Total Starch (Megazyme International Ireland Ltd., Wicklow, Ireland).

Total N was determined in duplicate in the faeces, in the composite digesta samples, and in the diets following the Dumas method. The samples were combusted at  $1050^{\circ}\text{C}$  in  $\text{O}_2$  gas. The N was then reduced to  $\text{N}_2$  by a catalyst, and this was measured using a Leco FP-2000 thermal conductivity cell (Leco Corp., St Joseph, MI).

The AA composition was determined on individual ileal digesta and faeces samples as follows: duplicate dried samples (5 mg) were hydrolysed in 1 ml of 6 M glass-distilled HCl containing 0.1% phenol for 24 h at  $110^{\circ}\text{C} \pm 2^{\circ}\text{C}$  in glass tubes sealed under vacuum. AA concentrations were then measured using a Waters ion exchange high-performance liquid chromatography (HPLC) system calibrated against a reference AA mixture with known concentrations. The peaks of the chromatograms were integrated using the dedicated software Millennium (Waters Millipore), which identifies the AA by retention time against a reference AA mixture. Cysteine, methionine, and tryptophan, being destroyed during acid hydrolysis, were not determined.

Titanium dioxide was determined by a colometric assay following the method of Short *et al.* (1996). The sample was ashed and dissolved in sulphuric acid. Hydrogen peroxide was subsequently added and absorbance measured at 410 nm using an automatic spectrophotometer (COBAS FARA 2, Roche Diagnostic, Basel, Switzerland).

### *Data Analysis*

Ileal and faecal starch flows were determined using the following equation:

(1) Starch flow (mg/100mg of dry matter intake (DMI)) =

$$\frac{\text{starch in digesta or faeces (mg/100 mg digesta or faeces)} \times \text{TiO}_2 \text{ in diet (mg/100 mg diet)}}{\text{TiO}_2 \text{ in digesta or faeces (mg/100 mg digesta or faeces)}}$$

Ileal and faecal starch digestibility determined using the following equation:

(2) Ileal or faecal starch digestibility (%) =

$$100 \times \frac{\text{dietary starch intake (mg/100 mg DMI)} - \text{ileal or faecal starch flow (mg/100 mg DMI)}}{\text{dietary starch intake (mg/100 mg DMI)}}$$

Endogenous N or AA flows were determined using the following equation:

(3) Endogenous flow (mg/100 mg DMI) =

$$\frac{\text{N or AA in digesta or faeces (mg/100 mg digesta or faeces)} \times \text{TiO}_2 \text{ in diet (mg/100 mg diet)}}{\text{TiO}_2 \text{ in digesta or faeces (mg/100 mg digesta or faeces)}}$$

The data were tested for homogeneity of variance using Bartlett's test using Minitab (version 14, Minitab Inc., State College, PA). The results were subjected to a one-way ANOVA using the computer programme SAS (version 8.2; SAS Institute Inc., Cary, NC). The food intake data were subjected to a one-way ANOVA for repeated measures. For  $P < 0.05$ , the significance of difference between means was determined using Tukey's test.

## **RESULTS**

The ANF activities in PVE and aPVE are given in **Table 3**. The lectin was completely destroyed in aPVE, whereas  $\alpha$ AI and trypsin inhibitor activities were reduced by 96 and 62%, respectively. Determined total dietary fibre contents were 34.9 and 36 g/100 g DM in PVE and aPVE, respectively. The protein profile of PVE (**Figure 1**) showed strong signals between 43 and 50 kDa and close to 30 kDa, and lower signals at 20 and 25 kDa. These signals were lower in aPVE. A band at 10 kDa was visible for both PVE and aPVE.

**Table 3.** Amylase inhibitor activity, trypsin inhibitor activity and lectin (PHA) content in the commercial *Phaseolus vulgaris* extract (PVE) before and after autoclaving.

	Undenatured PVE	Autoclaved PVE <sup>1</sup>	Pooled SE
Amylase inhibitor activity (AIU/g DM) <sup>2</sup>	89.9	3.6	3.8
Lectin (mg PHA/g DM) <sup>3</sup>	14.0	nd <sup>4</sup>	0.6
Trypsin inhibitor activity (TIU x 10 <sup>-3</sup> /g DM) <sup>5</sup>	56.9	21.3	0.7

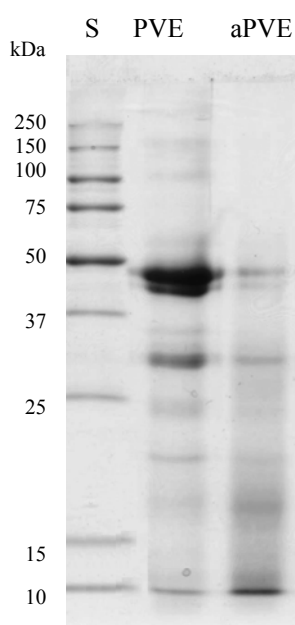
<sup>1</sup> aPVE.

<sup>2</sup> AIU, amylase inhibitor unit; DM, dry matter, n = 4.

<sup>3</sup> PHA, phytohaemagglutinin, i.e. *Phaseolus vulgaris* lectin, n = 4.

<sup>4</sup> not detectable.

<sup>5</sup> TIU= trypsin inhibitor unit, n = 6.



**Figure 1.** Protein profile of extracts from a crude commercial *Phaseolus vulgaris* extract (PVE) and an autoclaved PVE (aPVE). Aliquots from each extract containing 200 µg soluble proteins were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) followed by coloration with Coomassie Brilliant blue R-250 (Burbano *et al.*, 1999). Electrophoresis was run at 40 mA for 1.15 h. A molecular mass standard (S) was included (10–250 kDa).

The rats consumed the diets readily and remained healthy, although they lost body weight over the study. Body weight loss over the 14-day experimental period were (mean ± SE) 14.8 ± 2.0, 13.7 ± 1.3, 12.5 ± 1.5, and 12.4 ± 2.0 g for PF1, PF2, PF3, and PF4, respectively, and were not significantly different ( $P > 0.05$ ) between diets. Daily food intakes (days 10–23, mean ± SE) were 7.0 ± 0.1, 7.2 ± 0.1, 7.1 ± 0.1, and 7.1 ± 0.2



g for the diets PF1, PF2, PF3, and PF4, respectively, and were not significantly different ( $P > 0.05$ ) between diets.

### ***Starch digestibility***

Ileal and faecal measures of starch digestibility were close to 100% (99.2–100%) for all of the diets.

### ***Nitrogen and amino acid flows in the ileal digesta***

The endogenous AA flows at the terminal ileum are given in **Table 4**. There was a significant effect ( $P < 0.05$ ) of diet on the endogenous ileal AA flows for most of the AA except for alanine, proline, glycine and histidine. When there was a statistically significant effect of the diet, the endogenous ileal AA flows for rats fed diet PF3 were significantly higher ( $P < 0.05$ ) than those for rats fed the controls PF1 and PF4 and for rats fed diet PF2 except for the AA threonine and tyrosine, for which endogenous flows from rats fed diets PF2 and PF3 were not significantly different ( $P > 0.05$ ). Endogenous ileal AA flows were not different ( $P > 0.05$ ) among rats fed the controls PF1 and PF4 and rats fed diet PF2 except for the AA threonine and tyrosine, for which endogenous flows from rats fed diet PF2 were higher than those from rats fed the controls PF1 or PF4.

Endogenous ileal total N flows, determined on the composite ileal digesta samples, were 0.073, 0.070, 0.089, and 0.069 mg/100 mg DMI, for rats fed diets PF1, PF2, PF3, and PF4, respectively. As observed for the endogenous ileal AA flows, the endogenous ileal N flow for rats fed diet PF3 was numerically higher than those for rats fed diets PF1, PF2, or PF4.

### ***Nitrogen and amino acid flows in the faeces***

The endogenous AA and N flows in the faeces are given in **Table 5**. As observed for the ileal measures, the endogenous faecal AA flows for rats fed diet PF3 were significantly higher ( $P < 0.05$ ) than those for rats fed diet PF2 or the controls PF1 and PF4. Faecal endogenous AA flows were not significantly different ( $P > 0.05$ ) among rats fed the controls PF1 and PF4 and rats fed diet PF2. The endogenous faecal N flow for rats fed diet PF3 was significantly higher ( $P < 0.001$ ) than for rats fed diets PF1, PF2, or PF4. The endogenous faecal N flow for rats fed diet PF2 was significantly higher ( $P < 0.001$ ) than for rats fed the controls PF1 and PF4. The endogenous faecal N flows for rats fed diets PF1 or PF4 were not significantly different ( $P > 0.05$ ).

**Table 4.** Mean ileal endogenous amino acid flows (n = 8) at the terminal ileum of growing rats fed diets PF1, PF2, PF3, or PF4<sup>1</sup>.

	Diet				Pooled SE	Significance <sup>2</sup>
	PF1	PF2	PF3	PF4		
	<i>mg/100 mg dry matter intake</i>					
Aspartic acid <sup>3</sup>	0.049 <sup>a</sup>	0.049 <sup>a</sup>	0.060 <sup>b</sup>	0.049 <sup>a</sup>	0.002	**
Threonine	0.034 <sup>a</sup>	0.035 <sup>ac</sup>	0.040 <sup>bc</sup>	0.034 <sup>a</sup>	0.002	*
Serine	0.022 <sup>a</sup>	0.023 <sup>a</sup>	0.029 <sup>b</sup>	0.022 <sup>a</sup>	0.001	***
Glutamic acid	0.049 <sup>a</sup>	0.048 <sup>a</sup>	0.060 <sup>b</sup>	0.046 <sup>a</sup>	0.002	**
Proline	0.031	0.029	0.033	0.029	0.002	NS
Glycine	0.065	0.059	0.068	0.055	0.005	NS
Alanine	0.018	0.019	0.022	0.018	0.001	NS
Valine	0.017 <sup>a</sup>	0.018 <sup>a</sup>	0.022 <sup>b</sup>	0.017 <sup>a</sup>	0.001	**
Isoleucine	0.011 <sup>a</sup>	0.012 <sup>a</sup>	0.015 <sup>b</sup>	0.011 <sup>a</sup>	0.001	**
Leucine	0.018 <sup>a</sup>	0.020 <sup>a</sup>	0.025 <sup>b</sup>	0.018 <sup>a</sup>	0.001	**
Tyrosine	0.010 <sup>a</sup>	0.011 <sup>ac</sup>	0.012 <sup>bc</sup>	0.009 <sup>a</sup>	0.001	**
Phenylalanine	0.010 <sup>a</sup>	0.011 <sup>a</sup>	0.014 <sup>b</sup>	0.010 <sup>a</sup>	0.001	***
Histidine	0.021	0.021	0.025	0.021	0.001	NS
Lysine	0.015 <sup>a</sup>	0.016 <sup>a</sup>	0.019 <sup>b</sup>	0.015 <sup>a</sup>	0.001	*
Arginine	0.012 <sup>a</sup>	0.011 <sup>a</sup>	0.015 <sup>b</sup>	0.011 <sup>a</sup>	0.001	**

<sup>1</sup> PF1, PF2, PF3 contained 0, 4, and 11 g/kg commercial *Phaseolus vulgaris* Extract (PVE). PF4 contained 11 g/kg autoclaved PVE.

<sup>2</sup> \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; NS, non significant.

<sup>3</sup> Means within a row with a different superscript were significantly different (P < 0.05).

**Table 5.** Mean endogenous amino acid and total nitrogen flows (mg/100 mg dry matter intake, n = 8) in the faeces of growing rats fed diets PF1, PF2, PF3, and PF4<sup>1</sup>.

	Diet				Pooled SE	Significance <sup>2</sup>
	PF1	PF2	PF3	PF4		
	<i>mg/100mg dry matter intake</i>					
Aspartic acid <sup>3</sup>	0.063 <sup>a</sup>	0.066 <sup>a</sup>	0.080 <sup>b</sup>	0.067 <sup>a</sup>	0.002	***
Threonine	0.027 <sup>a</sup>	0.028 <sup>a</sup>	0.034 <sup>b</sup>	0.028 <sup>a</sup>	0.001	***
Serine	0.026 <sup>a</sup>	0.027 <sup>a</sup>	0.033 <sup>b</sup>	0.027 <sup>a</sup>	0.001	***
Glutamic acid	0.074 <sup>a</sup>	0.079 <sup>a</sup>	0.097 <sup>b</sup>	0.076 <sup>a</sup>	0.003	***
Proline	0.024 <sup>a</sup>	0.025 <sup>bd</sup>	0.031 <sup>c</sup>	0.025 <sup>ad</sup>	0.001	**
Glycine	0.032 <sup>a</sup>	0.033 <sup>a</sup>	0.040 <sup>b</sup>	0.033 <sup>a</sup>	0.001	***
Alanine	0.038 <sup>a</sup>	0.040 <sup>a</sup>	0.049 <sup>b</sup>	0.041 <sup>a</sup>	0.002	*
Valine	0.029 <sup>a</sup>	0.031 <sup>a</sup>	0.038 <sup>b</sup>	0.030 <sup>a</sup>	0.001	***
Isoleucine	0.023 <sup>a</sup>	0.024 <sup>a</sup>	0.029 <sup>b</sup>	0.024 <sup>a</sup>	0.001	***
Leucine	0.037 <sup>a</sup>	0.039 <sup>a</sup>	0.048 <sup>b</sup>	0.038 <sup>a</sup>	0.001	***
Tyrosine	0.019 <sup>a</sup>	0.021 <sup>ac</sup>	0.025 <sup>b</sup>	0.020 <sup>ac</sup>	0.001	***
Phenylalanine	0.021 <sup>a</sup>	0.023 <sup>a</sup>	0.028 <sup>b</sup>	0.021 <sup>a</sup>	0.001	***
Histidine	0.015 <sup>a</sup>	0.015 <sup>a</sup>	0.019 <sup>b</sup>	0.014 <sup>a</sup>	0.001	***
Lysine	0.031 <sup>a</sup>	0.034 <sup>a</sup>	0.041 <sup>b</sup>	0.033 <sup>a</sup>	0.001	***
Arginine	0.024 <sup>a</sup>	0.024 <sup>a</sup>	0.030 <sup>b</sup>	0.024 <sup>a</sup>	0.001	***
Nitrogen	0.089 <sup>a</sup>	0.105 <sup>b</sup>	0.116 <sup>c</sup>	0.095 <sup>a</sup>	0.003	***

<sup>1</sup> PF1, PF2, and PF3 contained 0, 4, and 11 g/kg commercial *Phaseolus vulgaris* Extract (PVE). PF4 contained 11 g/kg autoclaved PVE.

<sup>2</sup> \*\*\*, P < 0.001; \*\*, P < 0.01; \* P < 0.05.

<sup>3</sup> Means within a row with a different superscript were significantly different (P < 0.05).

## DISCUSSION

The primary aim of the present study was to assess the effects of a commercial *Phaseolus vulgaris* extract given at recommended concentrations on ileal endogenous protein losses in the growing rat. The current work showed that PVE, when included in the diet at 1.1%, was not only ineffective in reducing starch digestibility but also led to increased ileal and faecal endogenous protein losses. The ineffectiveness of a commercial PVE was reported earlier (Carlson *et al.*, 1983) and shown to be due to insufficient antiamylase activity (Pusztai *et al.*, 1995).

The present study did not show any specific effect of PVE either on body weight loss or on voluntary food intake, as previously observed with rats fed a commercial soyabean starch stopper product (Umoren & Kies, 1992). This finding is consistent with the absence of a PVE effect on starch digestibility, presumably due to its amylase inhibitor activity (89.8 units/g), which is in the lower range of that determined on other commercial *Phaseolus vulgaris* extracts (44.3–327.6 units/g) (Liener *et al.*, 1984). Moreover,  $\alpha$ AI was found to be unstable in the stomach and active only after preincubation with amylase in the absence of starch (Lajolo & Genovese, 2002), which cannot occur when PVE is ingested within a starchy meal.

Our results showed that endogenous protein losses increased in animals fed the PVE-supplemented diets, from determinations both at the terminal ileum and in the faeces. Although ileal endogenous losses are considered to be more representative of the gut endogenous losses, as the metabolic interference of the colonic bacteria is reduced, endogenous faecal losses, thus, are expected to follow a similar trend, giving supplementary information on the effect of dietary factors on endogenous protein losses. Endogenous protein losses, determined after administration of a protein-free diet, are known to be somewhat underestimated (Moughan, 2003). This method, however, is still useful and widely used for evaluating relative effects of dietary factors, such as in the present study. The PVE used in this study was included in the protein-free diets at low but nutritionally relevant concentrations (0.4 and 1.1% in PF2 and PF3, respectively). The actual consumption of PVE (30 and 75 mg/d/rat for diets PF2 and PF3, respectively) was equivalent to -36% and +53% of the average recommended consumption for a human subject (1.9 and 4.6 g/day, respectively). The actual consumption of PVE was less than first planned, due to a lower daily food intake than

that reported by Butts *et al.* (1992), but did not affect the objectives of this study. Diets PF2 and PF3 were not strictly protein-free but contained 0.45 and 0.59% crude protein, respectively. It can be shown from first principles (Deglaire, unpublished data) that such amounts of dietary protein would make only a negligible contribution to the ileal AA flows. When rats were fed diet PF4, which contained the same protein concentration as diet PF3 but from inactivated (autoclaved) PVE, ileal and faecal endogenous amino acid losses were not significantly different from those determined in rats fed the protein-free diet (PF1). This confirms that protein from PVE in diets PF2 and PF3 was a negligible proportion of the ileal and faecal protein losses and that AA and N determined in the digesta or in the faeces could reasonably be considered as endogenous.

When rats were fed the diet containing 1.1% PVE (PF3), they exhibited higher ileal and faecal endogenous AA and total N losses than rats fed the protein-free diet (PF1) or the diet containing 1.1% aPVE (PF4). This accords with the observations of Costa de Oliveira *et al.* (1986), who reported higher endogenous faecal protein losses in rats fed a diet containing 10% *Phaseolus vulgaris*. The diet used by Costa de Oliveira *et al.* is similar to the present diet at 1.1% PVE (PF3), as 1 g PVE is a water extract of 12 g of raw *Phaseolus vulgaris*.

As shown on the SDS-PAGE profile, the present PVE was not a purified extract and contained a large number of different proteins, the qualitative distribution of which was similar to the one found in raw *Phaseolus vulgaris* (Liener *et al.*, 1984). This has been previously observed in at least four other commercial PVE (Liener *et al.*, 1984). The signal observed at 30 kDa is characteristic for lectins, and the one at 10 kDa is typical for the trypsin inhibitor (8 kDa) (Pusztai *et al.*, 1995). The strong signals observed between 43 and 48 kDa appear to belong to phaseolin, the main storage protein reported to have subunits between 43 and 53 kDa (Carbonaro *et al.*, 2005) and which represents near 80% of the total bean proteins (Genovese & Lajolo, 1998). Some of the signals observed at 33 and 45 kDa might be subunits, reported to be at 12.4, 15.2, 33.6, and 45 kDa (Lee *et al.*, 2002). The profile of autoclaved PVE confirms that proteins, more specifically ANF, were denatured by thermal treatment, as observed with the measure of activities and concentrations. Trypsin inhibitor activity was not completely inactivated by the autoclaving step, which is in agreement with previous observations on autoclaved *Phaseolus vulgaris* (Carvalho & Sgarbieri, 1997). The trypsin inhibitor of most legume seeds is known to be difficult to completely inactivate by heat treatment, but is usually reduced to nonharmful concentrations (Liener, 1994), with autoclaving. Phaseolin, the

concentration of which was not determined here, is known to have an improved digestibility and thus a denatured structure after thermal treatment (Marquez & Lajolo, 1990), which is further indication to associate the signals between 43 and 48 kDa with phaseolin subunits.

The observed effect of PVE on the ileal and faecal endogenous N and AA losses is most likely due to the ANF, especially trypsin inhibitor and lectins, known to be the major ANF present in *Phaseolus vulgaris* (Pusztai *et al.*, 1982; Liener *et al.*, 1984). Lectins have been shown to bind to the microvilli of rat mucosa cells, leading to a degradation of brush border cells and to a faster renewal of cells. One result of this is cellular hyperplasia and increased gut endogenous losses as reviewed by Vasconcelos and Oliveira (2004). Trypsin inhibitors from legume seeds were shown to stimulate pancreatic secretions (Lajolo & Genovese, 2002), resulting in an increase of gut endogenous protein losses. More generally, ANF may adversely affect protein digestibility and AA availability (Gilani & Sepehr, 2003) and are reported to enhance endogenous ileal protein losses in pigs (Grala *et al.*, 1998 ; Jansman *et al.*, 1998). Phaseolin's effect on the gut is controversial: Santoro *et al.* (1999) reported an increase in the small intestinal dry weight and of faecal losses of endogenous N in rats, whereas Montoya *et al.* (2006) did not find any detrimental effect.

The total dietary fibre content was high in both PVE and aPVE, and dietary fibre may influence gut endogenous losses. Garcia *et al.* (1997) reported a value close to 20% in other varieties of raw *Phaseolus vulgaris*. The fibre content was not different between PVE and aPVE, however, which means that the level of dietary fibre was not responsible for the higher N and AA losses.

When rats were fed diet PF2, containing 0.4% PVE, most endogenous ileal and faecal AA flows were not different ( $P > 0.05$ ) from those of rats fed the control diets (PF1 and PF4), except for the ileal AA threonine and tyrosine. This suggests the presence of a threshold concentration of PVE, and probably of ANF, before major effects on endogenous protein losses are found. PVE from other manufacturers can be found with higher ANF concentrations, as reported by Liener *et al.* (1984), and are thus likely to enhance endogenous losses over and above that found with the present PVE.

In conclusion, the PVE (crude extract of *Phaseolus vulgaris*), when ingested at 1.1% of the diet, increased ileal and faecal protein losses in the growing rat, but did not affect carbohydrate digestion. The consumption of the present commercial *Phaseolus vulgaris*

extract is not without physiological effect, at least in the growing rat. This is likely to be due to the ANF, especially trypsin inhibitor and lectin. The replacement of gut proteins arising due to enhanced losses of gut endogenous proteins is energy demanding, and thus the PVE may have some effect on overall animal energetics. The potentially harmful effects of the *Phaseolus vulgaris* extract, when consumed at a nutritional level, on gut tissue integrity need to be investigated further.

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## CHAPTER III

# Feeding dietary peptides to growing rats enhances gut endogenous protein flows as compared to feeding protein-free or free amino-acid based diets

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The present study aimed to assess the influence of diets containing a source of N on ileal endogenous protein flows compared with that of a protein-free diet in the growing rat. Two forms of delivery of N, a casein hydrolysate or the corresponding free amino acid mixture, were compared.

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## ABSTRACT

The effect of dietary peptides on gut endogenous nitrogen (N) flow (ENFL) and amino acid (AA) flows (EAAFL) was studied. Semi-synthetic diets containing enzyme-hydrolysed casein (HC; 11%) or a free AA mixture devoid of aspartic acid and serine (A1) or glycine and alanine (A2) were formulated to have similar AA compositions except for the excluded AA and similar dietary electrolyte balances ( $\text{Na}^+ + \text{K}^+ - \text{Cl}^-$ ). A protein-free diet (PF) served as a control. Sprague-Dawley rats were given the diets 8 times a day for 10 min each hour over 7 days. Rats were killed and digesta were sampled (6 observations within each group) along the intestinal tract 6 h after the first meal on day 7. EAAFL and ENFL, estimated with reference to the dietary marker titanium dioxide, were determined directly (PF, A1 and A2) or after centrifugation and ultrafiltration of the digesta (HC). Endogenous flows of aspartic acid and serine or glycine and alanine did not differ ( $P > 0.05$ ) in any of the intestinal sections between rats fed PF and A1 or PF and A2, respectively, except in the stomach where serine flow was greater for rats fed A1. Ileal endogenous flows for most of the AA and for N were significantly higher ( $P < 0.05$ ) for rats fed diet HC compared to those for rats fed diets PF, A1 or A2, except for phenylalanine, tyrosine, lysine, which did not differ among the groups. Ileal EAAFL and ENFL were not influenced by body N balance *per se* but were affected by the presence in the gut of dietary peptides derived from casein.

## INTRODUCTION

It is important to be able to distinguish between amino acids (AA) of body origin (*i.e.* endogenous) present in digesta and those of dietary origin. Ileal endogenous AA flows (EAAFL), considered as losses to the body, are an important component of the daily dietary AA requirement in humans (Gaudichon *et al.*, 2002; Moughan *et al.*, 2005). A better understanding of factors influencing these endogenous losses is thus required.

Several methods are available for determining gut EAAFL. The protein-free diet (PF), although used routinely in dietary protein evaluation (FAO/WHO, 1991), creates a non-physiological state (Low, 1980) by inducing a negative body nitrogen (N) balance, which may lead to a decreased rate of whole body protein synthesis (Millward & Garlick, 1976) and thus lowered gut EAAFL compared with protein alimentation (Darragh *et al.*, 1990; Butts *et al.*, 1993; Moughan *et al.*, 2005). However, previous

results suggest that body N balance *per se* does not affect EAAFL (Skilton *et al.*, 1988; Darragh *et al.*, 1990; Butts *et al.*, 1993). Peptides arising from protein digestion may have a specific effect on gut protein secretion and reabsorption (Butts *et al.*, 1993). The enzyme-hydrolysed protein method was proposed as an alternative method to the PF diet for determining ileal endogenous protein losses routinely (Moughan *et al.*, 1990). This technique consists of measuring ileal endogenous N and AA under conditions in which the gut is supplied with dietary AA and peptides [hydrolysed casein, molecular weight (MW) < 5 kDa], mimicking the breakdown products of natural digestion. After digesta centrifugation and ultrafiltration (10 kDa, MW cut-off), endogenous protein is determined in the high fraction MW. Any undigested dietary AA (MW < 10 kDa) are discarded as well as any small endogenous peptides and free AA, leading to some degree of underestimation of EAAFL. Nevertheless, application of the enzyme hydrolysed protein method appears to lead to higher estimates of endogenous ileal protein losses than PF or synthetic AA diets (Darragh *et al.*, 1990; Butts *et al.*, 1993; Hodgkinson *et al.*, 2000), but this has not been assessed in a study whereby dietary electrolyte balance (DEB; Na<sup>+</sup> + K<sup>+</sup> - Cl<sup>-</sup>) and dietary AA composition have been controlled. Our aim was to assess the effect on ileal endogenous protein flows consequent on feeding animals diets supplying similar AA either in the form of peptides (HC diet) or free L-AA (A1 and A2 diet) when DEB was adjusted to be similar in the various diets. A PF diet served as a control. The HC, A1 and A2 diets had similar AA compositions, except that acid aspartic and serine were omitted from diet A1 and glycine and alanine were omitted from diet A2, to enable direct determination of their endogenous losses (Skilton *et al.*, 1988). The omitted dietary dispensable AA were chosen as representative of different AA absorption mechanisms (Rérat *et al.*, 1976). Endogenous N and AA losses in rats fed the HC diet were determined using the hydrolysed-protein/ultrafiltration technique (Moughan *et al.*, 1990).

## **MATERIALS AND METHODS**

### ***Animals and housing***

Forty-eight Sprague-Dawley male rats [242 ± 3 g body weight (BW), mean ± SE] were housed individually in raised stainless steel cages with wire mesh floors in a room maintained at 21 ± 2 °C with a 12 h light/dark cycle. Food was given during the light

cycle. Water was continuously available. Ethics approval was received from the Massey University Animal Ethics Committee (protocol 05/04).

### ***Diets***

Four semi-synthetic test diets (**Table 1**) were prepared including a PF diet and diets containing as the sole source of N enzyme-hydrolysed casein (HC diet) or a free L-AA mixture simulating the HC except for the omitted AA serine and Aspartic acid (A1 diet) or glycine and alanine (A2 diet). All diets were formulated to meet the nutrient requirements of the growing rat (National Research Council, 1995). Dietary AA contents (**Table 2**), although lower in diet HC than in diets A1 and A2, were in a comparable range. Sodium bicarbonate was added to diets PF, A1 and A2. The DEB in the final diets ranged from 158 (diet HC) to  $216 \pm 2$  mEq/kg (diets PF, A1, A2). Titanium dioxide was added to the diets as an indigestible marker. A standard casein-based preliminary diet was prepared.

HC was prepared (INRA, Rennes, France) from native micellar casein extracted by microfiltration followed by diafiltration of milk (Mahé *et al.*, 1994). Casein solution (1:10 w/v) was heated up to 50°C, pig pancreatin was added with an enzyme to substrate ratio of 0.0089 (pH 8.0, 50°C, 1 h 50 min) and was then inactivated (85°C, 20 min). The final product was freeze-dried.

The MW profile was determined using a high-performance liquid chromatography (HPLC) gel filtration column (TSKGel G2000SWXL 30 cm, Phenomenex, Auckland, NZ). The eluting solvent contained 36% acetonitrile and 0.1% trifluoroacetic acid, with detection at a wavelength of 205 nm; 21% of the peptides were between 1 and 5 kDa in size, and 79% were less than 1 kDa.

### ***Experimental design***

For the entire study rats received 8 meals daily given at hourly intervals and each for a 10-min period. The rats were acclimatized from days 0–9 while receiving the preliminary diet. The rats were then randomly allocated to the test diets ( $n = 12$  per treatment) which they received from days 10–17. Food intake was recorded daily. The rats were killed  $6 \text{ h} \pm 15 \text{ min}$  after the first meal as previously described (Deglaire *et al.*, 2006). The stomach, the final 20 cm of ileum, and the caecum/colon were first removed. The remaining section of the small intestine was divided into 2 equal parts labelled

proximal and medial intestine. Digesta were gently removed using ice-cold saline solution and immediately frozen at -20°C, then freeze-dried and finely ground.

**Table 1.** Ingredient compositions of the diets given to growing rats.

Ingredient	Diet				
	Preliminary	PF	A1	A2	HC
	<i>g/kg air dry weight</i>				
Corn starch <sup>1</sup>	634	723	627	622	628
Purified cellulose <sup>2</sup>	50	50	50	50	50
Vitamin mix <sup>3</sup>	50	50	50	50	50
Salt mix <sup>4</sup>	50	50	50	50	50
NaHCO <sub>3</sub>	/	19	23	23	/
Sucrose	70	70	70	70	70
Soya oil	35	35	35	35	35
Acid casein <sup>5</sup>	111	-	-	-	-
AA mixture 1 <sup>6</sup>	-	-	95	-	-
AA mixture 2 <sup>6</sup>	-	-	-	100	-
Hydrolysed casein	-	-	-	-	114
Titanium dioxide	-	3	3	3	3

<sup>1</sup> PF, A1 and A2 diets: Golden Harvest, Primary foods Ltd., Auckland, New Zealand. HC diet: Cerestar, Haubourdin, France.

<sup>2</sup> PF, A1 and A2 diets: Avicel PH101, Commercial Minerals Ltd., Auckland, New Zealand. HC diet: Medias Filtrants Durieux, Torcy, France.

<sup>3,4</sup> Crop & Food Research, Palmerston North, New Zealand. The mixtures were formulated to meet the vitamin and mineral requirements of the growing rat (National Research Council, 1995).

<sup>3</sup> The mixture supplied: (mg/kg diet) retinol acetate 5.0, DL- $\alpha$ -tocopheryl acetate 200, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20, folic acid 2.0, nicotinic acid 20, D-biotin 1.0, myo-inositol 200, choline chloride 1500; ( $\mu$ g/kg diet) ergocalciferol 25, cyanocobalamin 50.

<sup>4</sup> The mixture supplied: (g/kg diet) Ca 6.29, Cl 7.79, Mg 1.06, P 4.86, K 5.24, Na 1.97; (mg/kg diet) Cr 1.97, Cu 10.7, Fe 424, Mn 78.0, Zn 48.2; ( $\mu$ g/kg diet) Co 29.0, I 151, Mo 152, Se 151.

<sup>5</sup> New Zealand Milk Products, Wellington, New Zealand.

<sup>6</sup> Synthetic L-amino acids: Ajinomoto Co., Japan.

**Table 2.** Nutrient and selected mineral contents of the experimental diets given to the growing rats.

	Diet			
	PF	A1	A2	HC
<i>Amino acid</i>	<i>g/kg dry matter</i>			
Threonine	0.1	4.0	4.0	3.7
Valine	nd <sup>1</sup>	6.5	6.5	5.8
Isoleucine	nd	5.0	5.0	4.7
Leucine	0.1	9.5	9.5	8.7
Phenylalanine	nd	5.2	5.2	4.7
Tyrosine	nd	5.6	5.6	5.1
Lysine	0.1	7.9	7.9	7.2
Histidine	0.2	3.0	3.0	2.2
Glutamic acid	0.1	22.2	22.2	21.0
Proline	nd	11.2	11.2	10.3
Arginine	nd	3.4	3.4	3.2
Tryptophan	nd	1.3	1.3	1.3
Aspartic acid	0.1	-	6.9	6.4
Serine	0.1	-	4.8	4.6
Glycine	0.1	1.9	-	1.7
Alanine	nd	4.1	-	3.4
Cysteine	nd	0.3	0.3	0.3
Methionine	nd	2.7	2.7	2.8
<i>Nitrogen</i>				
$\alpha$ AN <sup>2</sup>	0.1	12.0	12.4	12.2
TN <sup>3</sup>	0.5	13.1	12.3	17.7
<i>Minerals</i>				
K	5.2	5.2	5.2	5.3
Na	6.9	8.1	8.1	5.9
Cl	7.8	9.7	9.7	8.3
	<i>mEq/kg dry matter</i>			
DEB <sup>4</sup>	216	215	215	158

<sup>1</sup>nd: not detectable. <sup>2</sup> $\alpha$ -amino nitrogen. <sup>3</sup>Total nitrogen. <sup>4</sup>Dietary electrolyte balance (Na<sup>+</sup> + K<sup>+</sup> - Cl<sup>-</sup>).



### ***Chemical analysis***

Digesta were pooled across randomly selected pairs of rats within each treatment to give 6 digesta samples per treatment for each intestine section. Ileal digesta from rats fed diet HC were divided into 2 portions to be analysed “as is” or after centrifugation and ultrafiltration (ultrafiltered digesta = precipitate + retentate). The latter portion was rehydrated overnight and then centrifuged (1400 g, 30 min,  $3 \pm 1^\circ\text{C}$ ). The supernatant was ultrafiltered (Centriprep-10 devices, 10 kDa MW cut-off; Amicon Inc., Beverly, MA) as described previously (Hodgkinson *et al.*, 2003). The resulting retentate (MW>10kDa) was added to the precipitate from the centrifugation step, freeze-dried, and finely ground.

Diets and digesta samples were analysed for  $\text{TiO}_2$ , total N and AA. Total N was determined using an elemental N analyser (NA 1500 series 2, Fisons Instruments, Manchester, UK) (Gausserès *et al.*, 1997). AA were determined after acid hydrolysis using a Waters ion exchange HPLC system (AOAC, 2003). Methionine and cysteine were measured as methionine sulfone and cysteic acid after performic acid oxidation (Moore, 1963). Methionine and cysteine were not determined in HC ileal digesta because of limited sample size.  $\text{TiO}_2$  was determined by a colorimetric assay after ashing the sample and digestion of the minerals (Short *et al.*, 1996).

Minerals were determined in hydrolysed casein. Na and K were analysed after acid digestion using inductively coupled plasma optical emission spectrometry (ICP-OES) (Fecher *et al.*, 1998). The chloride ion was analysed after weak acid extraction by potentiometric determination (Fecher *et al.*, 1998).

### ***Data Analysis***

Gut AA flow and N flow ( $\mu\text{g/g}$  dry matter intake, DMI) were determined as follows:

$$\text{N flow or AA flow} = \frac{\text{N or AA in digesta} \times \text{TiO}_2 \text{ in diet}}{\text{TiO}_2 \text{ in digesta}} \quad (\text{Eq. 1})$$

Total (dietary and endogenous) N flow (TNFL) were calculated using Eq. 1 in digesta from rats fed the HC diet before any processing. Endogenous N flow (ENFL) and EAAFL were calculated using Eq. 1 in digesta from rats fed the PF diet and in ultrafiltered digesta from rats fed the HC diet. EAAFL of the omitted AA were determined directly in digesta from rats fed diets A1 and A2.

Apparent ileal digestibility (AID; %) and standardized ileal digestibility (SID; %) of AA (or N) were calculated as follows:

$$\text{AID} = \frac{(\text{Diet AA intake} - \text{TAAFL})}{\text{Diet AA intake}} \times 100 \quad (\text{Eq. 2})$$

$$\text{SID} = \frac{[\text{Diet AA intake} - (\text{TAAFL} - \text{EAAFL})]}{\text{Diet AA intake}} \times 100 \quad (\text{Eq. 3})$$

The N contents of individually determined AA were summed to give an estimate of  $\alpha$  amino N ( $\alpha$ AN). The terminology standardized digestibility was used as defined by Stein *et al.* (Stein *et al.*, 2007) and relates to the previously used term “true digestibility”.

Data were tested for homogeneity of variance using Bartlett’s test and then subjected to a one-way ANOVA (SAS, SAS Institute Inc., version 8.2, Cary, NC). The food intake data were subjected to a one-way ANOVA for repeated measures. For  $P < 0.05$ , the significance of differences between means was determined using Tukey’s test. Results are given as means  $\pm$  SE.

## RESULTS

Hydrolysed casein contained 2.26, 0.06 and 0.04 g/100 g air-dried hydrolysed casein of sodium, potassium and chloride, respectively.

The rats receiving the PF diet lost BW ( $7.0 \pm 1.8$  g), whereas rats fed the A1, A2 and HC diets had similar ( $P > 0.05$ ) BW gains ( $17.2 \pm 2.4$ ,  $17.9 \pm 2.4$ ,  $17.4 \pm 1.5$  g, respectively). Daily food intakes were significantly lower ( $P < 0.01$ ) for rats fed diet PF ( $10.4 \pm 0.4$  g/d) than for those fed diets A1 ( $14.7 \pm 0.5$  g/d), A2 ( $14.8 \pm 0.5$  g/d) and HC ( $15.5 \pm 0.5$  g/d).

The endogenous flows of aspartic acid and serine were similar between rats fed diets PF and A1, but were significantly lower ( $P < 0.001$ ) than those for rats fed diet HC (**Table 3**). The endogenous flow of alanine was similar between rats fed diets PF and A2 but was significantly lower ( $P < 0.001$ ) compared with that of rats fed diet HC. On the contrary, the endogenous flow of glycine was significantly higher ( $P < 0.05$ ) for rats fed the PF and A2 diets than that for rats fed diet HC (Table 3). Endogenous flows of aspartic acid and serine or alanine and glycine were not significantly different ( $P > 0.05$ )

in any of the intestinal sections between rats fed diets PF and A1 or PF and A2, except for the flow of serine in the stomach, which was greater for rats fed diet A1 than for those fed diet PF (Table 3). EAAFL were considerable in the stomach and in the proximal intestine and thereafter declined to lower and relatively constant flows.

The AID of AA were not significantly different ( $P > 0.05$ ) between rats fed diets A1 and A2, except for the AID of histidine and arginine (data not shown). A similar result was found for the SID of AA. AA digestibilities were thus pooled across diets A1 and A2 (**Table 4**). Most AA (Table 4) had a SID close to 100%, except for cysteine (63.3%), indicating that they were fully absorbed prior to the end of the ileum. Therefore, we assumed that their ileal flows were endogenous (**Table 5**). For several of the AA, the pattern of their endogenous flows was similar to that found for the omitted AA, aspartic acid, serine, and alanine, with no significant difference between rats fed the PF, A1 and A2 diets but with a significantly higher ( $P < 0.05$ ) flow in those fed the HC diet. Flows of phenylalanine, tyrosine and lysine did not differ in the rats fed the different diets. For histidine, the endogenous flow was lower for rats fed diet HC than for those fed PF or A1, whereas for arginine the endogenous flows for rats fed diets HC, A1 and PF were similar. Of note, the endogenous methionine flows for rats fed diets A1 and A2 were significantly higher (about 2 times;  $P < 0.05$ ) compared with that for rats given diet PF.

Ileal ENFL were not significantly different ( $P > 0.05$ ) in rats fed diets PF, A1, and A2, but were significantly lower compared with rats fed diet HC (**Table 6**). A corresponding result was found for the endogenous flows of  $\alpha$ AN, the values for which were  $628 \pm 57$ ,  $637 \pm 29$ ,  $647 \pm 40$ , and  $1071 \pm 56$  for rats fed diets PF, A1, A2, and HC, respectively. The TNFL for rats fed diet HC was  $1428 \pm 194$   $\mu$ g/g DMI.

**Table 3.** Endogenous amino acid flows along the digestive tract of growing rats fed diets PF, A1, A2 and HC<sup>1</sup>.

	Diet				Pooled SE
	PF	A1	A2	HC	
	<i>µg/g dry matter intake</i>				
<i>Aspartic acid</i>					
Stomach	1218	1540	-	-	223
Proximal intestine	1950	2850	-	-	400
Medial intestine	990	1260	-	-	110
Terminal ileum	500 <sup>a</sup>	532 <sup>a</sup>	-	882 <sup>b</sup>	35
Cecum and colon	590	590	-	-	35
<i>Serine</i>					
Stomach	589 <sup>2,a</sup>	1434 <sup>b</sup>	-	-	131
Proximal intestine	910	1280	-	-	180
Medial intestine	470	490	-	-	60
Terminal ileum	254 <sup>a</sup>	256 <sup>a</sup>	-	1142 <sup>b</sup>	31
Cecum and colon	230	220	-	-	15
<i>Glycine</i>					
Stomach	1090	-	930	-	250
Proximal intestine	1920	-	2070	-	395
Medial intestine	1480	-	1420	-	175
Terminal ileum	695 <sup>b</sup>	-	561 <sup>b</sup>	284 <sup>a</sup>	45
Cecum and colon	290	-	280	-	20
<i>Alanine</i>					
Stomach	870	-	1020	-	310
Proximal intestine	1250	-	1785 <sup>2</sup>	-	205
Medial intestine	510	-	730	-	85
Terminal ileum	194 <sup>a</sup>	-	246 <sup>a</sup>	432 <sup>b</sup>	33
Cecum and colon	310	-	300	-	25

<sup>1</sup> Values are means, n=6 except when noted. Means in a row with superscripts without a common letter differ, P < 0.05.

<sup>2</sup> n = 5.

**Table 4.** Apparent and standardized ileal amino acid digestibility for growing rats fed the synthetic amino acid-based diets A1 and A2<sup>1</sup>.

Amino acid	Apparent digestibility	Standardized digestibility <sup>2</sup>	Pooled SE
	%		
Threonine	90.8	99.6	0.4
Valine	96.4	100.3	0.2
Isoleucine	96.7	99.9	0.2
Leucine	97.5	99.7	0.2
Phenylalanine	97.4	99.7	0.2
Tyrosine	97.3	99.5	0.2
Lysine	97.2	99.9	0.2
Histidine	90.4	102.4	0.3
Glutamic acid	96.7	98.9	0.3
Proline	97.4	99.7	0.2
Arginine	95.8	98.9	0.5
Cysteine	41.8	63.3	4.8
Methionine	97.6	98.5	0.1
Aspartic acid <sup>3</sup>	91.8	98.8	0.5
Serine <sup>3</sup>	94.4	99.7	0.3
Glycine <sup>4</sup>	69.0	105.8	1.9
Alanine <sup>4</sup>	95.9	100.7	0.3

<sup>1</sup> Values are means determined from pooled data from rats fed diets A1 and A2, n=12 except when noted.

<sup>2</sup> Calculated after correction for endogenous AA losses determined in rats fed diet PF.

<sup>3</sup> Data from rats fed diet A2 (n = 6).

<sup>4</sup> Data from rats fed diet A1 (n = 6).

**Table 5.** Endogenous ileal amino acid flows determined in rats fed diets PF, A1, A2 and HC<sup>1</sup>.

Amino acid	Diet				Pooled
	PF	A1	A2	HC	SE
	<i>µg/g dry matter intake</i>				
Threonine	350 <sup>a</sup>	363 <sup>a</sup>	369 <sup>a</sup>	514 <sup>b</sup>	22
Valine	255 <sup>a</sup>	247 <sup>a</sup>	221 <sup>a</sup>	634 <sup>b</sup>	19
Isoleucine	160 <sup>a</sup>	163 <sup>a</sup>	163 <sup>a</sup>	646 <sup>b</sup>	17
Leucine	210 <sup>a</sup>	230 <sup>a</sup>	239 <sup>a</sup>	384 <sup>b</sup>	21
Phenylalanine	116	134	132	135	11
Tyrosine	124	149	151	142	12
Lysine	215	235	205	265	21
Histidine	364 <sup>b</sup>	366 <sup>b</sup>	216 <sup>b</sup>	185 <sup>a</sup>	14
Glutamic acid	476 <sup>a</sup>	613 <sup>ab</sup>	841 <sup>b</sup>	2109 <sup>c</sup>	68
Proline	263 <sup>a</sup>	297 <sup>a</sup>	295 <sup>a</sup>	523 <sup>b</sup>	28
Arginine	105 <sup>a</sup>	113 <sup>a</sup>	173 <sup>b</sup>	148 <sup>ab</sup>	17
Methionine	27 <sup>a</sup>	66 <sup>b</sup>	67 <sup>b</sup>	-	4

<sup>1</sup> Values are means, n=6. Means in a row with superscripts without a common letter differ, P < 0.05.

**Table 6.** Endogenous flows and digestibility of nitrogen at the terminal ileum of rats fed diets PF, A1, A2 and HC<sup>1</sup>.

	Diet				Pooled
	PF	A1	A2	HC	SE
Endogenous N flows ( <i>µg/g dry matter intake</i> )	827 <sup>a</sup>	879 <sup>a</sup>	915 <sup>a</sup>	1352 <sup>b</sup>	61
Apparent digestibility (%)	-	93.3	92.6	92.0	0.7
Standardized digestibility <sup>2</sup> (%)	-	99.6	99.3	99.6	0.6

<sup>1</sup> Values are means, n=6. Means in a row with superscripts without a common letter differ, P < 0.05.

<sup>2</sup> Standardized N digestibilities for diets A1 and A2 were calculated after correction for endogenous N losses determined in rats fed diet PF. Standardized N digestibility for diet HC was calculated after correction for endogenous N losses determined in rats fed diet HC.

## DISCUSSION

Endogenous AA flows at the terminal ileum have been compared previously in rats fed PF, synthetic AA- or HC-based diets, revealing a possible stimulatory effect of peptides on gut secretions (Darragh *et al.*, 1990; Butts *et al.*, 1991; Butts *et al.*, 1993; Donkoh *et al.*, 1995; Rutherford & Moughan, 1998). EAAFL and ENFL have not, however, hitherto been directly compared where diet AA composition and DEB have been controlled.

DEB, in particular, may be an important consideration when determining endogenous AA losses, because it has been reported to influence the AID of N (Haydon & West, 1990). Enzyme-hydrolysed casein contains a high amount of sodium due to the hydrolysis process requiring substantial addition of NaOH, so in the present study, NaHCO<sub>3</sub> was added to the PF, A1 and A2 diets to ensure comparable DEB. The total N content of diets A1 and A2 was lower than that of diet HC, because AA were purposefully omitted from the diet AA mixtures and no correction was made for the N from the carboxamide groups of glutamine and asparagine lost during AA analysis of the HC. The  $\alpha$ AN content was similar across diets A1, A2 and HC.

Ileal endogenous flows of aspartic acid, serine, glycine and alanine were similar in rats fed diets A1 and A2 (devoid of these particular AA) and in rats fed diet PF, which is consistent with earlier data (Skilton *et al.*, 1988; Darragh *et al.*, 1990). When endogenous ileal AA flows were determined for the other AA, assuming a virtually complete absorption of the synthetic AA, flows were generally similar to those observed for diet PF. Methionine was an exception whereby the flow was much lower for diet PF. The ENFL for rats fed diet PF were in agreement with earlier data (Donkoh *et al.*, 1995; James *et al.*, 2002; Deglaire *et al.*, 2006) and were similar to those for rats fed diets A1 and A2. These findings confirm the observation that body nitrogen balance *per se* does not influence gut endogenous protein losses in the growing rat. A similar observation has been made in pigs fed a PF diet with a simultaneous intravenous AA infusion (de Lange *et al.*, 1989; Leterme *et al.*, 1996).

The ileal EAAFL in rats fed diet HC were in the range of previous estimates (Darragh *et al.*, 1990; Butts *et al.*, 1991; Donkoh *et al.*, 1995; Rutherford & Moughan, 1998; Hendriks *et al.*, 2002; Hodgkinson *et al.*, 2003). The HC diet generally resulted in higher EAAFL compared with the PF diet, which also accords with earlier findings in

rats (Darragh *et al.*, 1990; Butts *et al.*, 1991; Donkoh *et al.*, 1995; Rutherford & Moughan, 2003), pigs (Leterme *et al.*, 1996; Yin *et al.*, 2004; Steendam *et al.*, 2004), and humans (Moughan *et al.*, 2005). The present difference was of a lower magnitude than previously reported (Darragh *et al.*, 1990; Butts *et al.*, 1991; Donkoh *et al.*, 1995; Rutherford & Moughan, 2003).

A higher endogenous flow of glycine was observed for the PF feeding, which has also been noted previously (Skilton *et al.*, 1988; Moughan *et al.*, 1992; Hendriks *et al.*, 2002). This has been suggested to be directly related to the PF condition of the animals as an intravenous infusion of AA to pigs fed a PF diet numerically decreased glycine flow (de Lange *et al.*, 1989; Leterme *et al.*, 1996), which is consistent with the effect observed here when synthetic AA were ingested. It is important to note, however, that the differences were not statistically significantly different in either study. An underestimation of glycine flow may occur with the centrifugation and ultrafiltration technique, thus explaining the low glycine flow for the HC diet. Glycine is one of the predominant AA in bile acids (Juste, 1982) and whereas most of the bile salt conjugates are reabsorbed after intestinal bacteria hydrolysis, substantial amounts of deconjugated glycine escape reabsorption and are thus discarded after digesta ultrafiltration (Rutherford & Moughan, 2003). The overall degree of underestimation of endogenous N is, however, likely to be low in the present study, because only 7% of the total N was removed in ultrafiltered digesta, whereas previous studies reported values of 13–24% (Moughan *et al.*, 1992 ; Butts *et al.*, 1992; Leterme *et al.*, 1996).

The present findings suggest that gut endogenous protein losses are increased by peptides derived from casein but not by free AA. This presumably results from lower reabsorption and/or higher secretions of endogenous N and AA. The activity of the L-AA transporters or the di-/tri-peptide transporters (PepT1), regulated through complex mechanisms, is possibly influenced by dietary AA and N after several days of feeding (Kilberg *et al.*, 1993; Daniel, 2004). However, a similar brush-border AA and peptide uptake was reported in mice fed for 14 days diets based on peptides or free AA, both simulating casein (Ferraris *et al.*, 1988). The higher endogenous protein losses induced by dietary peptides are more likely due to enhanced proteic secretions. Endogenous protein losses are composed mainly of mucins, enzymatic secretions, sloughed cells, and bacteria (technically non-dietary rather than endogenous) (Fauconneau & Michel, 1970). Dietary peptides are known to stimulate pancreatic secretions more effectively than do free AA (Puigserver *et al.*, 1982; Temler *et al.*, 1984) and the degree of AA



polymerisation has been shown to influence small bowel mucosa growth rate (Poullain *et al.*, 1989; Zaloga *et al.*, 1991; Stoll *et al.*, 2006; Guay *et al.*, 2006). A higher distal (but not proximal) gut growth rate has been observed in rats fed for 10 days a diet based on casein-derived peptides as compared with rats fed a free AA-based diet (Zaloga *et al.*, 1991). This would theoretically result in higher mucosa sloughing and possibly higher ileal endogenous N losses in rats fed dietary peptides. However, a lower rate of mitosis per crypt was observed in the jejunum from rats fed for 4 days diets based on whey-derived peptides as compared with free AA (Poullain *et al.*, 1989). Recently, bioactive peptides, such as  $\beta$ -casomorphins (MW 0.5-1kDa), have been shown to induce mucus secretion in rat jejunum (Claustre *et al.*, 2002). Bioactivity, however, is related to the size and nature of the peptide (Froetschel, 1996) and is likely affected by protein hydrolysis conditions. There may be specific effects of dietary peptides from a pre-hydrolysed protein versus peptides naturally released during casein digestion (Yin *et al.*, 2004).

The present work demonstrates that feeding dietary peptides induces increased endogenous ileal N and AA flows compared with feeding a PF diet or diets containing only free AA, which yield similar estimates of endogenous ileal N and AA flows. This suggests that EAAFL and ENFL are not influenced by body N balance *per se* but rather by the presence of dietary peptides in the gut lumen. Further investigation is required to determine whether this is a specific effect of dietary peptides compared with peptides released during protein digestion in the gut and whether this is a specific effect of casein-derived peptides. The underlying mechanisms need to be understood.

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## CHAPTER IV

# A casein hydrolysate does not enhance gut endogenous protein flows compared with intact casein when fed to growing rats

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As a complement to the study presented in the preceding chapter, we aimed to assess in the present work the influence of a casein hydrolysate on gut endogenous protein flows compared with that of the parent intact casein. This was undertaken using the growing rat as an animal model.

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## ABSTRACT

The effect of dietary free peptides versus peptides released naturally during digestion on gut endogenous nitrogen flow (ENFL) and endogenous amino acid flows (EAAFL) was studied. Semi-synthetic diets containing 110 g/kg diet of the same casein, intact (C) or hydrolysed (HC), were formulated with TiO<sub>2</sub> as a dietary marker. Sprague-Dawley rats were given the diets hourly (0800-1500 h) for 10 min each hour for 7 days. Rats received unlabelled diets for 6 days and <sup>15</sup>N-labelled diets on day 7, whereby they were killed and digesta sampled (6 observations per group) along the intestinal tract. EAAFL and ENFL were determined by <sup>15</sup>N-isotope dilution for C or by isotope dilution or after centrifugation and ultrafiltration for HC. Ileal EAAFL and ENFL (isotope dilution) were not enhanced with diet HC compared to diet C. The amino acid compositions (g/16g N) of ileal ENFL did not differ between rats fed HC and C except for aspartic acid, phenylalanine, tyrosine and serine for which contributions were relatively lower ( $P < 0.05$ ) for rats fed C. Ileal EAAFL and ENFL (HC) were considerably lower ( $P < 0.05$ ) with the isotope dilution method than with the ultrafiltration method, but flows of glycine, phenylalanine and histidine were similar. There was no stimulatory effect of dietary peptides from HC on endogenous ileal protein flow compared with C, but the result is tentative given the high degree of dietary nitrogen found to be recycled within endogenous protein and which could have occurred at a differential rate between rats fed diets C and HC.

## INTRODUCTION

The amount of ileal digesta protein of non-dietary origin, commonly referred to as endogenous protein, is a fundamental measure in the study of protein digestion. Estimates of ileal endogenous amino acids (AA) are required to determine true coefficients of ileal AA digestibility (Hodgkinson *et al.*, 2003; Fuller & Tome, 2005) and the loss of endogenous AA from the small bowel is an important component of the daily AA requirement (Gaudichon *et al.*, 2002; Moughan *et al.*, 2005).

Although protein-free diets have traditionally been used to determine endogenous protein in digesta (FAO/WHO, 1991), such an approach has been criticized as leading to a physiologically abnormal metabolism (Fauconneau & Michel, 1970), resulting in lower ileal endogenous protein losses compared with protein-containing diets (Butts *et*



*al.*, 1993; Hodgkinson *et al.*, 2000; Moughan *et al.*, 2005; Deglaire *et al.*, 2007). The enzyme hydrolysed-protein/ultrafiltration technique (Moughan *et al.*, 1990) was developed as an alternative approach, whereby the gut is directly supplied with AA and peptides, assumed to be similar to those arising during digestion in the gut, to allow the determination of endogenous AA flows (EAAFL). After feeding enzyme-hydrolysed protein [usually casein, molecular weight (MW) < 5 kDa] to animals or human subjects, ileal digesta are centrifuged and ultrafiltered (10 kDa MW cut-off). Proteins and peptides larger than 10 kDa in size are assumed to be of endogenous origin and any undigested small peptides and unabsorbed AA are discarded in the ultrafiltrate. This method allows a direct simultaneous determination of endogenous flows for all AA and nitrogen (N) and is of practical interest for the development of routine digestion assays. Questions have been raised, however, as to whether potentially bioactive peptides present in the hydrolysate may enhance the loss of AA from the small bowel (Butts *et al.*, 1993; Jansman *et al.*, 2002; Yin *et al.*, 2004; Rutherfurd-Markwick & Moughan, 2005; Stein *et al.*, 2007) over and above that which may be found with the corresponding intact protein.

To our knowledge, no carefully controlled study has been conducted to date to specifically compare gut endogenous protein flow for a hydrolysed dietary protein (various sized oligopeptides) with its parent intact protein, whereby peptides are released naturally during digestion. The primary aim of the present work was to assess whether hydrolysed protein enhances gut endogenous protein flows compared with the parent intact protein. Semi-synthetic diets based on casein in either the intact or hydrolysed form were fed to growing rats. Both forms of casein originated from the same source of  $^{15}\text{N}$ -labelled milk so that endogenous protein flows could be determined and compared for both diets using the isotope dilution technique. Although the  $^{15}\text{N}$ -labelling of diets may lead to an underestimation of gut endogenous N loss, the method is considered a valid technique for making relative comparisons (Mahé *et al.*, 1994b; Hess *et al.*, 2000; Gaudichon *et al.*, 2002). Additionally, endogenous ileal protein flows were determined using the enzyme-hydrolysed protein/ultrafiltration technique to enable a comparison with the isotope dilution method.

## MATERIALS AND METHODS

This study was conducted as part of a larger study (Deglaire *et al.*, 2007).

### *Animals and housing*

Twenty-four Sprague-Dawley male rats [ $219 \pm 14$  g body weight] were housed individually in raised stainless steel cages with wire mesh floors in a room maintained at  $21 \pm 2^\circ\text{C}$ , and with a 12h-light/-dark cycle. Rats were fed during the light cycle and water was continuously available. Ethics approval was received from the Massey University Animal Ethics Committee (protocol 05/04).

### *Diets*

Two semi-synthetic iso-nitrogenous (17.7 g N/kg dry matter) test diets (**Table 1**) were prepared containing as a sole source of N either uniformly  $^{15}\text{N}$ -labelled native phosphocaseinate referred to as intact casein (C diet) or a  $^{15}\text{N}$ -labelled casein-derived (derived from the former native phosphocaseinate) hydrolysate (HC diet). Adaptation diets had the same composition as the test diets except that the proteins were unlabelled. Sodium bicarbonate was added to diet C as previously described (Deglaire *et al.*, 2007) to equalise the dietary electrolyte balances. Titanium dioxide ( $\text{TiO}_2$ ) was included in the diets as an indigestible marker. A preliminary diet was also prepared (Table 1). Diets were formulated to meet the nutrient requirements of the growing rat (National Research Council, 1995).

$^{15}\text{N}$ -labelling of milk and dietary protein purification were performed as described previously (Mahé *et al.*, 1994a). Briefly, 3 lactating cows were perfused ruminally with ammonium sulphate ( $^{15}\text{NH}_4$ ) $\text{SO}_4$  (Eurisotop) for 5 days.  $^{15}\text{N}$ -labelled milk was collected 5 times over 3 days and then pooled.  $^{15}\text{N}$ -labelled native phosphocaseinate was extracted by microfiltration and then purified through water diafiltration. All processes were carried out at the National Institute of Agronomic Research (Rennes, France). The resulting casein was freeze-dried. Its global  $^{15}\text{N}$  isotopic enrichment was 0.5450 atom %, and the  $^{15}\text{N}$ -enrichments for individual AA are shown in **Table 2**. An aliquot of  $^{15}\text{N}$ -casein was hydrolysed with pig pancreatin as described earlier (Deglaire *et al.*, 2007). The MW profile, determined by HPLC gel filtration (Deglaire *et al.*, 2007), indicated that 21% of the peptides were between 1 and 5 kDa in size and 79% were less than 1 kDa. In comparison, the MW profile of the commercial hydrolysed casein used in the

adaptation diet and similar to that previously used in our work (Hodgkinson *et al.*, 2000; Yin *et al.*, 2004; Moughan *et al.*, 2005), showed that 12% of the peptides were between 1 and 5 kDa, and 88 % were less than 1 kDa.

**Table 1.** Ingredient compositions of the diets given to growing rats.

Ingredient	Preliminary diet	Test diets	
		C	HC
<i>g/kg air dry weight</i>			
Corn starch <sup>1</sup>	634	612	628
Purified cellulose <sup>2</sup>	50	50	50
Vitamin mix <sup>3</sup>	50	50	50
Salt mix <sup>4</sup>	50	50	50
NaHCO <sub>3</sub>	-	20	-
Sucrose	70	70	70
Soyabean oil	35	35	35
Acid casein <sup>5</sup>	111	-	-
<sup>15</sup> N-casein	-	110	-
<sup>15</sup> N-hydrolysed casein	-	-	114
Titanium dioxide	-	3	3

<sup>1</sup> Cerestar, Haubourdin, France.

<sup>2</sup> Medias Filtrants Durieux, Torcy, France.

<sup>3,4</sup> Crop & Food Research, Palmerston North, New Zealand. The mixtures were formulated to meet the vitamin and mineral requirements of the growing rat (National Research Council, 1995).

<sup>3</sup> The mixture supplied: (mg/kg diet) retinol acetate 5.0, DL- $\alpha$ -tocopheryl acetate 200, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20, folic acid 2.0, nicotinic acid 20, D-biotin 1.0, myo-inositol 200, choline chloride 1500; ( $\mu$ g/kg diet): ergocalciferol 25, cyanocobalamin 50.

<sup>4</sup> The mixture supplied: (g/kg diet) Ca 6.29, Cl 7.79, Mg 1.06, P 4.86, K 5.24, Na 1.97; (mg/kg diet) Cr 1.97, Cu 10.7, Fe 424, Mn 78.0, Zn 48.2; ( $\mu$ g/kg diet): Co 29.0, I 151, Mo 152, Se 151.

<sup>5</sup> New Zealand Milk Products, Wellington, New Zealand.

**Table 2.** Amino acid composition of casein and  $^{15}\text{N}$ -enrichment of individual amino acids.

Amino acid	$^{15}\text{N}$ -labelled casein	
	%	atom%
Threonine	3.8	0.5653
Valine	6.0	0.5198
Isoleucine	4.6	0.5245
Leucine	8.9	0.5070
Phenylalanine	4.9	0.5101
Tyrosine	5.3	0.5132
Lysine	7.4	0.5478
Histidine	2.7	0.5081
Glutamic acid	20.7	0.5677
Proline	10.6	0.4942
Aspartic acid	6.4	0.5391
Serine	4.5	0.5397
Glycine	1.7	0.5295
Alanine	3.8	0.5265

***Experimental design***

On each day of the study the rats received 8 meals at hourly intervals commencing at 0800 h, each meal lasting 10 min. Food intake was recorded daily. The rats were acclimatized to the cages and to the feeding regimen from days 0 to 9 while receiving the preliminary diet. They were then randomly and equally allocated to the adaptation (unlabelled) diets from days 10 to 16. On day 17, they received the test (labelled) diets and were killed as described previously (Deglaire *et al.*, 2007). The entire digestive tract was dissected and digesta from the stomach, proximal intestine, medial intestine, the last 20 cm of the ileum and caecum/colon were collected as described previously (Deglaire *et al.*, 2007). Digesta were freeze-dried, ground and pooled across randomly selected pairs of rats within the same diet for each intestinal section such that there were 6 observations per treatment.

Pooled digesta were analysed for  $\text{TiO}_2$ , total N and  $^{15}\text{N}$ -enrichment. Ileal digesta samples were analysed for AA content and  $^{15}\text{N}$ -enrichment of AA. Ileal digesta from

rats fed diet HC were divided in 2 portions to be analysed “as is” or after centrifugation and ultrafiltration with a 10 kDa MW cut-off (ultrafiltered digesta = precipitate + retentate) as previously detailed (Deglaire *et al.*, 2007). The ultrafiltered digesta were analysed for TiO<sub>2</sub>, total N, AA, and total <sup>15</sup>N-enrichment (Deglaire *et al.*, 2007).

### ***Chemical analysis***

TiO<sub>2</sub> was determined by a colorimetric assay after ashing the sample and digestion of the minerals (Short *et al.*, 1996). AA were determined after acid hydrolysis using a Waters ion exchange high-performance liquid chromatography (HPLC) system (AOAC, 2003). Cysteine, methionine and tryptophan destroyed during acid hydrolysis, were not determined. The <sup>15</sup>N-enrichment and total N were determined on an isotopic ratio mass spectrometer (Optima, Fisons Instruments, Manchester, UK) coupled to an elemental N analyser (NA 1500 series 2, Fisons Instruments, Manchester, UK) as described previously (Mariotti *et al.*, 2001).

The <sup>15</sup>N-enrichments of individual AA were determined by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, Finnigan Delta S; Thermo Fisher Scientific Inc., Bremen, Germany) as described previously (Metges *et al.*, 1996; Metges & Petzke, 1997; Petzke *et al.*, 2005). Briefly, digesta samples were hydrolysed in 2 mL of 6 mol/L HCl (24 h, 110°C), dried under N at 60°C and dissolved in 0.1 mol/L HCl. AA were derivatized to their N-pivaloyl-i-propyl AA esters (Metges & Petzke, 1997) before being analysed for their individual <sup>15</sup>N-enrichments.

### ***Data analysis***

Total N and AA flows (TNFL, TAAFL), expressed in µg/g dry matter intake (DMI), were calculated as follows:

$$\text{NFL or TAAFL} = \frac{\text{N or AA in digesta} \times \text{TiO}_2 \text{ in diet}}{\text{TiO}_2 \text{ in digesta}} \quad (\text{Eq. 1})$$

Dietary N flow (DNFL) and endogenous N flow (ENFL) determined according to the isotope dilution (isotope dilution method) were calculated as follows (Hess *et al.*, 2000):

$$\text{DNFL}_{\text{ID}} = \text{TNFL} \times (E_s - E_0) / (E_{\text{meal}} - E_0) \quad (\text{Eq. 2})$$

$$\text{ENFL}_{\text{ID}} = \text{TNFL} - \text{DNFL} \quad (\text{Eq. 3})$$

where  $E_{\text{diet}}$  is the <sup>15</sup>N-enrichment in the diet (expressed as atom%),  $E_s$  is the <sup>15</sup>N-enrichment in digesta sample, and  $E_0$  is the basal enrichment. Dietary and endogenous

flows of single AA determined using the isotope dilution method were calculated as described above for N.

ENFL and EAAFL determined in the ultrafiltered digesta (diet HC) were determined as follows:

$$\text{ENFL}_{\text{UF}} \text{ or } \text{EAAFL}_{\text{UF}} = \frac{\text{N or AA in ultrafiltered digesta} \times \text{TiO}_2 \text{ in diet}}{\text{TiO}_2 \text{ in ultrafiltered digesta}} \quad (\text{Eq. 4})$$

with ultrafiltered digesta = precipitate + retentate (> 10 kDa).

$^{15}\text{N}$  recovered in the digesta was assumed to be unabsorbed dietary  $^{15}\text{N}$ . However, some  $^{15}\text{N}$  was detected in the > 10 kDa fraction of digesta, suggesting that some dietary  $^{15}\text{N}$  had been absorbed, incorporated into protein, and then recycled into the gut lumen. The latter  $^{15}\text{N}$  would be falsely considered as unabsorbed dietary N tracer.  $^{15}\text{N}$ -labelled endogenous protein due to tracer recycling was calculated based on the  $^{15}\text{N}$ -measurements in the endogenous N as determined in the ultrafiltered digesta and expressed as a proportion of endogenous N (MW>10 kDa, R, %) as follows:

$$R = 100 \times (E_{\text{UFS}} - E_0) / (E_{\text{meal}} - E_0)$$

where  $E_{\text{UFS}}$  is the  $^{15}\text{N}$ -enrichment in the ultrafiltered digesta (diet HC).

Ileal N digestibility (%) was calculated as follows:

$$\text{Apparent ileal digestibility (AID)} = \frac{(\text{Dietary N intake} - \text{TNFL}) \times 100}{\text{Dietary N intake}} \quad (\text{Eq. 5})$$

Standardized ileal digestibility (SID) =

$$\frac{[\text{Dietary N intake} - (\text{TNFL} - \text{ENFL})] \times 100}{\text{Dietary N intake}} \quad (\text{Eq. 6})$$

where dietary N intake is expressed as  $\mu\text{g/g}$  DMI. The terminology standardized digestibility was used as defined by Stein *et al.* (2007) and relates to the previously used term “true digestibility”.

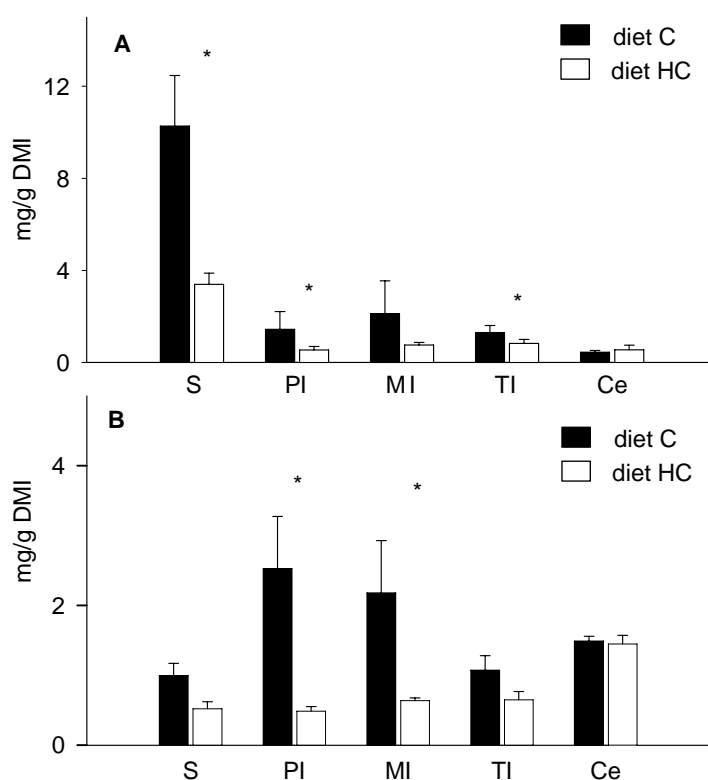
Data were tested for homogeneity of variance using Bartlett’s test, and if they were not homogeneous, they were  $\log_{10}$ -transformed. The daily food intake data were subjected to a one-way ANOVA for repeated measures. N and AA flows and apparent and true ileal N digestibility coefficients from rats fed different diets were subjected to a one-way ANOVA (SAS, version 8.2). Flows and standardized ileal N digestibility from rats fed diet HC and obtained either by the isotope dilution or ultrafiltration method were

compared using a paired t-test (SAS version 8.2, SAS Institute Inc., Cary, NC). Differences were considered significant at  $P < 0.05$ . Results are given as means  $\pm$  SE.

## RESULTS

The rats consumed the diets readily and remained healthy during the experiment. The body weight gains (days 9–16) were similar for rats fed diets HC ( $17.4 \pm 1.5$  g) and C ( $20.3 \pm 1.6$  g). Daily food intake was similar for rats fed diets HC ( $15.9 \pm 0.7$  g/d) and C ( $16.6 \pm 0.8$  g/d) from days 10 to 16, but was significantly higher ( $P < 0.05$ ) on day 17 for those fed diet HC ( $13.3 \pm 0.7$  g/d) than for those fed diet C ( $11.2 \pm 0.6$  g/d).

DNFL<sub>ID</sub> was significantly lower ( $P < 0.05$ ) for rats fed diet HC, in the stomach, proximal intestine and terminal ileum and ENFL<sub>ID</sub> was significantly lower ( $P < 0.05$ ) for rats fed diet HC, in the proximal and medial intestine (**Figure 1**). The proportion of digesta endogenous N to total N (**Table 3**) was similar for rats fed diets HC and C along the entire digestive tract except in the proximal intestine and caecum/colon, where it was somewhat higher ( $P < 0.05$ ) for rats fed diet C.



**Figure 1.** Dietary (A) and endogenous (B) nitrogen flows (isotope dilution,  $\mu\text{g/g}$  dry matter intake) along the digestive tract of rats fed diets C and HC. Mean  $\pm$  SE,  $n=4-6$ . Statistical analyses were performed on transformed data ( $\log_{10}$ ). Statistical significance: \*,  $P < 0.05$ . S: stomach, PI: proximal intestine, MI: medial intestine, TI: terminal ileum, Ce: caecum+colon

**Table 3.** Endogenous nitrogen (isotope dilution) expressed as a percentage of total nitrogen along the digestive tract of rats fed diets C and HC<sup>1</sup>.

	Diet		Pooled SE
	C	HC	
	%		
Stomach	9.5	13.1	1.6
Proximal intestine	59.9	47.7*	3.4
Medial intestine	49.4	44.6	1.7
Terminal ileum	43.3	42.6	3.4
Caecum+ colon	77.3	73.1*	1.2

<sup>1</sup> Values are means, n=4-6 due to limited amount for some of the digesta samples. Statistical significance: \*, different from C,  $P < 0.05$ .

Ileal TAAFL and TNFL were significantly lower ( $P < 0.05$ ) for rats fed diet HC than for those fed diet C (**Table 4**), except for alanine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine, for which there were no significant difference. Ileal EAAFL and ENFL, when determined using the isotope dilution technique, did not differ significantly ( $P > 0.05$ ) between rats fed diets HC and C (**Table 5**) except for glycine flow which was significantly lower ( $P = 0.05$ ) for rats fed diet HC. The AA compositions of these endogenous protein flows did not differ significantly between rats fed diets HC and C, except for aspartic acid, phenylalanine, tyrosine and serine for which the relative contributions were somewhat lower ( $P < 0.05$ ) for rats fed diet C (data not shown).

Ileal EAAFL and ENFL were significantly ( $P < 0.05$ ) lower when determined using the isotope dilution method compared with the ultrafiltration method (**Table 6**), except for glycine, phenylalanine and histidine which did not significantly differ ( $P > 0.05$ ). The AA composition of endogenous ileal protein flows from rats fed diet HC was significantly different ( $P < 0.05$ ) when determined using the ultrafiltration method compared with that determined using the isotope dilution method, except for aspartic acid, alanine and leucine for which there were no significant differences (data not shown).



**Table 4.** Ileal total amino acid and nitrogen flows determined in rats fed diets C and HC<sup>1</sup>.

	Diet		Pooled SE
	C	HC	
	<i>µg/g dry matter intake</i>		
Threonine	772	512*	77
Valine	966	635*	87
Isoleucine	982	585*	81
Leucine	679	449	90
Phenylalanine	289	218	49
Tyrosine	261	182	38
Lysine	556	378	85
Histidine	343	249	33
Glutamic acid	3529	2341*	275
Proline	729	508*	63
Arginine	375	229	62
Aspartic acid	1501	1010*	149
Serine	1664	1004*	119
Glycine	753	485*	76
Alanine	606	451	80
Nitrogen	2279	1428*	248

<sup>1</sup> Values are means, n=6. Statistical significance: \*, different from C, P < 0.05.

**Table 5.** Ileal endogenous amino acid and nitrogen flows determined using the isotope dilution method in rats fed diets C and HC<sup>1</sup>.

	Diet		Pooled SE
	C	HC	
	<i>µg/g dry matter intake</i>		
Threonine	429	285	56
Valine	159	127	42
Isoleucine	142	114	36
Leucine	329	212	62
Phenylalanine	182	138	35
Tyrosine	157	106	27
Lysine	298	206	60
Histidine	228	161	27
Glutamic acid	695	483	136
Proline	261	175	36
Aspartic acid	620	422	92
Serine	233	175	47
Glycine	443	273*	53
Alanine	229	180	54
Nitrogen	1038	634	167

<sup>1</sup> Values are means, n=6. Statistical significance: \*, different from C, P < 0.05.

**Table 6.** Ileal endogenous amino acid flows determined using the isotope dilution or ultrafiltration methods in rats fed diet HC<sup>1</sup>.

Amino acid	Method		Pooled SE
	Isotope dilution	Ultrafiltration <sup>2</sup>	
	<i>µg/g dry matter intake</i>		
Threonine	285	514*	33
Valine	127	634*	30
Isoleucine	114	646*	25
Leucine	212	384*	37
Phenylalanine	138	135	20
Tyrosine	106	142*	17
Lysine	206	265*	40
Histidine	161	185	13
Glutamic acid	483	2109*	84
Proline	175	523*	32
Aspartic acid	422	882*	56
Serine	175	1142*	35
Glycine	273	284	34
Alanine	180	432*	48
Nitrogen	634	1352*	122

<sup>1</sup> Values are means, n=6. Statistical significance: \*, different from C, P < 0.05.<sup>2</sup> The present values were presented in Deglaire *et al.* (2007)

The portion of dietary N recycled (R) within endogenous protein *i.e.* present in the >10 kDa fraction from rats fed diet HC (n=5) was  $64.7 \pm 2.9$  % of the N collected in this fraction.

Apparent ileal N digestibilities tended to be lower ( $P < 0.1$ ) for diet C than for diet HC (**Table 7**). Standardized ileal N digestibility, when corrected for ENFL determined using the isotope dilution method, was significantly lower ( $P < 0.05$ ) for rats fed diet C than for those fed diet HC. Standardized ileal N digestibility for rats fed diet HC, when corrected for ENFL determined using the ultrafiltration method, was  $99.9 \pm 1.0$  % and was significantly higher ( $P < 0.05$ ) than that corrected for ENFL determined using the isotope dilution method.

**Table 7.** Apparent and standardized ileal digestibility<sup>1</sup> of nitrogen in diets C and HC fed to growing rats<sup>2</sup>.

	Diet		Pooled SE
	C	HC	
	%		
Apparent ileal digestibility	87.1	92.0	1.5
Standardized ileal digestibility	93.0	95.6*	0.6

<sup>1</sup> Endogenous flows were determined using the isotope dilution technique.

<sup>2</sup> Values are means, n=6. Statistical significance: \*, different from C,  $P < 0.05$ .

## DISCUSSION

The enzyme-hydrolysed protein method allows for the determination of ileal endogenous protein losses under peptide alimentation, with an array of dietary peptides assumed to be similar to that arising naturally during digestion of the parent protein. However, a potential concern has been raised as a possible artificial stimulatory effect of dietary peptides on gut protein secretions with this method (Jansman *et al.*, 2002; Stein *et al.*, 2007). Most studies (Butts *et al.*, 1991; Butts *et al.*, 1993; Hodgkinson *et al.*, 2000; Moughan *et al.*, 2005; Deglaire *et al.*, 2007) have assessed only the impact of dietary peptides versus protein-free or synthetic AA-based diets. Yin *et al.* (2004) compared dietary casein peptides versus casein, but the comparison was somewhat biased by methodological factors as discussed below. The objective of the present work, therefore, was to determine within a controlled study the impact of <sup>15</sup>N-labelled casein

either in its intact form or after hydrolysis on ileal endogenous protein flows. Although the isotope dilution method is known to lead to some degree of underestimation of absolute gut endogenous protein flows (Leterme *et al.*, 1996), it was considered a useful tool to allow a comparison to be made using the same protein source.

Ileal ENFL and most EAAFL (isotope dilution method) were numerically lower for rats fed diet HC than for those fed diet C, although the differences did not reach statistical significance likely due to the large variability of the present data. In spite of this, there was no convincing evidence of a heightened ileal endogenous protein loss due to the dietary peptides *per se*. This was the case along the entire digestive tract, from the stomach to the colon. On the contrary, Yin *et al.* (2004) reported significantly higher ileal endogenous protein flows in growing pigs given hydrolysed-casein compared with an intact casein. However, the intact and hydrolysed caseins used were not from the same source. Additionally, endogenous flows were determined either using the hydrolysed protein-ultrafiltration method with the indigestible marker TiO<sub>2</sub> for the hydrolysed-casein diet or using the homoarginine method and the marker Cr<sub>2</sub>O<sub>3</sub> for casein (2004). Different dietary markers have been shown to behave differently (Jagger *et al.*, 1992) and the ultrafiltration method has been reported to give higher estimates of endogenous protein flows than the homoarginine method (Hodgkinson *et al.*, 2003). Endogenous ileal protein flows have been reported to be similar for pigs fed intact and hydrolysed pea protein when determined using the <sup>15</sup>N-infusion method (<sup>15</sup>N-labelling of endogenous protein) (Hess *et al.*, 1998), a finding that supports the present result.

The relative contribution of endogenous N to total N in ileal digesta was similar for rats fed diets C and HC, and a similar value was found in an identical study in our laboratory in which rats were fed a <sup>15</sup>N-labelled acid casein (average value of 46%; Deglaire, unpublished data). Also, the AA compositions of the endogenous protein flows were similar for rats fed diets HC and C. These results show that the ileal endogenous protein losses were qualitatively similar regardless of the dietary treatment. The AA composition of endogenous protein determined using the isotope dilution method was similar to that determined previously using a protein-free diet (Butts *et al.*, 1991; Donkoh *et al.*, 1995; Deglaire *et al.*, 2006), suggesting that the presently derived estimates of endogenous protein losses were qualitatively similar to minimal endogenous losses (Stein *et al.*, 2007). The exceptions were serine, valine and glycine for which the relative amounts were lower in the present study. The AA composition of

endogenous protein flows determined using the hydrolysed protein-ultrafiltration method exhibited a similar pattern to that previously reported using the same technique (Donkoh *et al.*, 1995; Hendriks *et al.*, 2002; Hodgkinson *et al.*, 2003).

ENFL and DNFL determined along the upper digestive tract were consistently lower for rats fed diet HC than for those fed diet C, suggesting a higher degree of absorption and reabsorption and/or faster digesta transit rates along the intestinal tract.

The present ileal ENFL and EAAFL for rats fed diet HC (ultrafiltration method) were in the range of previously comparable data (Donkoh *et al.*, 1995; James *et al.*, 2002; Hodgkinson *et al.*, 2003). Ileal ENFL and EAAFL (isotope dilution method) were on average 53% lower than with the ultrafiltration method. A similar difference has been observed in pigs (46%) and in rats (35%) (Hodgkinson *et al.*, 2003). The ultrafiltration method, however, has been shown to give similar estimates of ENFL or EAAFL compared with the  $^{15}\text{N}$ -infusion method (Schulze *et al.*, 1995) or to a diet based on zein, a maize protein naturally deficient in lysine, allowing a direct estimation of its endogenous flow (Butts *et al.*, 1993). It is likely that rapid recycling of  $^{15}\text{N}$  within the gut (van Leeuwen *et al.*, 1996; Leterme *et al.*, 1996; Fuller & Reeds, 1998; Hess *et al.*, 2000) led to a degree of underestimation of endogenous N with the isotope dilution method. This is highlighted here by the high proportion of  $^{15}\text{N}$  recovered in the endogenous protein fraction after ultrafiltration (MW > 10 kDa) for the HC fed rats. It is unlikely that the  $^{15}\text{N}$  could arise from incomplete separation of the unabsorbed dietary  $^{15}\text{N}$ -peptides, as dietary peptides have been reported to represent only 2% of the endogenous N (MW > 10 kDa) (Hodgkinson & Moughan, 2001). The recycled  $^{15}\text{N}$  might result from the luminal dietary  $^{15}\text{N}$  used for gut protein synthesis (Alpers, 1972; Stoll *et al.*, 1998) and subsequently re-excreted.  $^{15}\text{N}$  was found in pancreatic enzymes within 50 min following the ingestion of a labelled meal, in bile secretion after 90 min and in mucins after 4 h (Leterme *et al.*, 1996). The recycled dietary  $^{15}\text{N}$  may also arise due to incorporation of dietary AA into microbial proteins. Although not strictly endogenous, microbial proteins have been traditionally considered as part of the endogenous component. Microbial N has been reported to contribute from 15 to 50% of the total ileal N (Dugan *et al.*, 1994; Bartelt *et al.*, 1999; Caine *et al.*, 1999) and from 30 to 45% of the ileal endogenous N (Caine *et al.*, 1999). Evidence for a possible substantial use of dietary N for microbial N synthesis has been found in recent studies (Bartelt *et al.*, 1999; Libao-Mercado *et al.*, 2007).

The present data do not provide any information regarding the extent of the recycling in the upper parts of the intestine. It is also possible that different recycling rates occurred between diets HC and C. A degree of caution should be exercised, therefore, in our comparison of endogenous flows between the HC and C diets. The comparison is only truly valid if the relative rates of recycling were similar between the two diets and this remains unknown.

The present value for the standardized (true) ileal N digestibility of intact casein was similar to values reported previously (de Vrese *et al.*, 2000; Gaudichon *et al.*, 2002) but lower than values reported in other studies (Nyachoti, *et al.*, 1997; Yin *et al.*, 2004). Differences across studies are unlikely due to the different casein forms used, which were either the micellar form (native phosphocaseinate) (de Vrese *et al.*, 2000; Gaudichon *et al.*, 2002) such as in the present study or acid-precipitated casein (Nyachoti *et al.*, 1997; Yin *et al.*, 2004); rather, they probably reflect differences in methodology. In a similar study conducted within our laboratory (Deglaire, unpublished data), the ileal N digestibility of  $^{15}\text{N}$ -acid-precipitated casein ( $87.0 \pm 0.2\%$ , AID and  $92.9 \pm 0.2\%$ , SID) was similar to the value for the micellar (intact) casein reported here. The ileal ENFL for rats fed the acid casein in the latter unpublished work ( $939 \mu\text{g/g DMI}$ ) was also similar to that for rats fed the intact casein in the present study.

The present study has demonstrated that there was no specific heightened effect of dietary peptides from hydrolysed dietary casein on endogenous ileal protein flow in rats, when comparison was made with intact dietary casein. The latter conclusion is tentative, however, as the isotope dilution method (with  $^{15}\text{N}$ -labelled dietary protein) led to substantially lower estimates of endogenous ileal protein flows compared with the ultrafiltration method. The lower ENFL obtained with the isotope dilution method were assumed to be due to a recycling of  $^{15}\text{N}$  within the splanchnic bed and may have been maximized by the experimental design used (*i.e.* sampling of the ileal digesta samples after 8 hours of continuous feeding). In contrast, following single  $^{15}\text{N}$ -meals, which we frequently used in the past in humans (Gaudichon *et al.*, 2002; Bos *et al.*, 2005; Bos *et al.*, 2007), we calculated that the recycling would account for only a 4 to 9% error in the calculation of ileal endogenous N flow and a subsequent 0.6 to 1.6% error in the determination of true ileal protein digestibilities. It appears that the amount of gut protein recycled under the conditions of our study was considerable. The present results were obtained with growing rats using a frequent feeding regimen. The work needs to

be extended to other species of animal.

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## CHAPTER V

# A casein hydrolysate does not enhance ileal endogenous protein flows compared with the parent intact casein when fed to growing pigs

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We previously showed (Chapter IV) that a casein hydrolysate had no stimulatory effect on ileal endogenous protein flows compared with the parent intact casein in the growing rat, but the result was tentative due to the high degree of tracer recycling observed. The objective of the present study was to compare the influence of a casein hydrolysate on ileal endogenous protein flows with that of the parent intact casein within an experimental design expected to minimize the extent of tracer recycling (pig as animal model, meal-feeding as feeding regimen).

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## ABSTRACT

We studied the effect of dietary peptides on ileal endogenous nitrogen and amino acid flows (ENFL, EAAFL). Six pigs (34 kg) were equipped with a post-valve T-caecum (PVTC) cannula. Semi-synthetic test diets contained the same  $^{15}\text{N}$ -labelled casein, intact (C) or hydrolysed (HC), or the corresponding free L-amino acid (AA) mixture but devoid of aspartic acid and serine (A). Pigs received the test diets every six days following a duplicated 3x3 Latin Square design and the corresponding unlabelled diets in the intervening five days. Digesta were pooled over 4–10 h postprandially. EAAFL and ENFL, calculated with reference to the dietary marker  $\text{TiO}_2$ , were determined directly for A (for aspartic acid and serine), by isotope dilution for C and HC and additionally after digesta centrifugation and ultrafiltration for HC. As most AA from diet A had an apparent ileal digestibility above 95%, the ileal flows of these AA were assumed to be endogenous. Ileal EAAFL and ENFL did not differ ( $P > 0.05$ ) among pigs fed A, HC and C, except for threonine and glutamic acid for which there were lower ( $P < 0.05$ ) flows in pigs fed A compared with C. The enzyme-hydrolysed protein/ultrafiltration and isotope dilution methods generally led to similar endogenous protein flows. Some 20% of ileal endogenous protein (diet HC, ultrafiltered digesta) was  $^{15}\text{N}$ -labelled due to tracer recycling. Casein hydrolysate had no effect on ileal endogenous protein flows compared with intact casein.

## INTRODUCTION

After the ingestion of a protein-containing meal, undigested dietary and non-dietary (endogenous) proteins accumulate at the terminal ileum. It is important to be able to determine the amount of ileal endogenous amino acids (AA), as such a measure is required to determine true AA digestibility (Hodgkinson *et al.*, 2003; Fuller & Tome, 2005) and, moreover, is an important component of the daily AA requirement (Gaudichon *et al.*, 2002; Moughan *et al.*, 2005).

While protein-free diets have traditionally been used in the determination of digesta endogenous protein (FAO/WHO, 1991), such an approach has been criticized as leading to a physiologically abnormal metabolism (Fauconneau & Michel, 1970). The enzyme-hydrolysed protein/ultrafiltration technique (Moughan & Rutherford, 1990; Butts *et al.*, 1993) was developed as an alternative approach whereby animals or human subjects are fed an array of peptides [usually casein-derived, molecular weight (MW)  $< 5$  kDa],

mimicking the breakdown products of natural digestion. After digesta centrifugation and ultrafiltration (10 kDa molecular weight cut-off), endogenous protein is determined in the high molecular weight fraction. Any undigested dietary AA or peptides (molecular weight <10 kDa) are discarded. It has been questioned as to whether potentially bioactive peptides present in the hydrolysate used with this method, may enhance the loss of AA from the small bowel (Jansman *et al.*, 2002; Yin *et al.*, 2004; Stein *et al.*, 2007), over and above that which may be found with the corresponding intact protein.

The effect of dietary peptides versus intact protein on gut endogenous protein flow has been assessed directly (Butts *et al.*, 1993; Yin *et al.*, 2004). In the latter studies, however, dietary AA composition and electrolyte balance ( $\text{Na}^+ + \text{K}^+ - \text{Cl}^-$ ) were not controlled and sometimes different methodologies or different protein sources were used for determining endogenous AA and N flows (EAAFL, ENFL) between dietary treatments.

Previously, we have demonstrated in a controlled study using the  $^{15}\text{N}$ -isotope dilution method with the growing rat and a single source of protein that intact and hydrolysed casein have a similar influence on ileal endogenous protein flow (Deglaire *et al.*, 2008). The latter findings, however, were tentative because of a high degree of recycling of dietary  $^{15}\text{N}$  encountered in the frequently fed rat.

Our primary objective here, therefore, was to assess in the meal-fed growing pig, where the extent of the dietary  $^{15}\text{N}$  recycling was expected to be lower than in the frequently fed rat, the influence of a casein hydrolysate (diet HC), the parent intact casein (diet C) or a corresponding free AA mixture (diet A) on ileal endogenous AA flow. Dietary electrolyte balance was adjusted among diets and AA composition was controlled. Both forms of casein were  $^{15}\text{N}$ -labelled (same source of protein) so that endogenous protein flows could be determined and compared for both diets (C and HC) using the isotope dilution technique. Aspartic acid and serine were omitted from diet A to enable direct determination of their endogenous losses (Skilton *et al.*, 1988). Furthermore, endogenous ileal protein flows were determined using the enzyme-hydrolysed protein/ultrafiltration method to enable a comparison to be made with the isotope dilution method. The study also afforded the opportunity to determine whether the relative effects of the dietary treatments were the same when the diets were administered to the pigs either acutely (no adaptation) or after an adaptation period.

## MATERIALS AND METHODS

### *Animals and housing*

Eight 10-week-old Large White x Duroc entire male pigs were housed individually in steel metabolism crates in a room maintained at  $24 \pm 1^\circ\text{C}$ . Ethics approval was received from the Massey University Animal Ethics Committee (protocol 05/29).

### *Surgery*

Mean ( $\pm$  SE) body weight (BW) at the time of surgery (day 0 of the experimental period) was  $34.4 \pm 2.0$  kg. The pigs were fasted for 12 h before surgery. A post-valve T-caecum (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.* (van Leeuwen *et al.*, 1991). The cannulae were made of medical grade silastic tubing with an internal diameter of 24 mm and external diameter of 32 mm. Prior to the start of surgery, the pigs were given analgesics: Carprofen (Pfizer Laboratories Ltd., Manukau, UK; 3 mg/kg BW) administered by intravenous injection, and Methadone (David Bull Laboratories, Victoria, Australia; 0.2 mg/kg BW) administered by deep intramuscular injection. Anaesthesia was induced with an intramuscular injection of Midazolam (Roche Products Ltd., Auckland, NZ; 1 mg/kg BW) and Ketamine (Parnell Laboratories Ltd., East Tamaki, NZ; 10 mg/kg BW) followed by an intravenous injection of Propofol (Gensia Laboratories Ltd., Irvine, CA; 2 mg/kg BW). The anaesthesia was maintained via inhalation of isoflurane (Merial Ltd., Auckland, NZ; 1.5 to 2%) in O<sub>2</sub>. Intravenous crystalloids were infused throughout the anaesthesia period (5-10 mL/kg BW/h) to maintain hydration. Immediately after surgery, the pigs received an intramuscular injection of antibiotic (Duplocillin LA, Intervet International B.V., Boxmeer, The Netherlands; 2 mL). For the following 4 days, antibiotic powder (Mamyzim, Boehringer Ingelheim Ltd., Wiri, NZ) was dusted on the wound site daily. The site where the cannula was exteriorised was washed with water, and zinc ointment was applied daily throughout the experiment. The pigs regained consciousness within 1 h of surgery and were standing 7–8 h after surgery. There was a 14-day recovery period before the experimental diets were given.



### ***Diets***

In total, there were seven diets: a basal diet, 3 adaptation diets and 3 test diets (**Table 1**). The test diets contained as the sole source of N either uniformly  $^{15}\text{N}$ -labelled native phosphocaseinate (C diet), a  $^{15}\text{N}$ -labelled casein-derived hydrolysate (derived from the former native phosphocaseinate, HC diet) or a mixture of free L-AA simulating C and HC except for the omitted dietary non-essential AA, aspartic acid and serine (A diet). A highly digestible basal diet (Table 1) was prepared as described previously (Moughan *et al.*, 2006). The adaptation diets contained similar (but unlabelled) N sources to those included in the test diets (Table 1). All diets were formulated to meet the nutrient requirements of the growing pig (National Research Council, 1998). Sodium bicarbonate (diets C and A) and potassium bicarbonate (diet A) were added to the diets to equalize their dietary electrolyte balance to that of diet HC (Deglaire *et al.*, 2007). Titanium dioxide was added to the test diets as an indigestible marker. The determined total N and AA compositions of the diets are given in **Table 2**.

The  $^{15}\text{N}$ -casein was extracted by microfiltration followed by diafiltration (INRA Rennes) of  $^{15}\text{N}$ -labelled milk (Mahé *et al.*, 1994). The resulting casein was freeze-dried. Its isotopic enrichment was 0.5450%. There was a homogenous distribution of the  $^{15}\text{N}$ -label among individual AA for which enrichment ranged from 0.4942 to 0.5653 atom%. An aliquot of  $^{15}\text{N}$ -casein was hydrolysed with pig pancreatin as previously detailed (Deglaire *et al.*, 2007). The molecular weight profile, determined by HPLC gel filtration (Deglaire *et al.*, 2007), indicated that 21% of the peptides were between 1 and 5 kDa in size, and 79% were less than 1 kDa.

### ***Experimental design***

For 14 days post-surgery, all pigs were fed a standard pig weaner diet (Denver Stock Feeds) which was gradually introduced over the first 7 days up to a daily feeding level of 0.08 metabolic BW ( $\text{BW}^{0.75}$ ). This level of food intake was maintained for the remainder of the trial. Except on digesta collection days, the pigs received 3 meals daily (0800, 1200 and 1600 h) in equal portions. The diets were mixed with water (1:1, w/w) and water was freely available between meals. On digesta collection days, the pigs received (0900 h) the test diet (1/3 of the daily portion) mixed with water (2.3:1, w/w) and 200 mL water was given every 30 min. The pigs received the rest of their daily portion at 1900 h. The pigs were weighed every sixth day and food intake was adjusted accordingly. Mean ( $\pm$  SE) BW was  $39.8 \pm 0.9$  kg on day 14 and  $61.3 \pm 2.9$  kg on day 38.

**Table 1.** Ingredient compositions of the diets fed to growing pigs.

Ingredient	Basal diet					
	<i>g/kg air dry weight</i>					
Cooked wheat <sup>1</sup>	485.8					
Sucrose	168					
Casein <sup>2</sup>	159					
Skim milk powder <sup>2</sup>	80					
Soyabean oil	80					
Dicalcium phosphate	22					
Vitamin - mineral mix <sup>3</sup>	2.5					
Sodium chloride	1.6					
Synthetic methionine	0.7					
Calcium carbonate	0.2					
Antioxidant <sup>4</sup>	0.2					

Ingredient	Adaptation diets			Test diets <sup>9</sup>		
	C	HC	A	C	HC	A
	<i>g/kg air dry weight</i>					
Maltodextrin <sup>5</sup>	369.3	364	369.5	453	448	479
Sucrose	161	161	161	161	161	161
Soya bean oil	154	154	154	154	154	154
Casein <sup>6</sup>	211	-	-	211	-	-
Hydrolysed casein <sup>6</sup>	-	234	-	-	234	-
AA mixture <sup>7</sup>	-	-	177	-	-	177
Purified cellulose <sup>8</sup>	50	50	50	-	-	-
Vitamin - mineral mix <sup>3</sup>	2.5	2.5	2.5	-	-	-
Dicalcium phosphate	25	25	25	-	-	-
Potassium Bicarbonate	4	4	12	-	-	8
Magnesium sulphate	4	4	4	-	-	-
Sodium bicarbonate	18	-	18	18	-	18
Sodium chloride	1.2	-	-	-	-	-
Potassium chloride	-	1.5	-	-	-	-
Titanium dioxide	-	-	-	3	3	3

<sup>1</sup> Weet-bix, Sanitarium, Auckland, New Zealand.

<sup>2</sup> NZMP, Palmerston North, New Zealand.

<sup>3</sup> Vitalean, Vitec Nutrition Ltd., Auckland, New Zealand. Vitamins provided: (g/kg of diet) vitamin A 3; (mg/kg diet) cholecalciferol 500.0, choline 83.3, niacin 12.5, panthothenic acid 8.3, riboflavin 2.1, vitamin B6 1.7, vitamin E 41.7, vitamin K 1.7; ( $\mu\text{g}/\text{kg}$  of diet) biotin 8.3, folic acid 417, thiamin 833, vitamin B-12 8.3. Minerals provided: (mg/kg diet) Cu 104, Fe 83, Mn 38, Zn 100; ( $\mu\text{g}/\text{kg}$  diet) I 833, Co 417, Se 250.

<sup>4</sup> Ethoxyquin, Kemin Industries Ltd., Auckland, New Zealand.

<sup>5</sup> Fieldose 17, Penford NZ Ltd., Auckland, New Zealand.

<sup>6</sup> Non-labelled proteins in the adaptation diets (NZMP, New Zealand). <sup>15</sup>N-labelled proteins in the test diets (INRA Rennes, France).

<sup>7</sup> Free L-amino acids (Ajinomoto Ltd., Tokyo, Japan).

<sup>8</sup> Avicel. Commercial Minerals Ltd., Auckland, New Zealand.

<sup>9</sup> Some ingredients in the adaptation diets were excluded from the test diets as the test diets were subsequently given to human subjects to allow an inter-species comparison.

**Table 2.** Amino acid and nitrogen contents of the test diets

Amino acid	Diet		
	C	HC	A
	<i>g/kg air dry weight</i>		
Threonine	7.7	7.2	7.5
Valine	12.3	11.1	11.7
Isoleucine	9.4	9.0	9.2
Leucine	18.2	16.6	17.4
Phenylalanine	10.1	9.0	9.6
Tyrosine	11.0	9.9	10.4
Lysine	15.1	13.8	14.8
Histidine	5.5	4.3	4.9
Serine	9.2	8.8	- <sup>1</sup>
Glutamic acid	42.5	40.3	41.5
Alanine	7.9	6.6	7.3
Proline	21.7	19.6	20.7
Arginine	6.6	6.2	6.4
Aspartic acid	13.2	12.3	- <sup>1</sup>
Glycine	3.5	3.2	3.4
Cysteine	0.6	0.6	0.6
Methionine	5.8	5.4	5.6
Tryptophan	2.5	2.2	2.4
Nitrogen	32.6	31.8	20.5

<sup>1</sup> omitted from diet A.

### ***Acute feeding***

On days 14–37, the test diets were administered using a duplicated 4x4 Latin Square design such that within a Latin Square every test diet followed every other test diet once only. A diet based on rapeseed protein isolate was included in the Latin Square but as part of a separate study and data are not presented here. The pigs were randomly allocated to the Latin Square and were fed their respective test diet every sixth day after having been fed the basal diet in the intervening 5-day periods. On the sixth day of each test period, ileal digesta were collected continuously for 10 h following ingestion of the

test diet, using plastic bags attached to the cannula. The bung of the cannula was removed 2 h before the collection commenced as described 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. Digesta collection commenced 30 min prior to the ingestion of the test diet in order to determine the basal  $^{15}\text{N}$ -enrichment of the digesta. Plastic bags were removed every 30 min, and digesta were immediately frozen ( $-20^{\circ}\text{C}$ ) after addition of benzoic sodium (2.3 mol/L) and phenylmethylsulphonyl fluoride (PMSF, 70 mmol/L). This procedure was adopted to prevent bacterial and protease activity, respectively in the digesta samples (Salgado *et al.*, 2002).

### ***Adaptation feeding***

On days 38–54, the rapeseed diet was no longer included in the Latin Square. In this period, the test diets (C, HC and A) were administered using a duplicated 3x3 Latin Square design. The pigs were randomly allocated to the Latin Square and were fed their respective test diets every sixth day after having been fed the corresponding adaptation diet (containing similar, but unlabelled N) in the intervening 5-day periods. The digesta collection was performed as described for the acute feeding regimen.

### ***Chemical analysis***

Digesta were freeze-dried, ground and pooled for each diet and pig over the collection time period between 4 and 10 h after ingestion of the test diet (Leterme *et al.*, 1996b; Hess *et al.*, 2000). Pooled digesta were analyzed for  $\text{TiO}_2$ , total N, AA and, when applicable, for  $^{15}\text{N}$ -enrichment of the total N and single AA. Pooled digesta from pigs fed diet HC were divided in two portions to be analyzed “as is” or after centrifugation and ultrafiltration with a 10 kDa molecular weight cut-off (ultrafiltered digesta = precipitate + retentate) as described previously (Deglaire *et al.*, 2007). The ultrafiltered digesta were analysed for total N, AA, the  $^{15}\text{N}$ -enrichment of total N and single AA, and  $\text{TiO}_2$ . Diets were analyzed for  $\text{TiO}_2$ , total N, AA and the  $^{15}\text{N}$ -enrichment of total N and single AA.

$\text{TiO}_2$  was determined using a colorimetric assay after ashing the sample and digestion of the minerals (Short *et al.*, 1996). AA were determined in diets and digesta samples after acid hydrolysis (HCl, 6 mol/L containing 0.1% phenol) using a Waters ion exchange HPLC (AOAC, 2003). In the diets, cysteine and methionine were measured as

methionine sulphone and cysteic acid after performic acid oxidation (Moore, 1963) and tryptophan was determined after alkaline hydrolysis (AOAC, 1990). Cysteine, methionine and tryptophan were not determined in ileal digesta due to limited sample size.  $^{15}\text{N}$ -enrichment and total N contents were measured on an isotopic ratio mass spectrometer (Optima, Fisons Instruments, Manchester, UK) coupled to an elemental N analyzer (NA 1500 series 2, Fisons Instruments, Manchester, UK) (Gausserès *et al.*, 1997).

The  $^{15}\text{N}$ -enrichments of individual AA were determined using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, Finnigan Delta S; Thermo Fisher Scientific Inc., Bremen, Germany) as described previously (Metges *et al.*, 1996; Petzke *et al.*, 2006; Deglaire *et al.*, 2007)

### **Data analysis**

Total N and AA flows ( $\mu\text{g/g}$  dry matter intake, DMI) were calculated as follows:

$$\text{Total N or AA flow} = \frac{\text{N or AA in digesta} \times \text{TiO}_2 \text{ in diet}}{\text{TiO}_2 \text{ in digesta}} \quad (\text{Eq. 1})$$

Dietary N flows and ENFL ( $\mu\text{g/g}$  DMI) determined according to isotope dilution were calculated as described in Eq. 2 and Eq. 3, respectively (Hess *et al.*, 2000):

$$\text{Dietary N flow} = \text{Total N flow} \times \frac{(E_s - E_0)}{(E_{\text{diet}} - E_0)} \quad (\text{Eq. 2})$$

$$\text{ENFL}_{\text{ID}} = \text{Total N flow} - \text{Dietary N flow} \quad (\text{Eq. 3})$$

where  $E_{\text{diet}}$  is the  $^{15}\text{N}$ -enrichment in the diet (expressed as atom%),  $E_s$  is the  $^{15}\text{N}$ -enrichment in the digesta sample and  $E_0$  is the basal enrichment in the digesta sample. Dietary and endogenous flows of single AA determined using the isotope dilution method were calculated as described above for N.

ENFL and EAAFL ( $\mu\text{g/g}$  DMI) determined in the ultrafiltered digesta (diet HC) were determined as follows:

$$\text{ENFL}_{\text{UF}} \text{ or } \text{EAAFL}_{\text{UF}} = \frac{\text{N or AA in ultrafiltered digesta} \times \text{TiO}_2 \text{ in diet}}{\text{TiO}_2 \text{ in ultrafiltered digesta}} \quad (\text{Eq. 4})$$

$^{15}\text{N}$  recovered in the digesta was assumed to trace unabsorbed dietary N. However, some  $^{15}\text{N}$  was detected in the  $> 10\text{-kDa}$  fraction of digesta following centrifugation and ultrafiltration, suggesting that some dietary N had been absorbed, incorporated into

protein and then recycled into the gut lumen. The latter  $^{15}\text{N}$  would be falsely considered as unabsorbed dietary N tracer. An estimate of the amount of  $^{15}\text{N}$ -labelled endogenous protein present due to tracer recycling was calculated based on the  $^{15}\text{N}$ -measurements in the endogenous N as determined in the ultrafiltered digesta and was expressed as a proportion of endogenous N (R, %) as follows:

$$R = 100 \times \frac{(E_{\text{UF}} - E_0)}{(E_{\text{diet}} - E_0)}$$

where  $E_{\text{UF}}$  is the  $^{15}\text{N}$ -enrichment in the ultrafiltered (MW > 10 kDa) digesta (diet HC).

The underestimation (U, %) of the endogenous N losses when determined using the isotope dilution method and due to tracer recycling was calculated using the following equation:

$$U = 100 \times \frac{\text{ENFL}_{\text{UF}} \times \frac{R}{100}}{\text{ENFL}_{\text{ID}} + \text{ENFL}_{\text{UF}} \times \frac{R}{100}}$$

Apparent ileal digestibility (%) of AA from diet A was calculated as follows:

$$\text{Apparent ileal digestibility} = 100 \times \frac{(\text{Dietary AA intake} - \text{Total AA flows})}{\text{Dietary AA intake}} \quad (\text{Eq. 5})$$

where dietary AA intake was expressed as  $\mu\text{g/g DMI}$ .

### ***Statistical analysis***

All the statistical analyses were performed using SAS (version 9.1, SAS Institute Inc., Cary, NC). The data set was subjected to the outlier test of Dixon (1950; 1953) with  $P < 0.05$  and data were analyzed within each experiment using the following general linear model:

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

where  $\alpha_i$ ,  $\beta_j$  and  $\delta_k$  represented the effects due to pig, day of collection and diet, respectively. Diet was a fixed effect, and pig and day of collection were random effects. When the effect of the diet was significant ( $P < 0.05$ ), Tukey's test was used for multiple comparisons of the means. Ileal ENFL and EAAFL from pigs fed diet HC and obtained using either the isotope dilution or ultrafiltration method were compared using a paired t-test. Results are given as means  $\pm$  SE.

## RESULTS

The pigs remained healthy and grew normally throughout the study, except for one pig which was removed from the study because of internalization of the cannula. This animal was replaced by a spare cannulated pig of a similar age and BW. Minimal leakage occurred during digesta collections. Average pig live weight at the completion of the trial was  $73.9 \pm 2.2$  kg. At dissection post-mortem, no signs of adverse effects due to the cannulation were observed.

For each diet, one observation each for AA and total N was removed from the data set, as detected by application of a statistical outlier test ( $P < 0.05$ ). The data removed ranged from being 60 to 150% higher than the corresponding mean values.

Generally, the pigs ate the test diet completely within 15 min. For the pigs which were adapted to the test diets, ileal total AA and N flows were not significantly different ( $P > 0.05$ ) between pigs fed diets C and HC (**Table 3**). Ileal endogenous flows of aspartic acid and serine were numerically lower by 16 and 23%, respectively, for the adapted pigs fed diet A compared to those for adapted pigs fed diets C and HC, but the differences did not attain statistical significance ( $P > 0.05$ , **Table 4**). The difference for aspartic acid was significant at  $P = 0.10$ . All the crystalline AA included in diet A had an apparent ileal digestibility above 95% (**Table 5**), except for threonine (91%) and glycine (85%). Ileal AA flows for this diet were thus assumed to be endogenous and on this basis were compared with EAAFL for pigs fed diets C and HC determined using the isotope dilution method (**Table 6**). Ileal EAAFL and ENFL were not significantly different ( $P > 0.05$ ) for the adapted pigs fed diets HC and C. Most of the ileal EAAFL and ENFL for adapted pigs fed diet A were numerically lower compared to those for pigs fed diets C and HC, but were significantly ( $P < 0.05$ ) so only for threonine and glutamic acid.

Endogenous N was a similar ( $P > 0.05$ ) proportion of the total N in ileal digesta for pigs receiving diets C and HC. When the isotope dilution method was compared directly with the ultrafiltration method there was no effect ( $P > 0.05$ ) of method on endogenous flows (**Table 7**), except for the AA leucine, lysine, histidine and glycine for which the ultrafiltration method yielded lower ( $P < 0.05$ ) endogenous flows.

The amount of  $^{15}\text{N}$ -labelled dietary N recycled (R) within endogenous protein (i.e. present in the  $>10$  kDa fraction of the ultrafiltered digesta) from the adapted pigs fed

diet HC (n = 5) was  $21.3 \pm 3.1\%$  of the N collected in this fraction. It was calculated that this would lead to an underestimation of ileal ENFL (diet HC, isotope dilution method) due to the tracer recycling of  $13.2 \pm 2.7\%$ .

When pigs were not adapted to the test diets (acute feeding), the relative effects of the dietary treatments differed, with diet A inducing significantly higher ( $P < 0.05$ ) ileal endogenous flows of aspartic acid and serine than diet HC (isotope dilution method, **Table 8**).

**Table 3.** Total ileal amino acid and nitrogen flows in growing pigs fed diets C or HC after 5-day adaptation to the diets<sup>1</sup>

	Diet		Pooled SE	Significance <sup>2</sup>
	C	HC		
	<i>µg/g DMI</i>			
Threonine	1322	1319	68	NS
Valine	740	959	87	NS
Isoleucine	583	622	64	NS
Leucine	822	947	90	NS
Phenylalanine	312	442	74	NS
Tyrosine	470	445	52	NS
Lysine	476	518	67	NS
Histidine	393	644	118	NS
Glutamic acid	2034	2634	168	NS
Proline	858	1082	65	NS
Glycine	752	704	65	NS
Alanine	741	769	73	NS
Aspartic acid	1232	1443	122	NS
Serine	1025	1116	37	NS
Nitrogen	2488	2752	435	NS

<sup>1</sup> Values are means, n = 5.

<sup>2</sup> NS: not significant. Pig and day effects were not significant ( $P > 0.05$ ).



**Table 4.** Ileal endogenous flows of aspartic acid and serine in pigs fed diets C<sup>1</sup>, HC<sup>1</sup> and A<sup>2</sup> after 5-day adaptation to the test diets.

	Diet			Pooled SE	Significance <sup>3</sup>
	C	HC	A		
	<i>µg/g DMI</i>				
Aspartic acid	950	879	736	67	NS <sup>4</sup>
Serine	631	584	522	46	NS

Values are means, n = 5.

<sup>1</sup> Endogenous flows determined using the isotope dilution method.

<sup>2</sup> A was devoid of aspartic acid and serine.

<sup>3</sup> NS: not significant ( $P > 0.05$ ). Pig and day effects were not significant ( $P > 0.05$ ), except for serine whereby pig effect was significant ( $P < 0.05$ ).

<sup>4</sup> A P-value of 0.10 was observed.

**Table 5.** Apparent ileal amino acid digestibility for growing pigs fed diet A after 5-day adaptation to the diet<sup>1</sup>

	Apparent ileal digestibility (%)	
	Mean	SE
Threonine	90.8	0.9
Valine	95.8	0.5
Isoleucine	96.7	0.3
Leucine	96.9	0.3
Phenylalanine	96.4	0.4
Tyrosine	96.7	0.4
Lysine	97.9	0.2
Histidine	94.5	0.6
Glutamic acid	98.0	0.3
Proline	97.3	0.3
Glycine	85.4	2.5
Alanine	94.2	0.6

<sup>1</sup> Values are means, n = 5.

**Table 6.** Ileal endogenous amino acid and nitrogen flows in pigs fed diets C, HC or A<sup>1</sup> after 5-day adaptation to the test diets<sup>2</sup>

Amino acid	Diet			Pooled SE	Significance <sup>4</sup>
	C <sup>3</sup>	HC <sup>3</sup>	A		
	<i>µg/g DMI</i>				
Threonine <sup>5</sup>	995 <sup>a</sup>	910 <sup>ab</sup>	691 <sup>b</sup>	58	*
Valine <sup>5</sup>	507	587	494	47	NS <sup>6</sup>
Isoleucine	392	399	300	33	NS <sup>6</sup>
Leucine	673	681	548	48	NS <sup>6</sup>
Phenylalanine	256	310	346	31	NS
Tyrosine	464	323	338	119	NS
Lysine	421	361	304	49	NS
Histidine	369	468	271	56	NS <sup>6</sup>
Glutamic acid <sup>5</sup>	1170 <sup>a</sup>	945 <sup>ab</sup>	816 <sup>b</sup>	70	*
Proline <sup>5</sup>	582	565	553	49	NS
Glycine	608	522	497	85	NS
Alanine	531	481	425	46	NS
Nitrogen	1912	1828	1378	453	NS <sup>6</sup>

<sup>1</sup> For diet A values were determined assuming complete absorption of the crystalline AA.

<sup>2</sup> Values are means, n = 5.

<sup>3</sup> Flows determined using the isotope dilution method.

<sup>4</sup> Statistical significance: \*: P < 0.05, NS: not significant. Pig and day had no significant effect (P > 0.05), except when noted. Means in a row with superscripts without a common letter differ, P < 0.05.

<sup>5</sup> Pig had a significant effect (P < 0.05).

<sup>6</sup> P-values ranging from 0.05 to 0.15 were observed.

**Table 7.** Ileal endogenous amino acid and nitrogen flows determined using the isotope dilution or ultrafiltration method in pigs fed diet HC after 5-day dietary adaptation<sup>1</sup>.

Amino acid	Method		Pooled SE	Significance <sup>2</sup>
	Isotope dilution	Ultrafiltration		
	<i>µg/g DMI</i>			
Threonine	910	701	155	NS
Valine	587	464	92	NS
Isoleucine	399	295	55	NS
Leucine	681	480	103	*
Phenylalanine	310	275	54	NS
Tyrosine	323	240	52	NS
Lysine	361	266	60	*
Histidine	468	218	78	*
Glutamic acid	945	801	136	NS
Proline	565	590	100	NS
Glycine	522	333	70	*
Alanine	481	409	80	NS
Aspartic acid	879	674	124	NS
Serine	584	510	88	NS
Nitrogen	1828	1424	275	NS

<sup>1</sup> Values are means, n=5.

<sup>2</sup> Statistical significance: \*: P < 0.05, NS: not significant (P > 0.05).

**Table 8.** Ileal endogenous amino acid and nitrogen flows in growing pigs fed diets C, HC or A without prior adaptation to the test diets (acute feeding)<sup>1</sup>

	Diet			Pooled SE	Significance <sup>4</sup>
	C <sup>2</sup>	HC <sup>2</sup>	A <sup>3</sup>		
	<i>µg/g DMI</i>				
Threonine	1207	944	-	97	NS
Valine	692	505	-	84	NS
Isoleucine	464	384	-	55	NS
Leucine	851	731	-	72	NS
Phenylalanine	363	306	-	25	NS
Tyrosine	372	364	-	48	NS
Lysine	518	417	-	52	NS
Histidine	456	493	-	95	NS
Glutamic acid	1385	1122	-	158	NS
Proline	775	643	-	79	NS
Glycine	733	727	-	65	NS
Alanine	643	524	-	90	NS
Aspartic acid <sup>5</sup>	1183 <sup>ab</sup>	973 <sup>a</sup>	1513 <sup>b</sup>	130	*
Serine <sup>5</sup>	774 <sup>ab</sup>	619 <sup>a</sup>	1038 <sup>b</sup>	88	*
Nitrogen	2543	2092	-	291	NS

<sup>1</sup> Values are means, n = 7.<sup>2</sup> Flows determined using the isotope dilution method.<sup>3</sup> Flows determined directly for the AA omitted from the diet.<sup>4</sup>\*, P < 0.05; NS: not significant. Pig and day had no significant effect.<sup>5</sup> Statistical analyses were conducted across diets C, HC and A.

## DISCUSSION

While a possible artificial stimulatory effect of dietary peptides on gut protein secretions with the enzyme-hydrolyzed protein/ultrafiltration method has been discussed (Jansman *et al.*, 2002; Yin *et al.*, 2004; Stein *et al.*, 2007), this was not found in a previous study with the growing rat conducted within our laboratory (Deglaire *et al.*, 2008). The latter findings are confirmed by the present work, where similar ileal endogenous protein flows were found in meal-fed pigs receiving either a casein hydrolysate or the intact parent casein.

The PVTC-cannulation (van Leeuwen *et al.*, 1991), used in the present study, offers the advantage over other cannulation methods of allowing collection of ileal digesta without transection of the small intestine, thus minimizing effects on small intestinal muscle function. Previous studies have reported that long-term PVTC cannulation (12 weeks) in the pig does not greatly alter metabolism (Kohler *et al.*, 1992a; Kohler *et al.*, 1992b). In the present study, pigs remained healthy and grew normally. Representative samples of digesta were assumed to be collected through the PVTC-cannula, as the method allows collection of relatively large samples and dietary marker recoveries determined over a 24-h period have been routinely reported to range from 70 to 100% (Kohler *et al.*, 1990; Kohler *et al.*, 1991; Hodgkinson *et al.*, 2000). In the present work where recovery was calculated over a 10 h-period only, a recovery value of  $37 \pm 2\%$  was found. Digesta were pooled between 4 and 10 h of collection so as to maximize the recovery of the dietary  $^{15}\text{N}$  and to limit the extent of tracer recycling (Leterme *et al.*, 1996b). Moreover, the amount of ileal endogenous N moving with food dry matter flow has been reported to be relatively constant from 4 h following ingestion of a hydrolyzed casein-based meal in PVTC-cannulated pigs, based on an observed constant ratio of endogenous N/dietary marker (Hodgkinson *et al.*, 2002). In the present study and as expected (Darcy *et al.*, 1980; Hodgkinson *et al.*, 2002), only small quantities of digesta were collected during the first 3 h following meal ingestion.

The ileal endogenous flows of aspartic acid and serine were considerably lower, numerically, for the adapted pigs fed diet A (devoid of the particular AA) compared to those for the adapted pigs fed diets C and HC, however the differences did not reach statistical significance. Most AA included in the crystalline AA based diet (diet A) had an apparent ileal digestibility above 95%, and on this basis we assumed that their ileal flows were primarily endogenous (Skilton *et al.*, 1988; Darragh *et al.*, 1990; Deglaire *et*

*al.*, 2007). These EAAFL and ENFL (diet A, adapted pigs) were also generally lower compared with the HC and C diets, but statistically significant ( $P < 0.05$ ) differences were only found for threonine and glutamic acid. The results of the present study are generally in line with trends previously observed in rats and pigs fed synthetic AA-based and HC-based or C-based diet (Chung & Baker, 1992; Butts *et al.*, 1993; Deglaire *et al.*, 2007).

The present ENFL and EAAFL were in the range of previously published estimates (Jansman *et al.*, 2002; Hodgkinson *et al.*, 2003; Steendam *et al.*, 2004b). The present findings show that ileal endogenous protein flows are not enhanced by peptides supplied directly from a dietary hydrolysate of casein in comparison with the parent intact casein, and this finding corroborates earlier results obtained in the rat using similar diets (C and HC) and the same isotope dilution method (Deglaire *et al.*, 2008). To the contrary, Yin *et al.* (2004) reported higher ileal endogenous protein flow in growing pigs given hydrolysed-casein compared with an intact casein. Their comparison, however, was somewhat biased as different methods (ultrafiltration method versus homoarginine method) and different dietary markers ( $\text{TiO}_2$  versus  $\text{Cr}_2\text{O}_3$ ) were used between diets. When intact and hydrolysed pea proteins were compared in  $^{15}\text{N}$ -labelled pigs, similar ileal endogenous protein flows were reported (Hess *et al.*, 1998), a finding in support of the present result. The data in the present study were often highly variable, meaning that some differences between means that were numerically large, were not found to be statistically significant. This did not, however, compromise the testing of our primary hypothesis that hydrolysed casein elicits a higher ileal endogenous protein flow compared with intact casein. Further study involving a higher number of animals should be undertaken to assess whether any of these observed differences are real effects. In spite of this, and taking the data together, there is no convincing evidence that hydrolysed casein is associated with a heightened ileal endogenous protein flow.

The ileal ENFL and EAAFL determined using the ultrafiltration method in the presently reported study were similar to those previously reported (Hodgkinson *et al.*, 2003; Steendam *et al.*, 2004a), except for the present endogenous flow of glutamic acid which was two times lower than previous determinations (Hodgkinson *et al.*, 2003; Steendam *et al.*, 2004a). There was no apparent reason for this lower flow for glutamic acid. Estimates of ileal ENFL and EAAFL were generally numerically lower when

determined using the ultrafiltration method compared with the isotope dilution although the differences reached statistical significance ( $P < 0.05$ ) for four of the AA only. This is in contrast to previously reported pig and rat studies whereby the isotope dilution method, in comparison with the ultrafiltration method, resulted in 35 to 53% lower ileal ENFL (Hodgkinson *et al.*, 2003; Deglaire *et al.*, 2008), probably due to a rapid recycling of  $^{15}\text{N}$ -labelled dietary N into the intestinal secretions (Leterme *et al.*, 1996b; Deglaire *et al.*, 2008). The present study was designed (pig as animal model, meal feeding and defined digesta collection period) to minimize the extent of recycling. Interestingly, the proportion of  $^{15}\text{N}$ -labelled ileal endogenous protein in the high MW fraction ( $>10\text{kDa}$ ) was three times lower than that reported earlier (Deglaire *et al.*, 2008). While in the present work pigs received a single  $^{15}\text{N}$ -labelled meal, in the previous studies rats and pigs had access to the  $^{15}\text{N}$ -labelled meal 5–6 times/d for 10 min each hour (continuous feeding) (Hodgkinson *et al.*, 2003; Deglaire *et al.*, 2008). In the present study, recycling may have contributed to an underestimation of ENFL of around 13%. Similarly, the ultrafiltration method has been reported to underestimate ileal ENFL by 13 to 24 % due to the removal of small endogenous peptides and free AA in the high molecular weight ( $<10\text{ kDa}$ ) fraction (Moughan & Schuttart, 1991; Butts *et al.*, 1992; Leterme *et al.*, 1996a).

The present study afforded the opportunity to compare the relative effects of the dietary treatments for adapted (*i.e.* pigs that had received the adaptation diets for 5 days) and non-adapted (acute feeding) animals. Experimental protocols adopted with human subjects often require an acute feeding regimen and it was thus of interest to make such a comparison (although being uncontrolled). While the relative effect of diets C and HC on ileal EAAFL and ENFL were similar in pigs adapted or otherwise, this was not observed for diet A versus diets C and HC. This could result from a differential metabolic adaptation of the gut mucosa to the diets (Poullain *et al.*, 1989; Zaloga *et al.*, 1991; Daniel, 2004; Guay *et al.*, 2006). The present observation indicates that adaptation to the diet is potentially important and this needs to be investigated in a controlled study.

In conclusion, the present work demonstrates that dietary casein-derived peptides support similar endogenous protein flows at the terminal ileum of the pig as the intact parent casein. It does not appear that the feeding of a casein hydrolysate to the growing pig increases ileal endogenous protein loss. There was some evidence, in line with previous findings, that a synthetic AA mixture simulating the AA composition of casein

generally supports lower EAAFL compared with protein feeding. The relative effect of the dietary treatments on EAAFL was found to differ depending upon whether pigs were adapted or not to the test meal. The study provides an estimate of the dietary  $^{15}\text{N}$ -recycling within ileal endogenous protein (21%) after ingestion of a single  $^{15}\text{N}$ -labelled meal in the growing pig. The enzyme-hydrolysed protein/ultrafiltration and isotope dilution methods yielded similar estimates of ileal endogenous protein flows.

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# CHAPTER VI

## Validation of the intubation method for intestinal digesta sampling in the conscious adult human

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The objective was to validate the intubation method used in conscious adult humans to sample digesta from the intestinal lumen. The study reports an internal validation based on estimated intestinal fluid flow rates and determined marker concentrations and draws on previously unpublished data collected from a number of studies.

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## ABSTRACT

The intubation method has been used in conscious subjects to sample intestinal digesta, in particular for protein digestibility measurement. The present work aimed to intrinsically validate this method. Data were obtained from 5 studies (n = 75 healthy adult humans, 9 protein-based test meals) undertaken within the same laboratory, following the same design with the same analytical techniques, but conducted at different times by different experimenters. An indigestible marker, polyethylene (PEG)-glycol 4000, had been added to each test meal. Another non-absorbable marker (phenolsulphonphthalein, PSP) had been infused above the sampling site to allow estimation of intestinal fluid flow rate. Ileal digesta had been sampled through a triple-lumen tube (inserted through the nose) for 8 h postprandially and had been analysed for PEG, nitrogen (N) and PSP. Integrated PEG flow (%), *i.e.* the overall estimated ileal PEG flow relative to PEG ingested, and PEG recovery (%), *i.e.* the overall PEG collected in digesta samples relative to PEG ingested, were computed. PEG recovery was  $55.6 \pm 1.8\%$  indicating that a relatively large part of the total ileal digesta had been sampled through the tube. Integrated PEG flow was  $102.6 \pm 1.7\%$ ; *i.e.* very close to the expected value (100%). This demonstrated, assuming that dietary PEG flow through the upper digestive tract was complete after 8 h from meal ingestion, that the estimated PEG flows and in turn intestinal fluid flows were accurate. The data support the accuracy of determination of ileal nitrogen flows. Based on the inter-subject variability within and between studies, the intubation method also appeared to be reproducible. In conclusion, the intubation method is a valid method to sample intestinal digesta and determine overall intestinal flows of nitrogen, and could be used further for studying the flow of virtually any ingested or secreted materials in the intestinal lumen.

## INTRODUCTION

Measurement of protein digestibility at the ileal level is now recognized as being more meaningful than measurement at the faecal level (Darragh & Hodgkinson, 2000; Moughan, 2003; Fuller & Tomé, 2005) because, when undigested dietary proteins and unabsorbed dietary amino acids (AA) enter the hindgut, they are profoundly modified by the high metabolic activity of the microflora. Although it remains unclear as to whether the colon may absorb AA (Blachier *et al.*, 2007), most dietary AA are absorbed

in the small intestine (Krawielitzki *et al.*, 1990; Fuller & Reeds, 1998). Therefore, when studying the availability of nitrogen (N) derived from protein foodstuffs under different circumstances, oro-ileal absorption is the main measure used. Although animal models have been developed to allow prediction of ileal protein digestibility for humans, human data, when available, remain the most valuable. In humans, ileal digesta can be collected from subjects with ileostomies (Fuller *et al.*, 1994). The validity of this approach has, however, been questioned in as much as firstly the ileostomates may have altered digestive function, which may vary according to the exact disease that required the ileostomy and the nature of the surgical intervention, and secondly the ileal microflora of ileostomates is known to be more prolific than that of intact subjects (Gorbach *et al.*, 1967; Rowan *et al.*, 1994).

The intubation method has been developed to allow samples of small intestinal digesta from subjects with a normal entire digestive tract so as to assess digestive flows of both nutrients and endogenous components (Schedl & Clifton, 1961; Modigliani *et al.*, 1973). The method relies on a triple-lumen tube that allows (1) a continuous infusion into the small intestine of a solution containing a non-absorbable soluble marker, to estimate the intestinal fluid flow rate, and (2) the simultaneous collection of intestinal contents downstream of the site of infusion. The third thin tube lumen is used to inflate a terminal balloon, after the tube has passed the stomach, to facilitate the progression of the tube in the small intestine. The intubation method has been widely applied and recognized as a useful method for the study of food digestion (Mahé *et al.*, 1992; Champ *et al.*, 1998; Mariotti *et al.*, 1999; Bos *et al.*, 2007). Whether such a method allows for a representative sampling of intestinal digesta is a critical point for judging its accuracy and in particular the accuracy of the determination of the total amount of N that passes the terminal ileum over the collection period.

Few studies, aiming to determine starch digestibility, have attempted to validate the intubation method by quantitatively comparing digesta samples collected in intubated pigs or humans with those obtained from cannulated pigs (Noah *et al.*, 1998) or human ileostomates (Langkilde *et al.*, 1994). None of these methods, however, affords a perfect control.

Over the last few years, the intubation method has been applied by the Human Nutrition laboratory, INRA-AgroParisTech (France) for assessing intestinal dietary, or endogenous, N flows under different circumstances. Several studies have controlled internally the accuracy of the sampling using a non-absorbable marker [polyethylene



glycol (PEG)-4000] added to the meal, but most of these data have not been reported and now constitute a considerable data set. The objective of the present study was to use the latter data to investigate the accuracy of the intubation method for determining intestinal N fluxes.

Data from five studies, including 75 subjects in total, were analysed statistically. Assuming that the oro-ileal transit of dietary PEG is complete within 8 hours, the integrated PEG flow, which is the estimated total amount of PEG flowing through the terminal ileum over the 8-h collection period relative to the total amount of PEG ingested in the meal, provides an indicator of the accuracy of the intestinal fluid flow rates and of the method overall. In addition, an inter-subject comparison of this ratio within and between studies provides information regarding the precision of the intubation method. Finally, the amount of PEG recovered in the digesta samples over the 8-h collection period relative to the total amount of PEG ingested in the meal (marker recovery) indicates the proportion of total intestinal effluent, associated with the dietary flow, sampled through the tube.

## MATERIAL AND METHODS

### *Experimental data*

Data were collected from 5 studies which in total included 75 healthy adult humans from whom ileal digesta were collected using the intubation method (Bos *et al.*, unpublished data; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000; Mariotti *et al.*, 2001; Mariotti *et al.*, 2002). A description of the subjects is given in **Table 1**. All of these studies were undertaken within the same laboratory and followed the same design with the same analytical techniques, but were conducted at different times by different experimenters.

**Table 1.** Characteristics of the subjects included in the different intubation studies<sup>1</sup>.

Study <sup>2</sup>	Meal <sup>3</sup>	n	M/F <sup>4</sup>	Body weight	Age	Tube position
				kg	y	cm <sup>5</sup>
A	DFM	6	n.a. <sup>6</sup>	68	28	193 ± 15
B	MP1	7	13M/10F	n.a.	n.a.	189 ± 30
B	MP2	9		n.a.	29 ± 6	186 ± 43
B	MP3	7		n.a.	n.a.	n.a.
C	SP1	11	7M/4F	72 ± 11	26 ± 5	183 ± 29
C	SP2	9	7M/2F	65 ± 9	28 ± 5	189 ± 42
D	PGA	8	5M/3F	72 ± 15	27 ± 6	192 ± 21
D	PG	11	7M/4F	77 ± 17	28 ± 7	191 ± 22
E	LF	7	7M	79 ± 12	28 ± 7	191 ± 12

<sup>1</sup> Values are mean ± SD.

<sup>2</sup> A, Bos *et al.* (unpublished data); B, Gaudichon *et al.* (1999); C, Mariotti *et al.* (2000); D, Mariotti *et al.* (2001); E, Mariotti *et al.* (2002).

<sup>3</sup> Meals contained: defatted milk (DFM), milk protein (MP1), milk protein + carbohydrates (MP2), milk protein + fat (MP3), soya protein (SP1), soya protein + carbohydrates (SP2), pea protein (globulin and albumin) + carbohydrates + fat (PGA), pea protein (globulin) + carbohydrates + fat (PG), dehulled lupin flour + carbohydrates + fat (LF).

<sup>4</sup> Male/Female.

<sup>5</sup> Distance from the mouth.

<sup>6</sup> Data not available.

### ***Experimental methods***

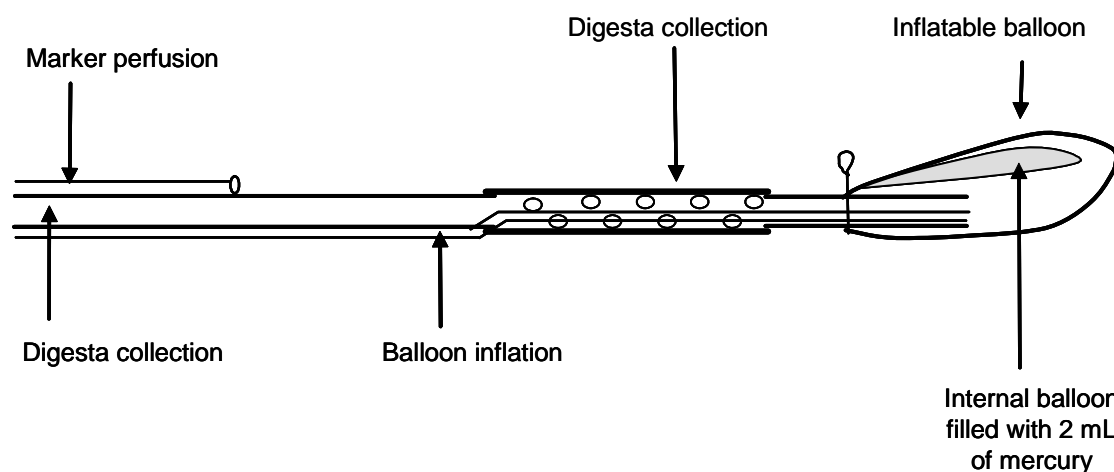
All studies were conducted within the same laboratory (UMR914, INRA, Paris, France) using the same experimental design. In total, 9 meals were tested (**Table 2**). In each test meal, dietary protein had been labelled uniformly and intrinsically with <sup>15</sup>N. Polyethylene glycol (PEG)-4000 was dissolved in each meal and used as an indigestible dietary marker of the liquid phase.

**Table 2.** Composition of the meals tested in the different intubation studies.

Study <sup>1</sup>	Meal	Protein		Carbohydrate		Fat		PEG	Final volume	Energy
		source	g	source	g	source	g	g	mL	kJ
A	DFM	defatted milk	16	defatted milk	20	-		15	480	617
B	MP1	milk protein	30	-		-		12	500	502
B	MP2	milk protein	30	sucrose	100	-		12	500	2173
B	MP3	milk protein	30	-		milk fat	43	12	500	2152
C	SP1	soya protein	30	-		-		15	500	502
C	SP2	soya protein	30	sucrose	100	-		15	500	2294
D	PGA	pea protein (albumin + globulin)	30	maltodextrin sucrose	100 20	sunflower oil	15	15	500	3105
D		PG	pea protein (globulin)	30	maltodextrin sucrose	100 20	sunflower oil	15	15	500
E	LF	dehulled lupin flour	28	lupin flour maltodextrin sucrose	46 100 20	sunflower oil	15	15	500	3853

<sup>1</sup> A, Bos *et al.* (unpublished data); B, Gaudichon *et al.* (1999); C, Mariotti *et al.* (2000); D, Mariotti *et al.* (2001); E, Mariotti *et al.* (2002).

Ileal digesta were collected using a naso-intestinal triple-lumen tube (**Figure 1**). The tube was made of 3 polyvinyl chloride (PVC) tubes, with different internal diameters: a first tube (2.0 mm bore), terminating with a 10-cm pierced (2 mm holes) zone, was used to collect digesta, a second radio-opaque tube (1.5 mm bore) was used to infuse the flow rate marker and ended 20 cm upstream from the collection zone while the third radio-opaque tube (1.0 mm bore) served to inflate the balloon 5 cm downstream from the collection zone.



**Figure 1.** Schematic drawing of the triple-lumen tube used to sample intestinal digesta in conscious humans.

The volunteers were admitted to hospital for 2 days. In the morning of day 1, a 3-m PVC triple-lumen tube was inserted via the nose under local anaesthesia and then swallowed by the subject so as to progress to the terminal ileum. The progression of the tube was facilitated by inflating the terminal balloon after the tube had passed the pyloric valve. The subjects were then given a standard hospital meal and received a second one at 1900 h before fasting overnight. The position of the tube was checked under X-ray. On day 2, the protocol started at 1000 h. A saline solution (NaCl 130 mmol/L, KCl 5 mmol/L, D-mannitol 30 mmol/L) containing a non-absorbable marker of the liquid phase, phenosulphophtalein (PSP), was infused continuously through one of the tube's lumen at a constant rate of 1 mL/min so as to determine the effluent flow rate. After baseline collections of intestinal fluid, the subjects ingested the test meal within a maximum time period of 30 min. The experiment was performed while the subjects were resting in a semi-recumbent position and no other food was ingested until the end of the study period. Water was given hourly.

Digesta samples were collected on ice and pooled for each 30-min period over 8 h postprandially. For each pooled sample, a 4 mL sub-sample was kept frozen (-20°C) for PEG determination and the remainder was freeze-dried and finely ground before being analyzed for PSP, total N and <sup>15</sup>N-enrichment. PEG was determined by turbidimetry, PSP by colorimetry, and total N and <sup>15</sup>N-enrichment using an elemental analyser coupled to an isotope ratio mass spectrometer (EA-IRMS).

The total intestinal fluid flow rate ( $F_{\text{tot}}$ , mL/30 min), derived for each 30-min period, was determined as follows:

$$F_{\text{tot}} = (\text{PSP}_i / \text{PSP}_s) \times F_i \times t$$

where  $\text{PSP}_i$  is the PSP concentration in the infused solution,  $\text{PSP}_s$  the PSP concentration in the digesta sample,  $F_i$  is the PSP infusion rate (1 mL/min), and  $t$  is the duration of the collection period ( $t=30$  min).

Total N flow rate ( $N_{\text{tot}}$ , g/30 min) was determined for each 30-min period using the following equation:

$$N_{\text{tot}} = N_s \times \text{DM}_s \times F_{\text{tot}}$$

where  $N_s$  is the N content in the digesta sample (g/100 g) and  $\text{DM}_s$  the dry matter in the digesta sample (g/100 mL).

Exogenous ( $N_{\text{exo}}$ ) and endogenous ( $N_{\text{endo}}$ ) N flows (g/30 min) were calculated for each 30-min period as follows:

$$N_{\text{exo}} = N \times (E_s - E_0) / (E_{\text{meal}} - E_0)$$

$$N_{\text{endo}} = N_{\text{tot}} - N_{\text{exo}}$$

where  $E_{s(T)}$  the <sup>15</sup>N-enrichment (atom %) in the digesta sample,  $E_{\text{meal}}$  the <sup>15</sup>N-enrichment in the meal and  $E_0$  the basal <sup>15</sup>N-enrichment of the digesta..

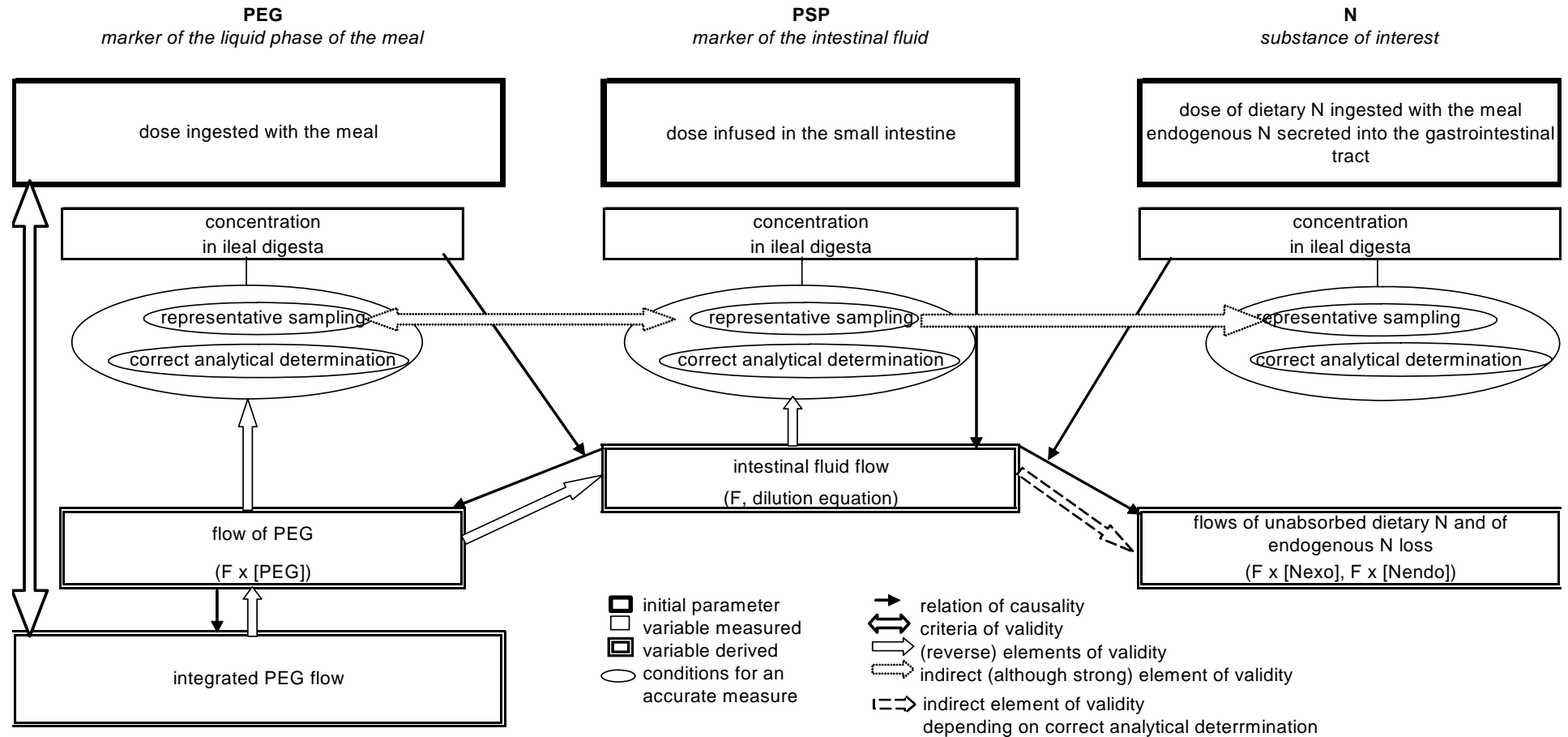
PEG flow (PEGFL, g/30 min) was calculated for each 30-min period from the following equation:

$$\text{PEGFL} = \text{PEG}_s \times F_{\text{tot}}$$

where  $\text{PEG}_s$  is the PEG concentration in the digesta sample (g/mL).

### ***Data analysis and further calculations***

The integrated PEG flow, *i.e.* the estimated total amount of PEG passing the terminal ileum over the 8-h collection period, relative to the total amount of PEG ingested in the meal, provides an indicator of the accuracy of the intestinal fluid flow rates, and thus indirectly of the intestinal N fluxes, as illustrated in **Figure 2**.



**Figure 2.** Rationale for the intrinsic validation of the intubation method. Integrated polyethylene-glycol (PEG)-4000 flow is determined and expressed relative to the dose ingested with the meal. If there is equality between these two parameters, *i.e.* integrated PEG flow (%) is 100%, (1) this proves that PEG flow and intestinal fluid flow ( $F$ ) were correctly determined, hence their conditions for an accurate measurement were met; (2) this indirectly validates the measure of intestinal nitrogen (N) flows. Intestinal fluid flow is determined based on the dilution of the intestinally perfused marker (phenolsuphonphthalein, PSP).

The integrated PEG flow over the 8-h collection period was calculated for each subject as follows:

$$\text{Integrated PEG flow (\%)} = 100 \times \sum \text{PEGFL} / \text{PEG}_{\text{ingested}}$$

where  $\sum \text{PEGFL}$  (mg/8 h) is the cumulative PEG flow over the 8 h postprandial period, derived from the estimated intestinal fluid flow rate and the PEG content in the fluid.  $\text{PEG}_{\text{ingested}}$  was expressed in mg.

The PEG collected in the digesta samples over the 8-h collection period relative to the total amount of PEG ingested in the meal indicates the proportion of digesta that has been sampled from the total intestinal digesta through the tube. This refers to the PEG recovered that was calculated as follows:

$$\text{PEG recovery (\%)} = 100 \times \sum (\text{PEG}_s \times \text{Vol}_s) / \text{PEG}_{\text{ingested}}$$

where  $\text{Vol}_s$  (mL/30 min) and  $\text{PEG}_s$  (mg/mL) are the volume and the PEG concentration in the digesta sample over each 30-min period and  $\sum (\text{PEG}_s \times \text{Vol}_s)$ , expressed in mg/8 h, is the cumulative amount of PEG recovered in the digesta over the 8-h collection period.

### ***Statistical analysis***

All statistical analyses were performed using SAS (version 9.1, SAS Institute Inc, Cary, NC). Differences in PEG recovery and integrated PEG flows among the different dietary treatments were tested for the effect of meal and study using the following general linear model:

$$Y_{ij} = \mu + \alpha_i (\beta_j) + \beta_j + \varepsilon_{ij}$$

where  $\alpha_i$  and  $\beta_j$  represent the effects due to meal and study, respectively. The effect of study using the term III Mean Square (MS) for  $\alpha_i (\beta_j)$  as an error term was reported. The homogeneity of variance for PEG recovery and integrated PEG flows among meals was tested using Bartlett's test. Correlations between tube length and PEG flow or integrated PEG flow were determined using Pearson's coefficient. Differences were considered significant at  $P < 0.05$ .

## RESULTS

Subjects included in the different intubation studies were similar in body weight and age (Table 1). The mean length of tube inserted ranged from 182 to 195 cm with a 95% confidence interval. The coefficients of variation for length of tube inserted ranged from 6 to 23% across meals. There was no significant correlation ( $P > 0.05$ ) between tube length and amount of PEG collected or integrated PEG flow. Flows of intestinal fluid, PEG and endogenous and dietary N as determined in ileal digesta samples over the 8-h postprandial period in the different intubation studies are given in **Figure 3**.

Variances were homogeneous among meals ( $P > 0.05$ ) for the integrated PEG flow with coefficients of variation (CV) ranging from 7 to 18%. The CV between studies was 3%. The integrated PEG flow (relative to the total amount ingested) at the terminal ileum over the 8-h postprandial period did not differ significantly ( $P > 0.05$ ) among studies and meals (**Table 3**) with an overall mean ( $\pm$  SE) of  $102.6 \pm 1.7\%$ .

Variances were homogeneous among meals ( $P > 0.05$ ) for PEG recovery with coefficients of variation ranging from 18 to 37%. The PEG recovered at the terminal ileum over the 8-h postprandial period did not differ significantly ( $P > 0.05$ ) among studies (Table 3) with an overall mean ( $\pm$  SE) of  $55.6 \pm 1.8\%$ .

**Table 3.** Polyethylene glycol (PEG)-4000 recovery and integrated PEG flows in ileal digesta for adult humans 8 h postprandially<sup>1</sup>.

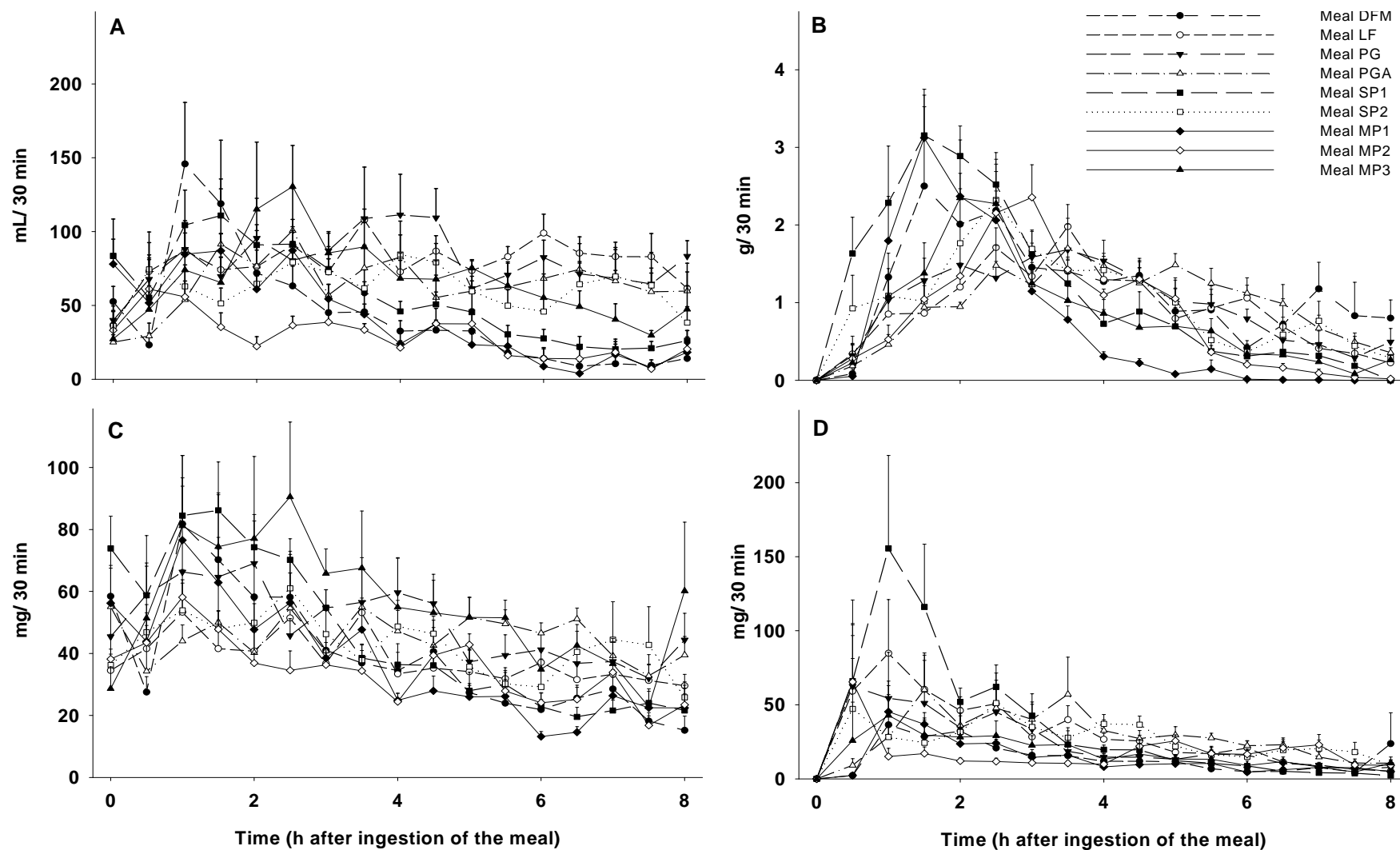
Study <sup>2</sup>	Meal <sup>3</sup>	n	PEG recovery	Integrated PEG flow
			%	%
A	DFM	6	$39.4 \pm 6.0$	$104.9 \pm 5.8$
B	MP1	7	-	$100.1 \pm 4.6$
B	MP2	9	-	$107.3 \pm 2.4$
B	MP3	7	-	$100.7 \pm 4.3$
C	SP1	11	$54.8 \pm 2.9$	$106.2 \pm 3.5$
C	SP2	9	$58.3 \pm 6.1$	$98.2 \pm 5.8$
D	PGA	8	$64.0 \pm 5.0$	$103.8 \pm 4.0$
D	PG	11	$59.4 \pm 4.5$	$100.9 \pm 5.5$
E	LF	7	$51.9 \pm 4.2$	$100.1 \pm 5.6$
Study			NS	NS

<sup>1</sup> Values are means  $\pm$  SEM. NS: non significant ( $P > 0.05$ ).

<sup>2</sup> A, Bos *et al.* (unpublished data); B, Gaudichon *et al.* (1999); C, Mariotti *et al.* (2000); D, Mariotti *et al.* (2001); E, Mariotti *et al.* (2002).

<sup>3</sup> Meals contained: defatted milk (DFM), milk protein (MP1), milk protein + carbohydrates (MP2), milk protein + fat (MP3), soya protein (SP1), soya protein + carbohydrates (SP2), pea protein (globulin and albumin) + carbohydrates + fat (PGA), pea protein (globulin) + carbohydrates + fat (PG), dehulled lupin flour + carbohydrates + fat (LF).





**Figure 3.** Estimated ileal flows of total fluid (A), PEG (B), endogenous N (C) and dietary N (D) as determined across different intubation studies in adult humans. Values are means  $\pm$  SE. Meals contained: defatted milk (DFM), milk protein (MP1), milk protein + carbohydrates (MP2), milk protein + fat (MP3), soya protein (SP1), soya protein + carbohydrates (SP2), pea protein (globulin and albumin) + carbohydrates + fat (PGA), pea protein (globulin) + carbohydrates + fat (PG), dehulled lupin flour + carbohydrates + fat (LF).

## DISCUSSION

The present study reports for the first time the results of a comprehensive intrinsic validation of the intubation method used for sampling intestinal digesta from conscious human subjects. The validation was based on data arising from an internal control (*i.e.* the non-absorbable marker PEG) included in different meals tested within our laboratory.

There are difficulties in externally validating the intubation method as there is no entirely suitable external control. Previous studies following starch digestion have compared the intubation method with the ileostomy model in humans (Langkilde *et al.*, 1994). In humans, the determined quantity of ileal resistant starch was higher (22%) with the intubation technique than with the ileostomy model (Langkilde *et al.*, 1994). This could have resulted from a potentially higher intestinal fluid flow rate with the intubation technique and/or a higher degree of carbohydrate degradation in the ileostomy model (Champ *et al.*, 2003). Compared to cannulated pigs, intubated pigs provided digesta samples relatively representative of the fraction reaching the large intestine, although particles of large size ( $\alpha$ -glucans of a high degree of polymerization) were excluded (Noah *et al.*, 1998). This is unlikely to be an issue in the present studies where homogenous liquid meals were fed to the subjects, and more generally in humans where more efficient chewing reduces digesta heterogeneity.

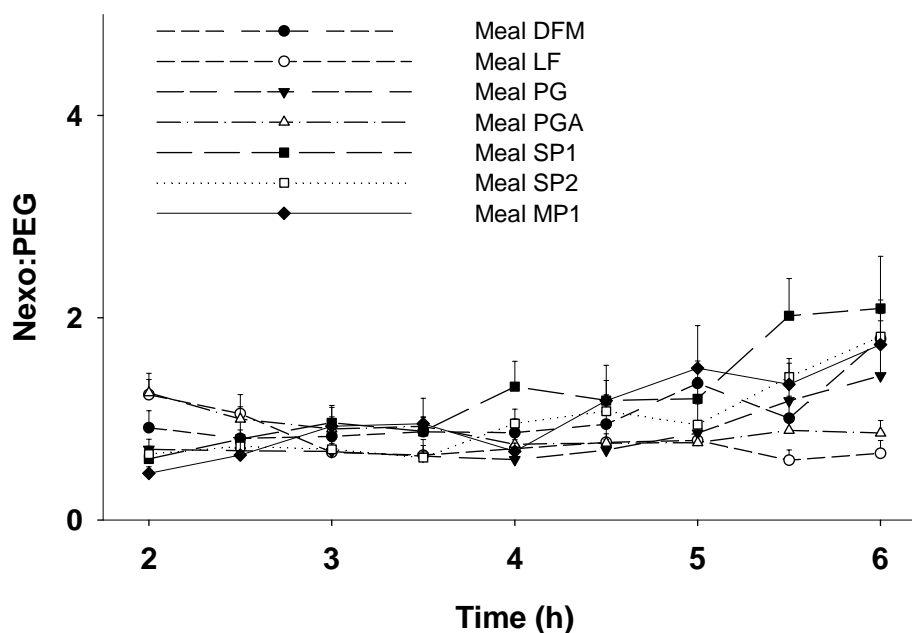
The present data indicate that a relatively large part of the actual flow of ileal effluent (more than 50% in most studies) was sampled through the tube over an 8-h sampling period. This enhances the probability of collecting digesta samples that are representative of the total digesta flowing through the intestinal lumen. The present marker recovery value was higher than that reported previously in a similar study from an independent laboratory (Kerlin & Phillips, 1983) where the PEG recovery ranged from 7 to 30% ( $n = 6$ ). The difference may arise from the structure of the tube (diameter of the tube, length of the aspiration site), although this is difficult to conclude in the absence of detailed description of the tube used in the study of Kerlin & Phillips (1983). The representativeness of the sampling also depends on the homogeneity of the intestinal mix across the intestinal lumen. In humans, pendular and peristaltic movements may favour this mixing (Modigliani *et al.*, 1973). In addition, the method of sampling, which in our case consisted either of collecting the spontaneous flow through

the collection lumen or gently aspirating through a syringe when the flow stopped, contributes to the collection of representative samples.

As illustrated in Figure 2, the integrated PEG flow is a useful indicator to assess accuracy (absence of bias) of the overall methodology, allowing validation of a series of variables (flows of intestinal fluid and of dietary or endogenous N) and conditions (sampling and analytical determination of the PSP and PEG concentrations). Assuming that the collection period used in our studies (8 h) allows for a complete passage of the liquid phase of the meal at the sampling site, the marker of the soluble phase of the meal (PEG), previously reported as an adequate marker (Wilkinson, 1971; Modigliani *et al.*, 1973), should, in theory, be completely recovered in the total digesta (*i.e.* the theoretical integrated PEG flow is 100%). Experimentally, as total digesta collection is not possible, PEG flows are determined from the PEG concentration in the digesta sample and from the ileal effluent flow rates (estimated from the dilution of the infused marker PSP in ileal digesta). Thus, the close agreement observed here between the experimental and theoretical integrated PEG flows ( $102.6 \pm 1.5\%$  versus 100%) demonstrates that PEG flows (*i.e.* PEG concentrations and intestinal flow rates) were determined accurately at least overall. This implies that both PEG and PSP concentrations were accurately determined, evidence of accurate analytical determination, and of a representative sampling for both compounds. The representative sampling for two different ileal compounds (PSP and PEG) strongly indicates that this would also pertain for N, the nutrient of interest. Consequently, and assuming that the analytical determination of N was accurate, the present results demonstrate that overall intestinal N fluxes, when determined using the intubation method, are accurate. It should be noted that an experimental integrated PEG flow close to 100% ( $101.8 \pm 0.1\%$ ,  $n = 6$ ) was also reported by Kerlin & Phillips (1983).

The accuracy of the intubation method overall provides evidence that the individual samples, collected over each 30-min period, were also representative or if not, measurement biases cancelled each other out after integration. This implies that the present kinetics of dietary or endogenous N flows were also likely to be accurate. Additionally, the relatively constant ratio of ileal dietary N to ileal PEG observed from 2 to 6 h after ingestion of the meal (**Figure 4**) suggests that ileal digesta were sampled in a representative manner. Experimental assessment of the validity of 30-min digesta

samples still needs to be performed, for instance by determining the recovery of a fast-transiting compound. This could be undertaken by infusing a bolus of a non-absorbable marker above the sampling site.



**Figure 4.** Ratio of unabsorbed dietary nitrogen (Nexo, % of the total amount recovered over 8 h) to polyethylene-glycol (PEG)-4000 (% of the total amount recovered over 8 h) as recovered in ileal digesta collected across different intubation studies in humans (Bos *et al.*, unpublished data; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000; Mariotti *et al.*, 2001; Mariotti *et al.*, 2002). Values are means  $\pm$  SE. Meals contained: defatted milk (DFM), milk protein (MP1), milk protein + carbohydrates (MP2), milk protein + fat (MP3), soya protein (SP1), soya protein + carbohydrates (SP2), pea protein (globulin and albumin) + carbohydrates + fat (PGA), pea protein (globulin) + carbohydrates + fat (PG), dehulled lupin flour + carbohydrates + fat (LF).

The present data also provide information concerning the precision of the method, although not direct evidence as digesta sampling was not repeated on the same subjects. Despite some variability among individuals (CV for integrated PEG flows within studies: 7–18%), the intubation method appears relatively repeatable across studies. The present data indicate that the intubation method is relatively reproducible (CV for integrated PEG flows among studies: 3%) among the different studies undertaken over more than 4 years by different workers within our laboratory.

A critical assumption in the present study is that all PEG should have transited to the terminal ileum by 8 h following the meal. Eight hours was considered sufficient time to allow complete passage of the liquid phase of the meal. Kerlin & Phillips (1983) reported that the oro-ileal transit of the soluble phase of a complete solid/liquid meal (600 kcal) was essentially complete 5 h after the meal in intubated healthy humans. In

the present work only a small amount of PEG was collected in the last 30 min collection period ( $1.2 \pm 0.2\%$  of total PEG collected over 8 h).

Indirect extrinsic evidence of the validity of the intubation method is provided by the values for ileal digestibility being in line with what is expected in humans based on global knowledge and a large body of data obtained with other methods. This has been fully discussed previously within each study (Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000; Mariotti *et al.*, 2001; Mariotti *et al.*, 2002).

The present study does not evaluate all aspects of the intubation technique and is thus not a complete validation. In particular, the present data do not address possible influences of the sampling tube on the intestinal transit rate of the food. As previously reported, a potential effect of the presence of an ileal tube might be a change in the gastric emptying kinetics, and hence in the kinetics of dietary AA inflow (Mariotti *et al.*, 2002). The results in the literature of the effect of an intestinal tube on gastric emptying of a mixed liquid or solid meal in human subjects are conflicting, reporting either no effect on gastric emptying (Longstreth *et al.*, 1975; Muller-Lissner *et al.*, 1982) or a delay in gastric emptying (Read *et al.*, 1983; Fone *et al.*, 1991; Medhus *et al.*, 1999), although this delay was reported to be followed by a shortening of the small intestinal transit time (Read *et al.*, 1983). However, the transit rate of food has been demonstrated to have no or little impact on overall AA absorption and hence on overall protein digestibility (Huge *et al.*, 1995; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000).

To conclude, the intubation method appears to be a valid method as it allows a precise and accurate sampling of intestinal digesta. Although the influence of the tube on digesta transit is difficult to assess, this remains the best method available to obtain samples of intestinal effluent from healthy humans with an entire digestive tract. The present demonstration was based on studies following the intestinal fate of N, but the validity of the intubation method goes beyond this nutrient of interest. The intubation method is useful for assessing intestinal flows of any intestinal compounds arising either from ingested or secreted materials. The intubation method offers the possibility of assessing the transit rate of different phases of a meal (e.g. using different indigestible markers) in different parts of the digestive tract.

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## CHAPTER VII

# Intact and hydrolysed casein lead to similar ileal endogenous protein flows and similar postprandial retention of dietary nitrogen despite different metabolic effects in adult humans

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We previously demonstrated in animal models that hydrolysed casein did not enhance ileal endogenous protein flows when compared with intact casein. The first aim of the present study was to assess whether a similar result would be found in adult humans. The second objective was to evaluate the impact of the form of delivery of dietary amino acids (peptide- or protein-bound) on postprandial metabolic partitioning and on the overall net nitrogen retention.

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## ABSTRACT

We studied the impact of the form of delivery of dietary amino acids (AA) on ileal endogenous protein flows and on postprandial metabolic orientation and net nitrogen (N) retention. Isotopically labelled  $^{15}\text{N}$ -casein (33–34 g), either in a hydrolysed (HC,  $n = 5$  subjects) or intact (C,  $n = 6$  subjects) form, was included as the sole source of N in a mixed meal (700 kcal) fed to healthy adult humans equipped with a triple-lumen sampling tube in the small intestine. An additional meal containing a free AA mixture (31 g,  $n = 5$  subjects) simulating the AA composition of casein was included. Serine was omitted from the AA mixture to determine directly its ileal endogenous flow. Dietary N kinetics were quantified, by measuring N in intestinal fluid, urine and blood sampled at regular intervals during the postprandial period. Endogenous N and AA flows did not differ ( $P > 0.05$ ) for C and HC with mean respective N flows of 728 and 617 mg/8 h ( $\pm$  pooled SD of 144 mg/8 h). Endogenous serine flow was similar ( $P > 0.05$ ) for C, HC and A [181, 169 and 191 mg/8 h ( $\pm$  56 mg/8 h)]. When compared to intact casein, hydrolysed casein-derived AA underwent more intense catabolism during the early postprandial phase (0–2 h) due to their fast systemic availability but these differences between meals were compensated for over the 8-h period. An hyperinsulinaemia and hyperleucinaemia was associated with HC ingestion that may have translated into an improved postprandial protein anabolism. The postprandial distribution of N of direct dietary origin at 8 h after the meal was similar ( $P > 0.05$ ) for C and HC with respective mean values of 13.4 and 10.6% ( $\pm$  4.0%) in body urea, 6.4 and 6.8% ( $\pm$  2.1%) in urinary urea and ammonia, and 8.6 and 7.6% ( $\pm$  1.7%) in plasma protein. The net postprandial dietary protein utilization [C, HC: 74.4 and 74.0% ( $\pm$  4.7%)] and the postprandial biological value [C, HC: 79.0 and 81.2% ( $\pm$  4.5%)] were similar ( $P > 0.05$ ) for C and HC. In conclusion, both forms of delivery (hydrolysed or intact casein) elicited similar ileal endogenous protein flows and overall nutritional value despite differences in the metabolic fate of the dietary N.

## INTRODUCTION

The form of delivery of dietary amino acids (AA) has been reported to influence their absorption and metabolism and to affect gastric emptying rate and the kinetics of absorption of AA (Moughan *et al.*, 1991; Rerat, 1995; Calbet & Holst, 2004), which in turn influence body protein turnover and potentially nitrogen (N) retention (Collin-Vidal *et al.*, 1994; Boirie *et al.*, 1997; Dangin *et al.*, 2001). Elucidating the modalities and mechanisms responsible for such effects is important to progress our understanding of the complex regulation of body N homeostasis with emphasis on the specific role of dietary protein. In addition, the ingestion of protein may affect gut endogenous protein secretion and reabsorption, thus influencing ileal endogenous protein flows.

The enzyme-hydrolysed protein/ultrafiltration technique was developed as an approach to determine ileal endogenous protein flows in animals and humans (Moughan *et al.*, 1990). It must be considered, however, as to whether the dietary peptides, present in the dietary protein hydrolysate used with this method, may potentiate AA losses from the small bowel (Jansman *et al.*, 2002; Yin *et al.*, 2004; Stein *et al.*, 2007) over and above those found with the corresponding intact protein. In previous controlled studies with intact and hydrolysed casein we found similar ileal endogenous protein flows when determined using animal models (Deglaire *et al.*, 2008; see Chapter V). That there is no effect of peptides *per se* from a dietary protein hydrolysate, however, remains to be demonstrated in humans, and this postulate gave rise to the first objective of the present study.

Several studies (Collin-Vidal *et al.*, 1994; Metges *et al.*, 2000; Daenzer *et al.*, 2001; Dangin *et al.*, 2001) suggest a higher nutritional value for the intact protein (casein) versus the corresponding hydrolysate or free AA mixture. However, this is contradictory to the observation of an absence of an effect on body N balance in long term studies (Moriarty *et al.*, 1985; Yamamoto *et al.*, 1985; Sales *et al.*, 1995) or a reported beneficial effect of hydrolysates versus complete proteins (Poullain *et al.*, 1989). The assessment of the nutritional value of hydrolysed proteins is of practical importance as they are used for infants with recognized or suspected milk allergy (Hernell & Lonnerdal, 2003) or within enteral formulas for patients with compromised digestive capacities (Meier *et al.*, 2006). The second objective of the present study was thus to compare the overall utilization of dietary N from hydrolysed and intact casein.

In the present work,  $^{15}\text{N}$ -casein, either in a hydrolysed or intact form, was included as the sole source of N in a mixed meal fed to healthy volunteers. Ileal digesta were collected through a triple-lumen intestinal tube and ileal endogenous protein flows were determined using the isotope dilution method. The extent of tracer recycling was assessed using the enzyme-hydrolysed protein/ultrafiltration method. An additional meal containing a free AA mixture simulating casein was included in the study. Serine was omitted from the AA mixture to determine directly its ileal endogenous flow. The study allowed an assessment of the impact of the form of delivery of dietary AA on their postprandial partitioning towards deamination or anabolic pathways and subsequent net body N retention.

## SUBJECTS AND METHODS

### *Subjects*

Twenty-six subjects (13 women, 13 men;  $28 \pm 9$  years of age) of mean ( $\pm$  SD) body weight  $66 \pm 12$  kg and of mean ( $\pm$  SD) body mass index  $22.3 \pm 3.3$  kg/m<sup>2</sup> were included in the study after a thorough medical examination and the conduct of routine blood tests. All subjects received detailed information on the protocol and gave their written informed consent to participate in the study. The protocol was approved by the Institutional Review Board for St-Germain-en-Laye Hospital, France. A description of the subjects is given in **Table 1**. There were no statistically significant differences among subjects fed the different test meals for any of the characteristics.

**Table 1.** Subject characteristics<sup>1</sup>.

	Jejunal group <sup>2</sup>		Ileal group <sup>2</sup>		
	C	HC	C	HC	A
Number (F/M) <sup>3</sup>	2F/2M	4F/2M	4F/2M	2F/3M	1F/4M
Age (y)	$27.3 \pm 10$	$28 \pm 8.1$	$35.7 \pm 6.6$	$28.4 \pm 8.5$	$24.6 \pm 8.1$
Body weight (kg)	$63.4 \pm 13.2$	$68.6 \pm 12.6$	$62.5 \pm 12.6$	$64.2 \pm 7.2$	$67.2 \pm 9.2$
BMI <sup>4</sup> (kg/m <sup>2</sup> )	$22.6 \pm 3.4$	$23.2 \pm 3.7$	$22.1 \pm 4$	$21.8 \pm 4.2$	$21.2 \pm 2.3$
Total body water (L) <sup>5</sup>	$38.6 \pm 5.1$	$41.3 \pm 2.6$	$38.1 \pm 4.8$	$39.8 \pm 2.6$	$41.8 \pm 4.3$

<sup>1</sup> Values are means  $\pm$  SD. There were no statistical differences among meals within each group for any of the characteristics.

<sup>2</sup> Group of subjects for whom the triple-lumen was inserted either at the jejunal or ileal level.

<sup>3</sup> F: female, M: male.

<sup>4</sup> Body mass index

<sup>5</sup> Predicted using the equation of Watson *et al.* (1980).

### ***Test meals***

Three semi-synthetic mixed meals were prepared with the respective sole source of N being  $^{15}\text{N}$ -micellar casein (C),  $^{15}\text{N}$ -hydrolysed casein (HC) or free L-AA (A). The three test meals had similar N contents and the C and HC meals provided 320 mmol N while the A meal provided 306 mmol N.

The  $^{15}\text{N}$ -labelling of milk and purification of the dietary protein were performed as described previously (Mahé *et al.*, 1994). Briefly, three lactating cows were perfused ruminally with ammonium sulfate ( $^{15}\text{NH}_4$ ) $\text{SO}_4$  (Eurisotop, St Aubin, France) for 5 days.  $^{15}\text{N}$ -labelled milk was collected five times over three days and then pooled.  $^{15}\text{N}$ -micellar casein was extracted by microfiltration and then purified using water diafiltration. All the processes were carried out at the National Institute of Agronomic Research (Rennes, France). The global  $^{15}\text{N}$  isotopic enrichment was 0.5450 atom% and there was a homogenous distribution of the  $^{15}\text{N}$ -label among individual AA for which enrichment ranged from 0.4942 to 0.5653 atom%. The  $^{15}\text{N}$ -hydrolysed casein was obtained by enzyme hydrolysis of the  $^{15}\text{N}$ -casein. The  $^{15}\text{N}$ -casein was dissolved in deionized water (1:10 w/v) with overnight stirring. The solution was then heated up to 50°C and pancreatin was added with an enzyme-to-substrate ratio of 0.0089 at pH 8.0 for 1 h 50 min at 50°C. The enzyme was inactivated by heating at 85°C for 20 min, and the product was then freeze-dried. The molecular weight profile of the  $^{15}\text{N}$ -labelled hydrolysed casein, determined by HPLC gel filtration (Deglaire *et al.*, 2007), indicated that 21% of the peptides were between 1 and 5 kDa in size, and 79% were less than 1 kDa. The A meal included free crystalline L-AA (Ajinomoto Co. Inc., Kawazaki, Japan) mimicking the AA composition of the  $^{15}\text{N}$ -casein, except that serine was voluntarily excluded, so as to allow direct determination of its ileal endogenous flow.

The test meals contained, as the sole respective sources of N, 33 g of  $^{15}\text{N}$ -labelled casein, 34 g of  $^{15}\text{N}$ -labelled hydrolysed casein or 31 g of the free crystalline L-AA mixture, to which were added 23 g soyabean oil and 92–94 g of carbohydrate (75% as maltodextrin and 25% as sucrose) to make up to a final air-dry weight of 150 g. Each meal was made up with water to reach a final volume of 550 mL. The total metabolisable energy content of each test meal was 700 kcal (2.93 MJ), equivalent to one third of the French daily dietary energy intake (Martin, 2001).

### **Protocol**

The volunteers were admitted to hospital for 2 days. In the morning of day 1, a 3-m PVC triple-lumen tube was inserted via the nose under local anaesthesia and then swallowed by the subject so as to progress down the gastrointestinal tract. The progression of the tube was facilitated by inflating a terminal balloon after the tube had passed the pyloric valve. The subjects were then given a standard hospital meal and received a second meal at 1900 h before fasting overnight. Following the meal the tube was restrained from further movement once it had reached either the jejunum or the terminal ileum to allow for digesta collection at these two intestinal sites. The final tube position was verified under X-ray, with mean tube length measured from the nose being either  $138 \pm 27$  or  $154 \pm 16$  cm, respectively. On day 2, the protocol started at 1000 h, when a saline solution (NaCl 130 mmol/L, KCl 5 mmol/L, D-mannitol 30 mmol/L) containing 20 g/L polyethylene glycol (PEG)-4000 was infused continuously through one of the tube's lumen at a constant rate of 1 mL/min. After baseline collections of intestinal fluid, blood, and urine samples, the subjects ingested the test meal (within 30 min). The study was performed while the subjects were resting in a semi-recumbent position and no other food was ingested until the end of the study period. Water was given hourly. Intestinal fluid, blood, and urine were sampled at regular intervals over the 8-h postprandial period. Digesta samples were collected on ice and pooled over 30-min periods. For each pooled sample, a 4 mL sub-sample was kept frozen ( $-20^{\circ}\text{C}$ ) for PEG determination and the remainder was freeze-dried, ground and stored until analysis. Freeze-dried digesta samples (30 min) were pooled over 8 h for each meal and subjected to analysis for AA and individual AA  $^{15}\text{N}$ -enrichments. In addition, 30-min freeze-dried digesta samples were pooled over 2 h between 0 and 4 h and every hour from 4 to 8 h postprandially for each subject fed meal HC. These pooled digesta were subsequently centrifuged and ultrafiltered with a 10 kDa MW cut-off (Deglaire *et al.*, 2007). N content and  $^{15}\text{N}$ -enrichment were determined in the precipitate and retentate fractions (molecular weight  $> 10$  kDa).

Blood was sampled every 30 min for 3 h and then every hour for the following 5 h. Plasma was immediately separated from whole blood (collected in fluoride-containing tubes) by centrifugation (2000 g, 12 min,  $4^{\circ}\text{C}$ ) and frozen at  $-20^{\circ}\text{C}$  until analysis. Serum was separated from whole blood (collected in dry tubes) after blood coagulation at room temperature and centrifugation (2000 g, 12 min,  $4^{\circ}\text{C}$ ). Total urine was collected every 2 h throughout the 8 h period. Urine samples were stored at  $-4^{\circ}\text{C}$  with thymol crystals and

paraffin added as preservatives to be processed within the next 48 h, or were immediately frozen (-20°C) depending on the chemical analysis.

### ***Analytical methods***

Plasma glucose was measured using a glucose oxidase method (Glucose GOD-DP kit; Kone, Evry, France). Serum urea, urinary creatinine and urinary urea were assayed by an enzymatic method (Dimension automate; Dupont de Nemours, Les Ulis, France). Urinary ammonia was measured by an enzymatic method (Kone automate; Kone, Evry, France).

AA concentrations in deproteinized serum samples were determined using HPLC after separation on a cation exchange resin and postcolumn ninhydrin derivatization (Biotek Instruments Inc., Winooski, VT), as previously described (Bos *et al.*, 2003). Norvaline and amino-guanidopropionic acid were used as internal standards to correct for AA losses occurring during freezing and sample preparation. The standard  $\gamma$ -amino-butyric acid was added just before analysis to control the injection volume. Tryptophan and sulphur AA concentrations were not determined. The concentration of PEG in the digesta was measured using a turbidimetric method (Hyden, 1955). Plasma insulin and glucagon concentrations were analysed using the Luminex system on a Bioplex system 200 (Bio-Rad, Hercules, CA) with the human endocrine Lincoplex kit (Linco Research Inc., Saint Charles, MO).

For isotopic determinations, urea and ammonia were isolated from urine on a Na<sup>+</sup> form of a cation exchange resin (Biorad Dowex AG50-X8, Interchim, Montluçon, France). For urinary ammonia extraction, 7 mL of urine were mixed with 2 mL of resin for 15 min. The supernatant was kept and the resin containing urinary ammonia was washed 5 times with distilled water. The supernatant (2 mL) was mixed with the resin (2 mL) and incubated for 2 h at 30°C in the presence of 20 mL of urease (Sigma-Aldrich, Lyon, France). The resin containing urinary urea-derived ammonia was then washed with distilled water and stored at 4°C for further isotopic determination. For serum AA and urea extraction, the serum proteins were precipitated by mixing 4 mL serum with 100 mg of 5-sulpho-salicylic acid (Prolabo, Paris, France). After centrifugation (2400 g, 25 min, 4°C), the pellet containing the serum proteins was freeze-dried and stored until analysis. The supernatant was kept and buffered at pH 7. The urea was isolated from free AA on 1 mL of resin in the presence of 8  $\mu$ L of urease. After incubation for 2 h at 30°C, the supernatant, containing free AA, was removed and freeze-dried. The resin

containing urea-derived ammonia from serum was washed with distilled water and stored at 4°C. Before isotopic determination, resins were eluted with KHSO<sub>4</sub> (2.5 mol/L). The <sup>15</sup>N:<sup>14</sup>N isotope ratio was determined by isotope-ratio mass spectrometry (Optima, Fisons Instruments, Manchester, UK) in the digesta, urinary urea and ammonia, serum protein, free N and urea. The N contents of the serum proteins and digesta were determined using an elemental N analyser (NA 1500 series 2, Fisons Instruments, Manchester, UK) with atropine as the standard.

### **Calculations**

#### *Intestinal nitrogen flows*

The total intestinal fluid flow rate ( $F_{\text{tot}}$ , mL/30 min), derived for each 30-min period from the dilution of PEG, was determined as follows:

$$F_{\text{tot}} = (\text{PEG}_i / \text{PEG}_s) \times F_i \times t$$

where  $\text{PEG}_i$  and  $\text{PEG}_s$  are the PEG concentrations in the infusion solution and sample, respectively,  $F_i$  is the PEG infusion rate (1 mL/min), and  $t$  is the duration of the collection period (30 min).

The actual intestinal fluid flow rate ( $F$ , mL/30 min), i.e. corrected for the infusion flow, was calculated using the following equation:

$$F = F_{\text{tot}} - F_i \times t$$

The jejunal and ileal total N flow rates ( $N_{\text{tot-digesta}}$ , mmol N/30 min) were derived from the following formula:

$$N_{\text{tot-digesta}} = (N_s \times \text{DM}_s \times F_t) / 14$$

where  $N_s$  is the N content (mg/g) measured in the freeze-dried digesta,  $\text{DM}_s$  is the dry matter of the sample (g/mL) and 14 is the molecular mass of N (mg/mmol).

Dietary and endogenous N flow rates ( $N_{\text{exo-digesta}}$ ,  $N_{\text{endo-digesta}}$ , mmol N/30 min) were determined for subjects fed C and HC diets according to the following equations:

$$N_{\text{exo-digesta}} = N_{\text{tot-digesta}} \times (E_s - E_0) / (E_{\text{meal}} - E_0)$$

$$N_{\text{endo-digesta}} = N_{\text{tot-digesta}} - N_{\text{exo-digesta}}$$

where  $N_{\text{tot-digesta}}$  is the total N flow rate,  $E_s$  is the <sup>15</sup>N-enrichment (expressed as atom %) in the freeze-dried digesta,  $E_{\text{meal}}$  the <sup>15</sup>N-enrichment in the meal and  $E_0$  the basal <sup>15</sup>N-enrichment of the digesta.

Ileal endogenous N flows (mg/8 h) were determined by summing  $N_{\text{endo-digesta}}$  for the ileal digesta samples collected over the 8-h postprandial period. Ileal endogenous AA flows



(mg/8 h) were calculated based on the AA concentrations and their individual  $^{15}\text{N}$ -enrichments determined in the pooled digesta over the 8-h postprandial period and using the general equations detailed above.

Daily ileal endogenous N losses were estimated assuming that endogenous N recovered in the ileal digesta would be irreversibly lost to the body and that the experimental meal represented one-third of the daily food intake. The 8-h ileal endogenous N flows were multiplied by a factor 3.

### *Tracer recycling*

$^{15}\text{N}$  recovered in the digesta was assumed to trace unabsorbed dietary N. However, some  $^{15}\text{N}$  was detected in the  $> 10$  kDa fraction of the ultrafiltered digesta (the precipitate + retentate), suggesting that some dietary N had been absorbed, incorporated into protein and then recycled into the gut lumen. The latter  $^{15}\text{N}$  would be falsely considered as unabsorbed dietary N tracer. An estimate of the amount of  $^{15}\text{N}$ -labelled endogenous protein present due to tracer recycling was calculated based on the  $^{15}\text{N}$ -measurements in the endogenous N as determined in the ultrafiltered digesta and was expressed as a proportion of endogenous N (R, %) as follows:

$$R = 100 \times (E_{\text{UFS}} - E_0) / (E_{\text{meal}} - E_0)$$

where  $E_{\text{UFS}}$  is the  $^{15}\text{N}$ -enrichment in the ultrafiltered (molecular weight  $> 10$  kDa) digesta (meal HC).

### *Incorporation of N into N body pools*

The time course of dietary N incorporation into the pools of serum free AA, serum protein, body urea, urinary urea and ammonia ( $N_{\text{exo-pool}}$ , % of ingested N), was evaluated for C and HC groups using the following general equation:

$$N_{\text{exo-pool}} = 100 \times [N_{\text{tot-pool}} \times (E_s - E_0) / (E_{\text{meal}} - E_0)] / N_{\text{ingested}}$$

where  $N_{\text{tot-pool}}$  is the N content of the pool (mmol N) at the collection time,  $E_s$  the  $^{15}\text{N}$  enrichment of the sample, and  $N_{\text{ingested}}$  is the N ingested within the meal (mmol).  $N_{\text{exo-pool}}$  was expressed as % of N ingested. For urinary urea,  $N_{\text{tot}}$  was calculated as the product of the urinary urea N concentration and the volume of urine excreted.  $N_{\text{tot}}$  in the serum protein pool was determined as the serum concentration of protein N multiplied by the serum volume, estimated to be 5% of body weight (Ganong, 1969).

The N in the body urea pool was calculated assuming that urea was uniformly distributed throughout the total body water (TBW) and according to the following equation:

$$N_{\text{body urea}} = C_{\text{urea}} \times \text{TBW} / 0.92$$

where  $C_{\text{urea}}$  is the urea N concentration in the plasma sample at the collection time and 0.92 is the correction factor for the water content of plasma. TBW was determined according to the equation of Watson *et al.* (1980).

#### *Urea production*

Total urea production levels (mmol N/kg/2 h) were calculated for the C, HC and A groups for the four 2-h periods following meal ingestion by summing the urinary urea N excreted and the increase in body urea N for each period. Dietary and endogenous urea productions were determined for the C and HC groups.

#### *Net postprandial protein utilization and postprandial biological value*

At the end of the 8-h experimental period, the amount of dietary N retained in the body, or net postprandial protein utilization (NPPU, % of ingested N), was calculated as follows:

$$\text{NPPU} = [N_{\text{ingested}} - \sum N_{\text{exo-ileal}} - \sum N_{\text{exo-urinary}} - N_{\text{exo-body urea}} (8 \text{ h})] / N_{\text{ingested}}$$

where  $\sum N_{\text{exo-ileal}}$  is the cumulative recovery over 8 h of dietary N collected in ileal digesta,  $\sum N_{\text{exo-urinary}}$  is the cumulative recovery over 8 h of dietary N incorporated into urinary ammonia and urea and  $N_{\text{exo-body urea}} (8 \text{ h})$  is the dietary N incorporated into body urea at 8 h.

The postprandial biological value (PBV, % of ingested N) was calculated as the relative amount of dietary N absorbed that was not deaminated during the postprandial period:

$$\text{PBV} = (\text{NPPU}/\text{TID}) \times 100$$

where TID is the true ileal digestibility (% of ingested N) determined from the cumulative recovery of dietary N in ileal digesta ( $\text{TID} = 100 \times [N_{\text{ingested}} - \sum N_{\text{exo-digesta}}] / N_{\text{ingested}}$ ).

#### *Area under the curve*

The area under the curve (AUC) was computed for the concentrations of serum AA and plasma glucose, insulin and glucagon determined from 0 to 8 h postprandially and referred to as the area between the curve and the baseline.

### *Statistical analysis*

All statistical analyses were carried out using SAS (version 8.2, SAS Institute Inc., Cary, NC). Differences among the test meals for subject characteristics, ileal endogenous flows of AA and N (mg/8 h), NPPU, PBV and AUC were tested by a one-way analysis of variance using a general linear model. Differences between urea production and excretion within each group was tested using a paired t-test. Differences over time for dietary  $^{15}\text{N}$  recycling (meal HC) were tested using a repeated-measures analysis of variance with a mixed model. Differences among the test meals during the 8-h postprandial period were tested using a repeated-measures analysis of variance with a mixed model. For each variable, 6 different covariance structures for random statements (Compound Symmetry, Heterogeneous Compound Symmetry, Unstructured, Auto Regressive 1, Auto Regressive Moving Average 1,1 and Toeplitz) were tested, and the most appropriate structure was selected based on the objective criteria. *Post hoc* tests were performed using linear contrast estimates. A P-value < 0.05 was considered as statistically significant.

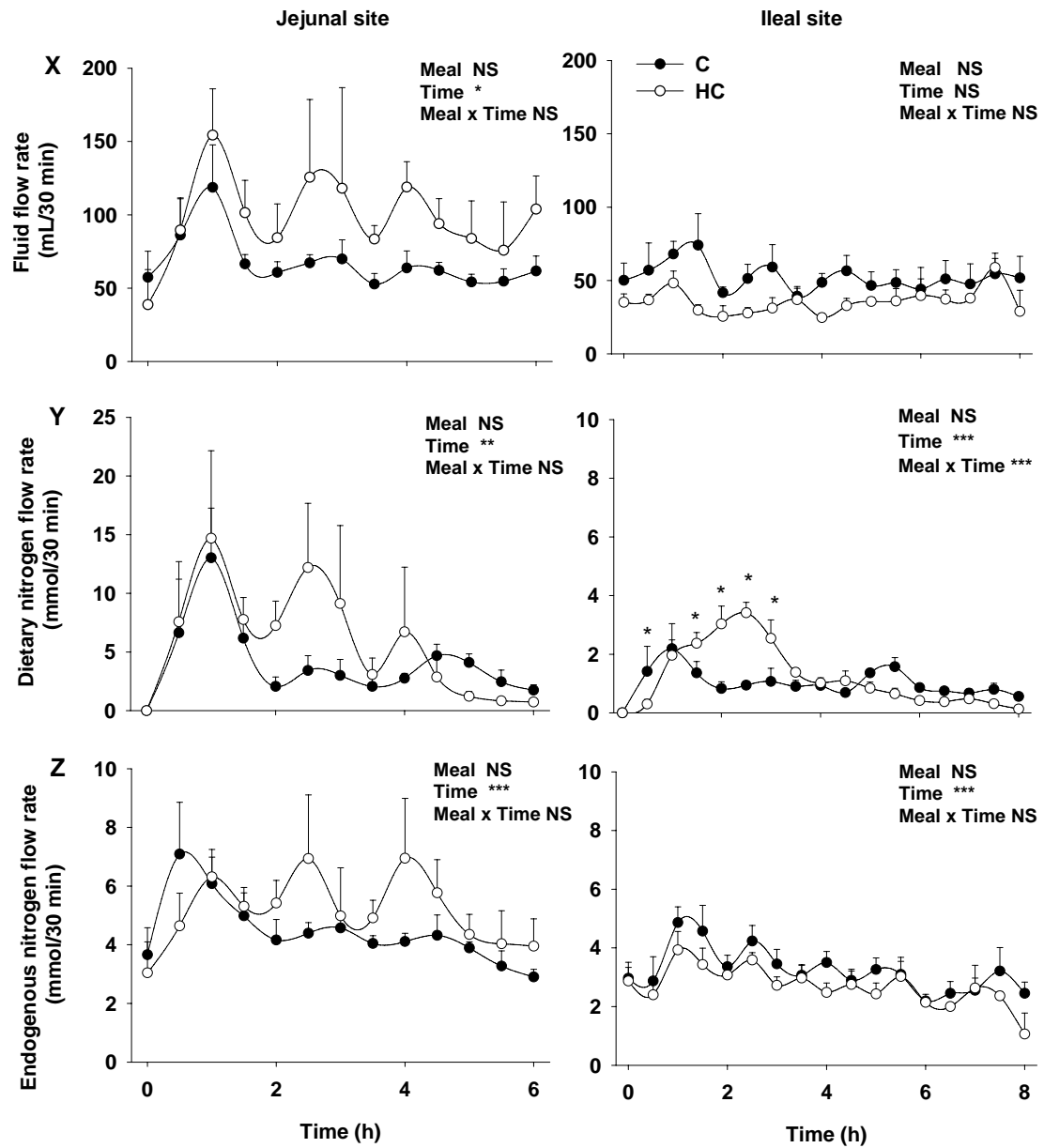
### *Curve fittings*

Different curvilinear models were used to fit the amount of dietary N incorporated into body urea (1), plasma amino acids (2), plasma proteins (3) and urinary urea or ammonia (4) during the postprandial period. The curve took the form  $y = a \cdot (t^b \cdot e^{-ct})$  for (1) and (2) and the form  $y = a \cdot (1 - e^{-bt})^c$  for (3) and (4), where t is time, a, b and c are estimated regression constants. Curve fittings were performed using Sigma Plot 10 (version 10.0, Systat Software Inc., Chicago, IL).

## **RESULTS**

### *Endogenous and dietary nitrogen in intestinal digesta*

The jejunal and ileal fluid flow rates did not differ significantly ( $P > 0.05$ ) among subjects fed meals C, HC (**Figure 1.X**) and A (data not shown). Dietary N flow rates (**Figure 1.Y**) did not differ significantly ( $P > 0.05$ ) between subjects fed meals C and HC when determined at the jejunal site, but were significantly higher ( $P < 0.05$ ) for HC at the ileal site at around 3 h postprandially. Jejunal and ileal endogenous N flow rates (**Figure 1.Z**) were similar ( $P > 0.05$ ) between subjects fed meals C and HC for the entire postprandial period, but were significantly higher ( $P < 0.001$ ) than the basal value at time 0.5, 1, 1.5 h and 1, 1.5, 2.5 h, respectively (statistical significance not shown). The overall average daily ileal endogenous N loss was estimated to be  $2026 \pm 441$  mg/d.



**Figure 1.** Postprandial kinetics of actual intestinal fluid flow rate (X) and dietary (Y) and endogenous (Z) nitrogen flows in the jejunum (C, n = 4; HC, n = 4) and ileum (C, n = 6; HC, n = 5) after ingestion by adult humans of a single mixed meal containing intact casein (C) or hydrolysed casein (HC). Values are means ± SD. Time is given as hours from ingestion of the test meal. \*, values significantly different (P<0.05) from each other at a given time. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported for each variable in the insert with \*\*\*, P < 0.001; \*\*, P < 0.01; \*P < 0.05; NS, non significant.

Ileal endogenous AA flows over the 8-h period (**Table 2**) were not significantly different ( $P > 0.05$ ) between subjects fed meals C and HC. The mean endogenous flow of serine (Table 2) did not differ ( $P > 0.05$ ) among subjects fed meals C, HC and A. In addition, similar ( $P > 0.05$ ) ileal total (endogenous and dietary) N flow rates (**Figure 2**) were observed for meals C, HC and A.

**Table 2.** Ileal endogenous amino acid and nitrogen flows in adult humans fed a single mixed meal containing intact casein (C,  $n = 6$ ), hydrolysed casein (HC,  $n = 5$ ) or free L-amino acids (A,  $n = 5$ )<sup>1</sup>.

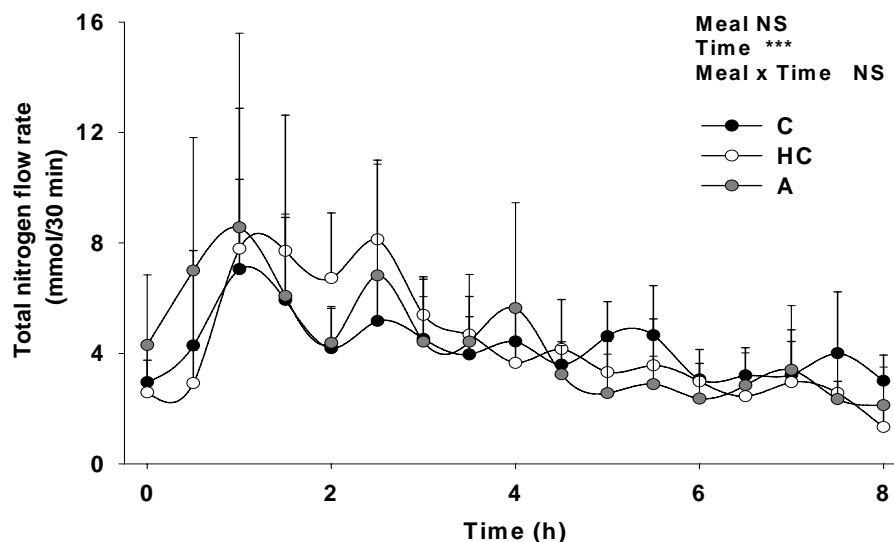
Amino acid	Meal			Pooled SD <sup>2</sup>	Significance
	C	HC	A		
		<i>mg/8 h</i>			
Threonine	189	186	-	60	NS <sup>3</sup>
Valine	157	152	-	56	NS
Isoleucine	136	126	-	40	NS
Leucine	184	172	-	59	NS
Phenylalanine	104	104	-	42	NS
Tyrosine	131	128	-	46	NS
Lysine	118	117	-	51	NS
Histidine	107	122	-	32	NS
Glutamic acid	260	235	-	90	NS
Proline	156	147	-	48	NS
Glycine	399	326	-	141	NS
Alanine	120	116	-	40	NS
Aspartic acid	289	289	-	98	NS
Serine	181	169	191	56	NS
Nitrogen	728	617	-	144	NS

<sup>1</sup> Values are means. For C and HC the values are based on measurement of <sup>15</sup>N while for A the value for serine is a direct value.

<sup>2</sup> Root Mean Square Error.

<sup>3</sup> Non significant ( $P > 0.05$ ).

The amount of tracer recycled within endogenous protein (R) from subjects fed meal HC (**Table 3**) was significantly lower ( $P < 0.05$ ) over 0–2 h compared with 2–5 h postprandially. For the values obtained between 2 and 8 h, for which there were no statistically significant differences, R was on average  $11.3 \pm 2.2$  %.



**Figure 2.** Postprandial kinetics of digesta total N flows in the ileum of subjects equipped with intestinal tubes after the ingestion of a mixed meal containing intact casein (C, n = 6), hydrolysed casein (HC, n = 5) or free amino acids (A, n = 5). Values are means ± SD. Time is given as hours from ingestion of a test meal. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported in the insert with \*\*\*, P < 0.001; NS, non significant.

**Table 3.** Estimate of dietary <sup>15</sup>N recycled within gut endogenous protein (R, %, molecular weight >10 kDa) determined in ileal digesta after ingestion of a single mixed meal containing hydrolysed casein (HC, n = 5) in adult humans<sup>1</sup>.

Time	R
h	%
0-2	8.7 ± 2.4 <sup>a</sup>
2-4	11.9 ± 1.7 <sup>b</sup>
4-5	11.7 ± 2.1 <sup>b</sup>
5-6	10.7 ± 1.7 <sup>ab</sup>
6-7	11.4 ± 2.3 <sup>ab</sup>
7-8	10.8 ± 3.7 <sup>ab</sup>

<sup>1</sup> Values are means ± SD. Time effect was tested using a mixed model for repeated measures. Values with superscripts without common letters were significantly different (P < 0.05).

**Kinetics of plasma glucose, insulin and glucagon**

There was a significant (P < 0.05) meal x time interaction for plasma glucose concentration (**Figure 3.X**). Plasma glucose concentrations differed significantly (P < 0.05) among meals (C, HC and A) at time 1, 3, 4 and 5 h postprandially. In particular, the peak of glucose concentration for meal HC (1 h postprandially) was higher (P < 0.05) than that for meals C and A at the same time, whereas at the later times (3 and 4 h) glucose concentration was higher for meal A than for meal C and HC, respectively. The overall AUC were, however, similar (P > 0.05) among meals.

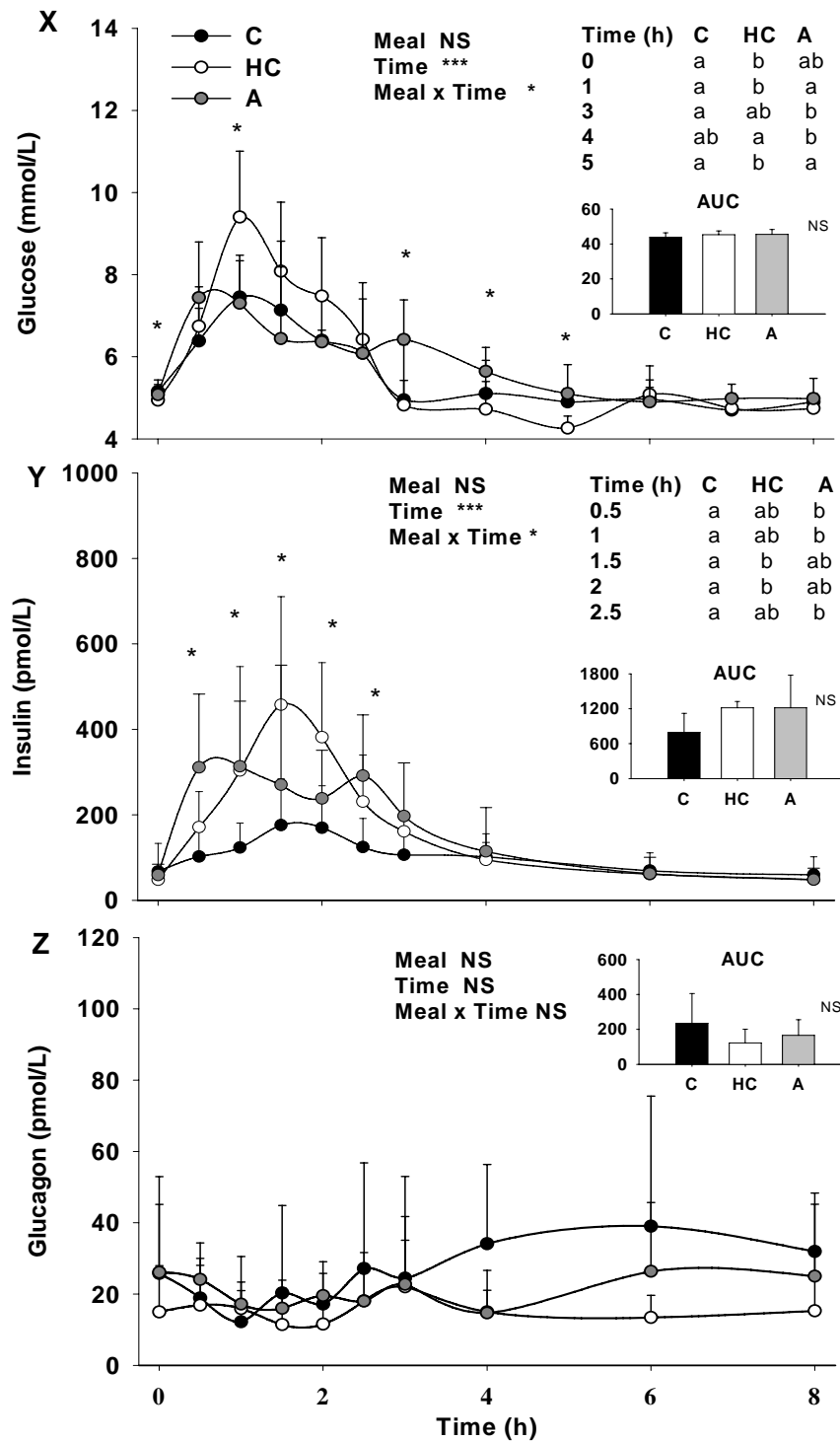
Similarly, there was a significant ( $P < 0.05$ ) meal  $\times$  time interaction for plasma insulin concentration (**Figure 3.Y**). Plasma insulin concentrations differed significantly ( $P < 0.05$ ) among meals from 0.5 to 2.5 h postprandially. In particular, the insulin concentration for meal HC (1.5 h postprandially) was higher ( $P < 0.05$ ) than that for meal C at the same time. However, the AUC did not differ significantly ( $P > 0.05$ ) among meals. Plasma glucagon concentrations were not significantly different ( $P > 0.05$ ) among meals C, HC and A (**Figure 3.Z**).

#### ***Kinetics of plasma AA***

The kinetics of the plasma AA concentrations are presented in **Figure 4**. There was a significant ( $P < 0.05$ ) meal  $\times$  time interaction. The total plasma AA concentrations did not differ significantly ( $P > 0.05$ ) for the first 3.5 h, but were significantly higher ( $P < 0.05$ ) from 4 to 7 h postprandially for meal C compared with meals HC and A. A similar trend was observed for the dispensable AA. In contrast, the indispensable AA concentrations for meals HC and A were significantly higher ( $P < 0.05$ ) than that for meal C for the first 3 h, but decreased thereafter. The indispensable AA concentrations for C observed a bimodal profile with subsequent increase and decrease. Finally, a similar ( $P > 0.05$ ) concentration of indispensable AA was reached among meals C, HC and A at 8 h. A similar trend was observed for the branched-chain AA and especially for leucine. The AUC did not differ significantly ( $P > 0.05$ ) among meals for any AA category (total, indispensable, dispensable and branched-chain).

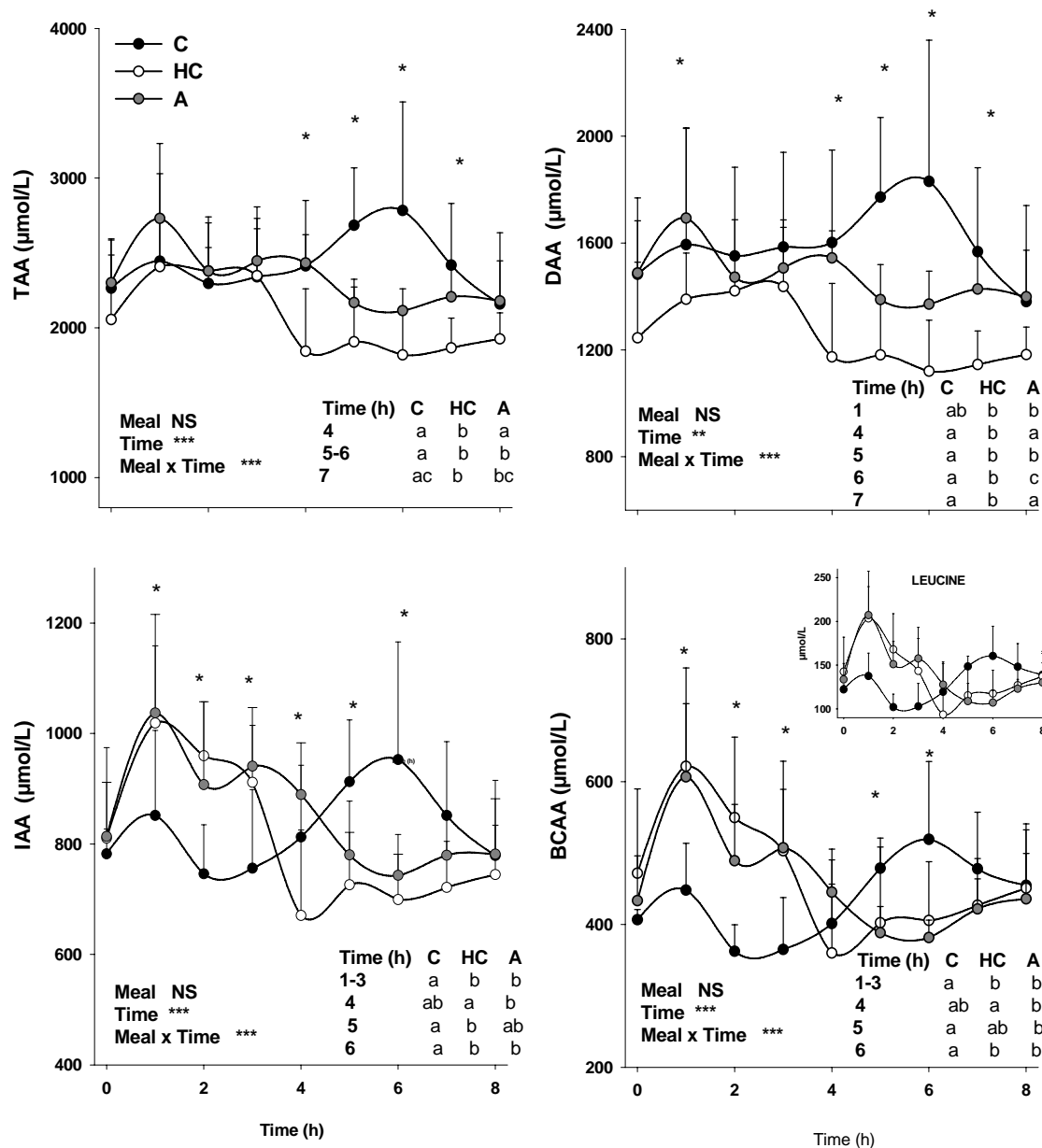
#### ***Dietary N incorporation into plasma free AA and protein pools***

The dietary N incorporation into the plasma free AA pool (**Figure 5.X**) reached a plateau between 2 and 3 h postprandially for HC (predicted value of 0.24% of ingested N), while this occurred between 6 and 7 h postprandially for C (predicted value of 0.22% of ingested N). The dietary N incorporation into the plasma protein pool (**Figure 5.Y**) was faster ( $P < 0.05$ ) for HC than for C, especially between 3 and 4 h postprandially, but was similar ( $P > 0.05$ ) between meals at 8 h postprandially, with an average predicted value of 7.9% of ingested N.

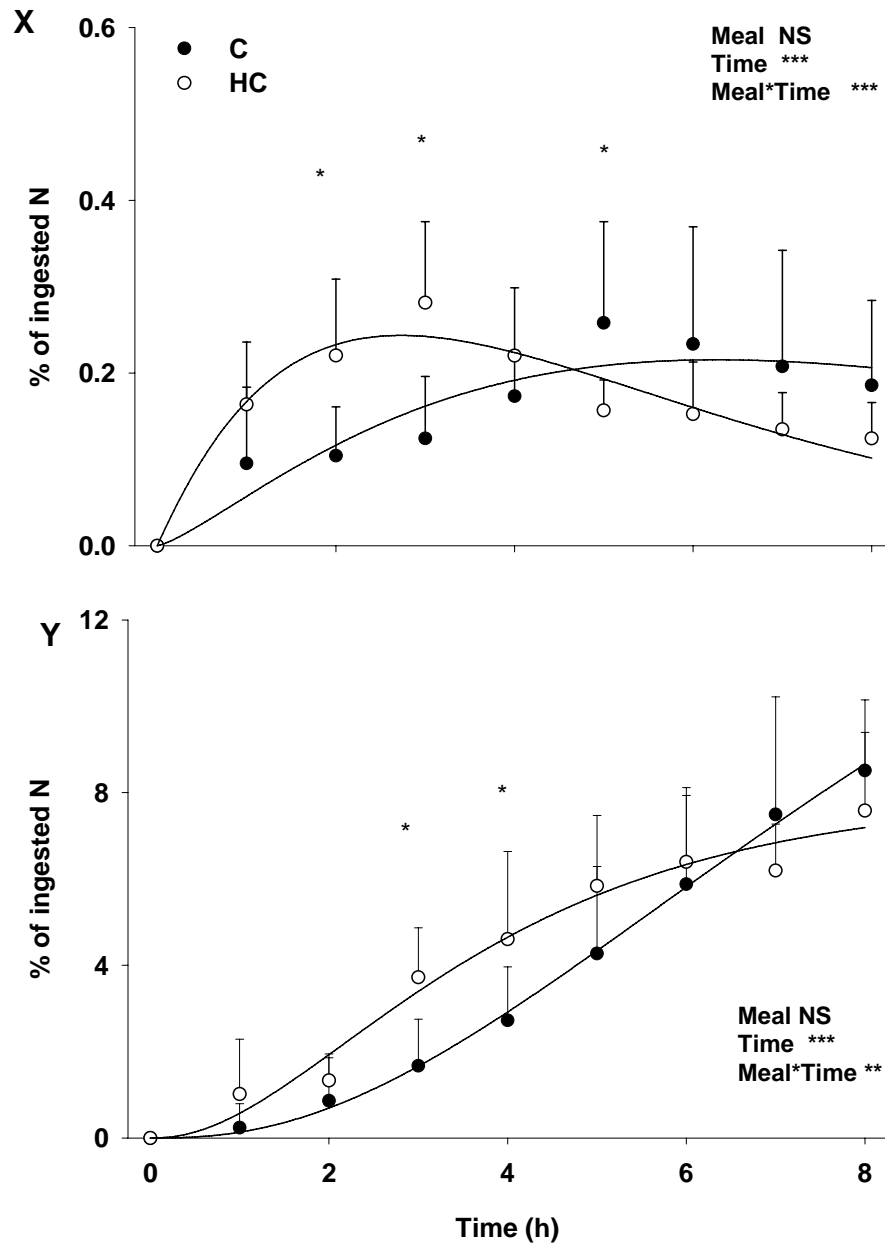


**Figure 3.** Plasma concentrations of glucose (X), insulin (Y) and glucagon (Z) after the ingestion of a mixed meal containing intact casein (C, n = 6), hydrolysed casein (HC, n = 5) or free amino acids (A, n = 5) in adult humans. Values are means ± SD. Time is given as hours from ingestion of the meal. \*, values significantly different (P<0.05) from each other at a given time with differences (P<0.05) among meals reported in the insert. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported for each variable in the insert with \*\*\*, P < 0.001; \*P < 0.05; NS, non significant. The area under the curve and above the baseline (AUC) is presented for each variable. NS: non significant (P>0.05).





**Figure 4.** Plasma concentrations of total (TAA), dispensable (DAA), indispensable (IAA) and branched-chain (BCAA) amino acids after ingestion of a mixed meal containing intact casein (C, n = 6), hydrolysed casein (HC, n = 5) or free amino acids (A, n = 5) in adult humans. Values are means ± SD. Time is given as hours from ingestion of the test meal. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported for each variable. \*, values significantly different (P<0.05) from each other at a given time with differences (P<0.05) among meals reported in the insert.



**Figure 5.** Incorporation of dietary nitrogen into plasma free amino acids (X) and proteins (Y) after ingestion of a mixed meal containing intact casein (C, n = 6) or hydrolysed casein (HC, n = 5) in adult humans. Values are means ± SD. Time is given as hours from ingestion of the test meal. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported for each variable. \*, values significantly different (P<0.05) from each other at a given time.

### ***Dietary N deamination and urea production***

Kinetics of dietary N incorporation into body urea and cumulative excretion into urinary urea and ammonia are presented in **Figure 6**. The incorporation of dietary N for meal HC into body urea increased faster ( $P < 0.05$ ) than that for meal C and reached a plateau between 3 and 4 h (predicted value of 15.2% of ingested N). Dietary N from the C meal was transferred to body urea more slowly and reached a maximum at 8 h (predicted value of 14.1% of ingested N). Cumulative dietary N excretions as urea and ammonia were similar ( $P > 0.05$ ) for the C and HC meals.

Total urea production (**Figure 7**) was significantly higher ( $P < 0.05$ ) for the HC and A meals between 0 and 2 h postprandially, and decreased more rapidly for meal A, with values significantly lower ( $P < 0.05$ ) between 4 and 8 h compared to meal C.

Compared to meal C, urea production of direct dietary origin (**Figure 8**) for meal HC was significantly higher ( $P < 0.05$ ) between 0 and 2 h but significantly lower ( $P < 0.05$ ) between 4 and 6 h and was thus similar ( $P < 0.05$ ) over the entire 8-h period. The 8-h production of urea of direct dietary origin for meal HC was significantly higher ( $P < 0.001$ ) by  $25 \pm 12\%$  than the 8-h urinary urea excretion, whereas these two parameters were similar ( $P > 0.05$ ) for meal C (data not shown). The endogenous urea production was not significantly different ( $P > 0.05$ ) between meals C and HC or between collection times.

### ***Postprandial N retention and biological value***

Protein quality measures are shown in **Table 4**. NPPU and PBV were not significantly different ( $P > 0.05$ ) for meals C and HC.

**Table 4.** Postprandial metabolic utilization of dietary nitrogen (N) 8 h after ingestion of a single mixed meal containing intact casein (C,  $n = 6$ ) or hydrolysed casein (CH,  $n = 5$ ) in adult humans<sup>1</sup>.

	% ingested N		Pooled SD <sup>2</sup>	Significance
	C	HC		
Ileal loss of dietary N <sup>3</sup>	5.9	7.7	1.4	NS <sup>4</sup>
Deamination of dietary N in body urea <sup>5</sup>	13.4	10.6	4.0	NS
urinary urea and ammonia <sup>3</sup>	6.4	6.8	2.1	NS
Total loss of dietary N at 8 h	25.6	26.0	4.7	NS
Net postprandial protein utilization	74.4	74.0	4.7	NS
Postprandial biological value	79.0	81.2	4.5	NS

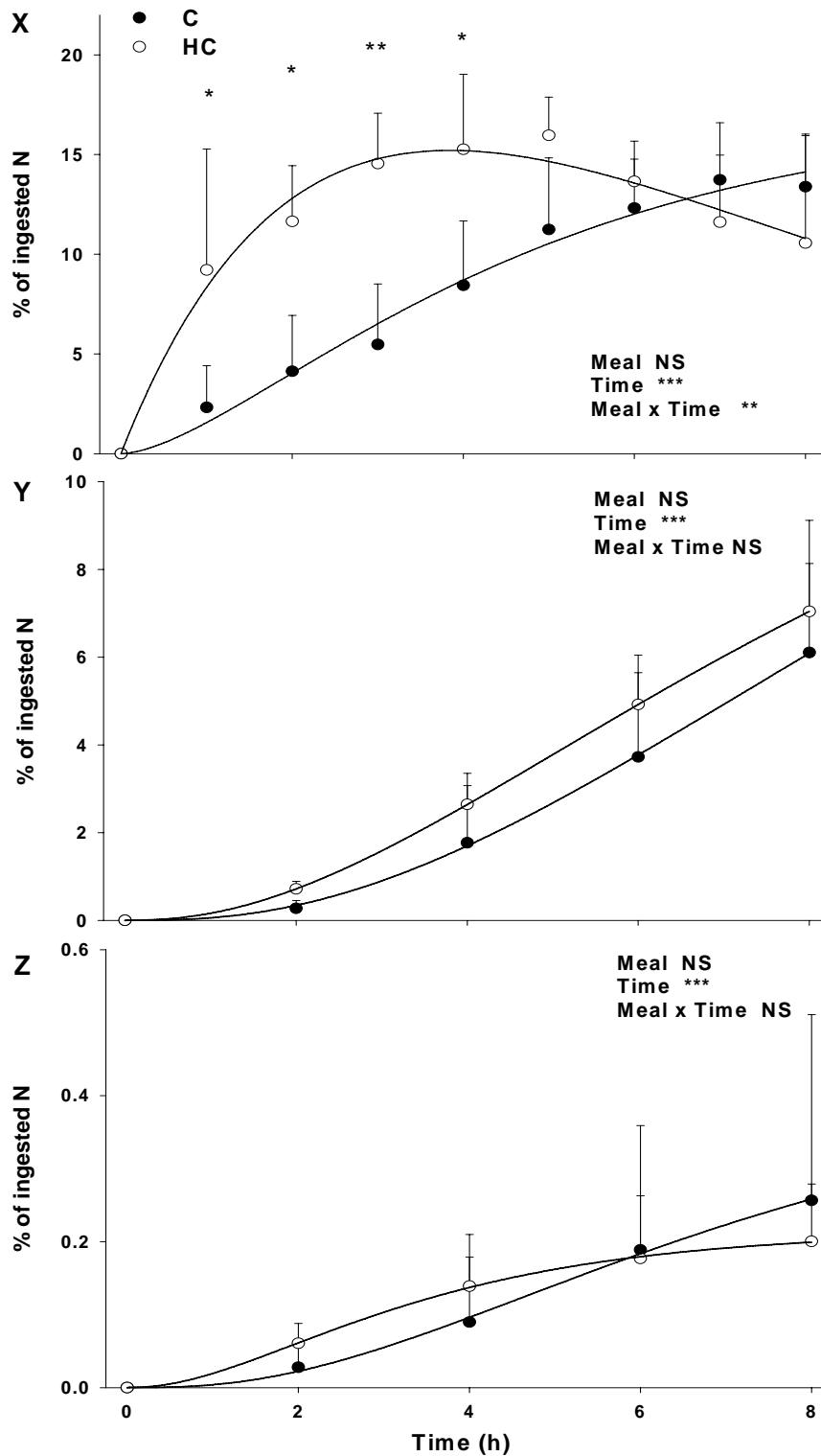
<sup>1</sup> Values are means.

<sup>2</sup> Root Mean Square Error.

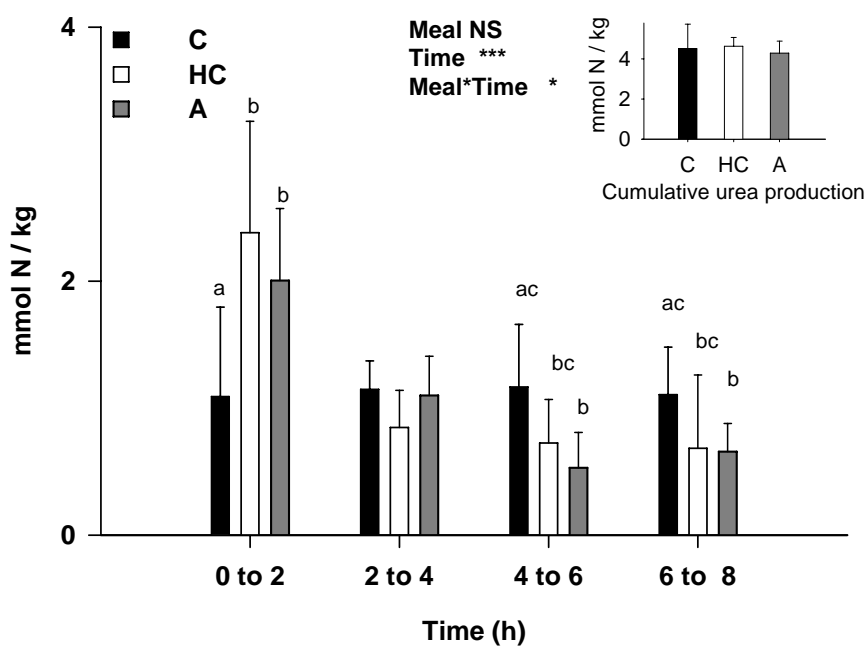
<sup>3</sup> Cumulative losses over 8 h.

<sup>4</sup> Non significant ( $P > 0.05$ ). For ileal loss of dietary N,  $P = 0.06$ .

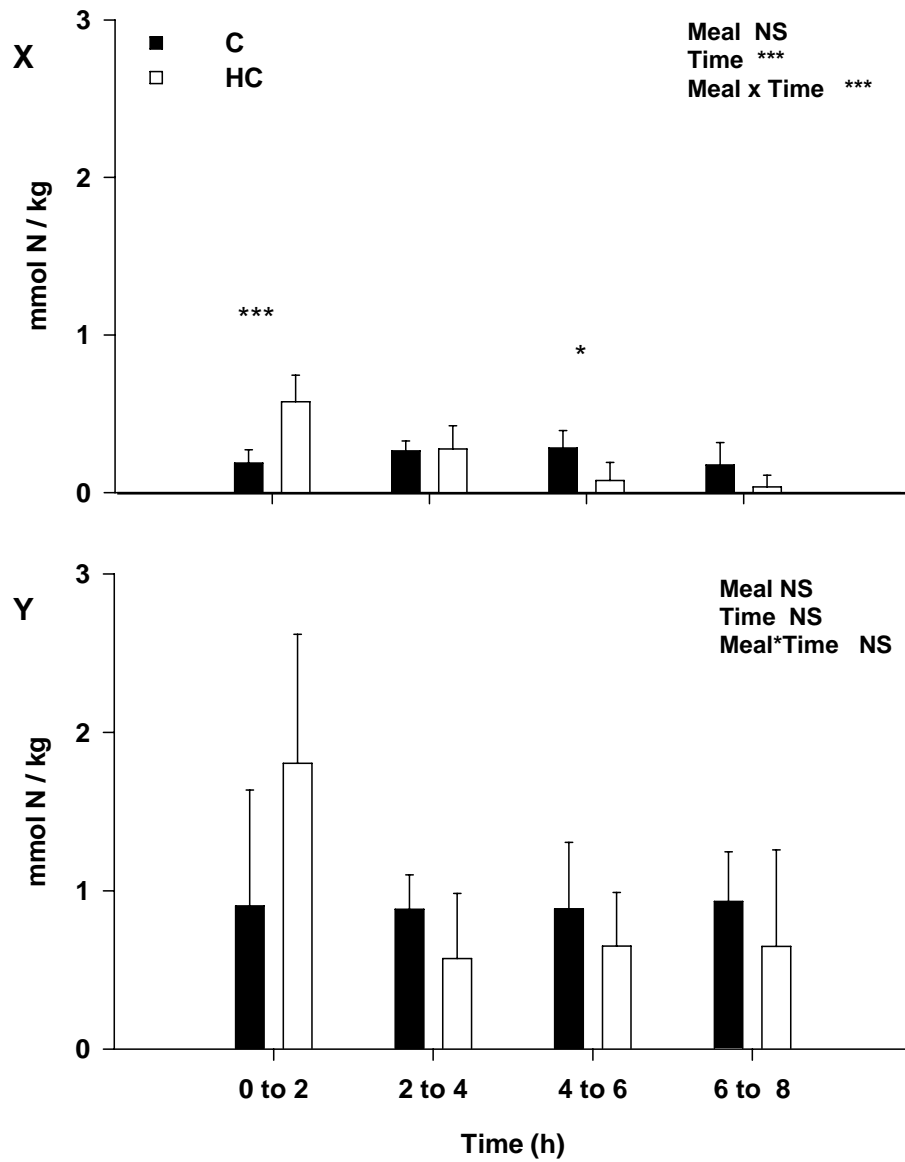
<sup>5</sup> Body urea N of dietary origin at 8 h.



**Figure 6.** Dietary nitrogen incorporation into body urea (X), cumulative excretion of urinary urea (Y) and cumulative excretion of urinary ammonia (Z) after the ingestion of a mixed meal containing intact casein (C, n = 6) or hydrolysed casein (HC, n = 5) in humans. Values are means ± SD. Time is given as hours from ingestion of the test meal. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported for each variable. \*, values significantly different (P < 0.05) from each other at a given time.



**Figure 7.** Rates of production of total urea after ingestion of a mixed meal containing intact casein (C,  $n = 6$ ), hydrolysed casein (HC,  $n = 5$ ) or free amino acids (A,  $n = 5$ ) in adult humans. Values are means  $\pm$  SD. Time is given as hours from ingestion of the test meal. The main statistical effects (Meal, Time, Meal  $\times$  Time) from a mixed model for repeated-measures over time are reported for each variable. Values within a time period with superscripts without a common letter were significantly different ( $P < 0.05$ ).



**Figure 8.** Rates of production of dietary-derived (X) and endogenous-derived (Y) urea after ingestion of a mixed meal containing intact casein (C, n = 6) or hydrolysed casein (HC, n = 5) in adult humans. Time is given as hours from ingestion of the test meal. The main statistical effects (Meal, Time, Meal x Time) from a mixed model for repeated-measures over time are reported for each variable. \*, values significantly different (P < 0.05) from each other at a given time.

## DISCUSSION

The present study had two main objectives. The first aim was to assess the specific effect of dietary peptides on ileal endogenous protein flows. Ileal endogenous protein flows were similar following ingestion of meals based either on hydrolysed casein or on the parent intact casein. The second aim was to determine the effect of feeding a protein hydrolysate on the postprandial metabolic utilization of dietary N. Whereas our results indicated differences in the kinetics of dietary AA uptake and metabolism from intact and hydrolysed casein, the overall net retention of dietary N was found to be similar for both forms of casein.

The endogenous AA and N flows were similar for both meals C and HC, which is in line with previous animal studies (Deglaire *et al.*, 2008; see Chapter V). The ingestion of dietary peptides exerted no specific effect on ileal endogenous protein flows compared to the natural peptides arising from the digestion of the same parent protein (casein) and thus undermines the hypothesis that dietary peptides potentiate the loss of AA from the small bowel (Jansman *et al.*, 2002; Yin *et al.*, 2004; Stein *et al.*, 2007). The daily ileal endogenous N losses estimated here were similar to those reported earlier for adult humans fed meals containing hydrolysed casein (Moughan *et al.*, 2005), milk or soya protein (Gaudichon *et al.*, 2002) and for which values ranged from 1640 to 2150 mg/d, but were higher than those reported for subjects fed a protein-free diet and for which values ranged from 700 to 845 mg/d (Rowan *et al.*, 1993; Fuller *et al.*, 1994; Moughan *et al.*, 2005).

Ileal endogenous flows of serine were similar ( $P > 0.05$ ) among the meals A, HC and C under our experimental conditions of acute feeding. Although not in disagreement with a previous pig study (See Chapter V), the present result is in contrast with previous findings in animals fed for more than 6 days diets based on free AA or hydrolysed or intact casein (Darragh *et al.*, 1990; Chung & Baker, 1992; Butts *et al.*, 1993; Deglaire *et al.*, 2007) and for which endogenous AA flows were generally lower with diets based on free AA. Adaptation to the test meal may have metabolic/physiological consequences on the gut, and this needs to be investigated further.

In the present study, tracer recycling within endogenous protein ( $11 \pm 2\%$ ) was approximately three times lower than that previously found for acutely fed pigs ( $29 \pm 12\%$ , Deglaire A., unpublished data). There may be a lower rate of N recycling in

human intestines than in pig intestines. The observed kinetics of tracer recycling suggest that after the first 2 hours postprandially, there was a constant proportion of the intestinal luminal dietary N incorporated into intestinal secretions and resecreted into the lumen. It was calculated that tracer recycling induced a slight underestimation of endogenous N flow ( $6.1 \pm 1.5\%$ ) and a negligible underestimation of TID of N ( $1.2 \pm 0.5\%$ ) for meal HC. Overall, this supports the use of the  $^{15}\text{N}$ -isotope dilution method for digestive studies in humans.

The present study confirmed the specific impact of the form of delivery of dietary AA on the rate of appearance of dietary N in the peripheral blood (Collin-Vidal *et al.*, 1994; Metges *et al.*, 2000; Dangin *et al.*, 2001; Calbet & Holst, 2004). We followed the kinetics of dietary N incorporation into the plasma AA pool, considered to reflect in part the intestinal digestion/absorption kinetics (Gaudichon *et al.*, 1999; Bos *et al.*, 2003). Although some transamination occurs during first-pass metabolism which may modify the pattern of dietary-derived AA, this bias was assumed to be the same between the meal types, hence allowing comparison. Dietary N from HC appeared in the systemic circulation in a transiently rapid manner in comparison with dietary N from C, suggesting a faster intestinal absorption rate, possibly related to a faster gastric emptying rate and/or intestinal transit for HC compared with C. Unlike hydrolysed casein, intact casein is known to clot at acidic pH in the stomach and hence has a likely slower gastric emptying (Mahé *et al.*, 1992; Mahé *et al.*, 1996). Independently of the latter effect, intact casein may slow down intestinal transit due to the action of opioid peptides, such as  $\beta$ -casomorphins that are released during digestion (Daniel *et al.*, 1990; Allescher *et al.*, 2000; Patten *et al.*, 2001). Hydrolysis of casein prior to its ingestion has been found to suppress opioid activity, potentially accelerating intestinal transit (Mihatsch *et al.*, 2005). In addition, hydrolysed casein and intact casein might trigger in a different manner hormones, such as CCK or GLP-1, that are involved in the gut transit time control. Overall, hydrolysed casein can be considered as a fast-digested protein and intact casein as a slow-digested protein, as first described by Boirie *et al.* (1997).

The sharp plasma appearance of dietary N from HC induced a transient higher deamination rate and a transient faster incorporation of dietary N into liver exported protein compared with meal C. This is in line with previous observations for slow- and fast-digested proteins (Collin-Vidal *et al.*, 1994; Boirie *et al.*, 1997; Metges *et al.*, 2000; Daenzer *et al.*, 2001; Dangin *et al.*, 2001; Bos *et al.*, 2003; Lacroix *et al.*, 2006). The



present findings confirm the impact of the form of delivery of dietary AA on their partitioning towards different metabolic pathways and show the important role of the splanchnic organs, especially the liver, in preventing hyperaminoacidaemia (Bos *et al.*, 2003; Lacroix *et al.*, 2006). As expected, the branched-chain AA were spared by the liver and rose sharply in the systemic circulation in the first hour following ingestion of the fast-digested proteins, the HC and its corresponding free AA mixture. Over the 8-h postprandial period, the excretion of urea from dietary origin was the same between the HC and C groups, which demonstrates that the observed differences in kinetics between meals in terms of AA catabolism were compensated for over the entire postprandial period, possibly through differences in urea recycling amplitude and kinetics between meals. Such a discrepancy has been noted previously (Jackson *et al.*, 1984; Fuller & Reeds, 1998). This will be investigated further using the compartmental model developed within our laboratory (Fouillet *et al.*, 2000; Fouillet *et al.*, 2002).

Insulin secretion after meal HC was considerably more stimulated than after meal C, as has been observed previously (Ziegler *et al.*, 1990; Collin-Vidal *et al.*, 1994), and exerted a higher peak but a similar AUC than that observed for meal A (Monchi & Rérat, 1993). The present data are consistent with other observations concerning the combined insulinotropic effect of glucose and other putative factors, such as plasma leucine (van Loon *et al.*, 2000; Koopman *et al.*, 2005). In addition, a different insulinotropic effect of peptides, compared with that of free or protein-bound AA, might have occurred (Monchi & Rérat, 1993; Calbet & Holst, 2004), possibly (though not determined in our study) through an increased release of glucose-dependent insulinotropic polypeptide (GIP) (Calbet & Holst, 2004), an hormone known to be a potent stimulator of insulin secretion (Baggio & Drucker, 2007). The synergistic effect of insulin with dietary branched-chain AA, especially leucine (Buse & Reid, 1975; Sherwin, 1978; Koopman *et al.*, 2005; Koopman *et al.*, 2006), on the regulation of protein turnover translates to an improved protein anabolism (Wolfe, 2002; Prod'homme *et al.*, 2004; Rennie, 2007) attributed to an inhibition of protein breakdown and/or stimulation of protein synthesis (Millward, 1990; de Feo, 1996; Matthews, 2005; Rennie, 2007). Thus, after HC ingestion, especially during the early postprandial period (0–4 h), there were probably two opposite effects that led on the one hand to an increased catabolism of dietary AA as a result of the rapid appearance of AA in the plasma and on the other hand to an improved protein anabolism as a result of the

hyperinsulinaemia and hyperleucinaemia.

Overall, the final postprandial retention of casein and its hydrolysate were alike. These results are consistent with body N balance studies in both healthy humans and rats which show no advantage of feeding protein in either hydrolysed or intact form (Moriarty *et al.*, 1985; Yamamoto *et al.*, 1985). Whereas similar N retention was observed in rats after liver or bowel resection (Chan *et al.*, 1993; Sales *et al.*, 1995), starved rats exerted a better N retention when fed hydrolysed casein compared to intact casein (Boza *et al.*, 1995). The present result contradicts the paradigm, based on the observation of whole body  $^{13}\text{C}$ -leucine kinetics, that slow-digested proteins promote protein retention as compared with fast-digested proteins (Collin-Vidal *et al.*, 1994; Boirie *et al.*, 1997; Metges *et al.*, 2000; Daenzer *et al.*, 2001; Dangin *et al.*, 2001).

In the absence of a specific labelling, the present study did not provide information regarding the partitioning of dietary AA from meal A. Such as assumed for meal HC, both insulin and branched-chain AA after meal A likely interacted to stimulate protein anabolism. Earlier studies reported higher urinary ammonia excretion (Yamamoto *et al.*, 1985) and lower net protein utilization (Monchi & Rérat, 1993) with free AA as compared to peptides, but this could not be corroborated here. The postprandial metabolic fate of dietary AA when ingested in the free form is, however, of particular interest as free AA are currently used with isotopic methods for determining AA requirements.

In conclusion, casein-derived peptides do not specifically enhance ileal endogenous protein losses when compared to peptides released naturally during *in vivo* digestion of the parent protein. Both forms of AA delivery (peptide- or protein-bound) elicited the same overall nutritional value with a similar net postprandial protein utilization despite differences in the metabolic fate of dietary N. When compared to intact casein, hydrolysed casein-derived AA underwent more intense catabolism during the early postprandial phase due to their fast systemic availability. This was compensated for over the entire postprandial period. The underlying mechanisms need to be investigated further.

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## CHAPTER VIII

### Ileal digestibility of dietary protein in the growing pig and adult human

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The pig has been reported to be a potentially useful animal model for predicting the ileal digestibility of dietary protein in the human although comparative studies are scarce. The present study aimed to compare apparent and true ileal digestibility values between the species for dietary proteins of animal and vegetable origin.

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## ABSTRACT

The suitability of the pig as an animal model for predicting protein digestibility in the human was evaluated. Healthy adult humans [mean bodyweight ( $\pm$  SD)  $67 \pm 12$  kg,  $n = 5-6$  per meal] and growing pigs [mean bodyweight  $39.8 \pm 2.6$  kg,  $n = 7-8$  per meal] were fed semi-synthetic mixed meals containing as a sole source of nitrogen (N), animal or vegetable proteins [casein (C), hydrolysed casein (HC) or rapeseed isolate (R)]. For each species, the feeding of the test meal was acute (without prior adaptation). Ileal digesta were sampled through a naso-ileal tube in humans or through a post-valve T-caecum (PVTC) cannula in pigs. The protein sources were  $^{15}\text{N}$ -labelled to allow for the correction of apparent to true digestibility. Amino acid (AA) digestibilities were not determined for meal R. Ileal apparent N digestibility was markedly lower (14-16%,  $P < 0.05$ ) in humans than in pigs (meals C, HC and R). Similarly, most apparent ileal AA digestibilities were lower (8% on average,  $P < 0.05$ ) in humans (meals C and HC). Ileal true N digestibility was slightly lower (3-5%,  $P < 0.05$ ) in humans than in pigs (meals C, HC and R) and most true ileal AA digestibilities were similar ( $P > 0.05$ ) between the species (meals C and HC). Exceptions were for phenylalanine, tyrosine, lysine, histidine and aspartic acid for which digestibilities were lower (3% on average,  $P < 0.001$ ) in humans. A similar protein ranking was observed between the species and the correlation between the species for true ileal AA digestibility ( $r = 0.83$ ) was significant ( $P < 0.05$ ). Overall, the present findings support the use of the growing pig as an animal model to allow prediction of true ileal protein digestibility.

## INTRODUCTION

The digestibility of dietary protein, an indirect measure of the extent of digestion and absorption of food protein as amino acids (AA), is a key determinant of protein bioavailability (Fuller & Tome, 2005 ; FAO/WHO/UNU, 2007). It is thus considered an important factor for nutritional quality assessment (FAO/WHO/UNU, 2007). It is important to determine protein digestibility with accuracy as it differs substantially among diets, in particular between those from developing or developed countries whereby values of 54-78% (diets from India, Guatemala or Brazil) versus 88-94% (diets from North America) have been reported (Gilani *et al.*, 2005). Measurement of digestibility at the ileal level is now recognized as being more accurate than its

measurement at the faecal level (Darragh & Hodgkinson, 2000; Moughan, 2003; Fuller & Tome, 2005), due to the high metabolic activity of the hindgut microflora leading to modification of the undigested dietary AA profile. Most dietary AA are absorbed in the small intestine (Krawielitzki *et al.*, 1990; Fuller & Reeds, 1998), although it remains unclear if the colon may also absorb AA to some degree (Blachier *et al.*, 2007).

In humans, ileal digestibility can be determined in subjects with ileostomies (Moughan *et al.*, 2005) or by sampling via a naso-ileal triple-lumen tube (Bos *et al.*, 2007). None of these methods, however, is suitable for routine application due to technical and economic constraints. Animal models have thus been developed to determine ileal protein digestibility for humans. While the laboratory rat has been considered as a suitable animal model (FAO/WHO, 1991), the pig, the digestive tract of which is more similar to that of humans both anatomically and physiologically (Pond & Houpt, 1978; Miller & Ullrey, 1987; Moughan & Rowan, 1989; Moughan *et al.*, 1992), may be a better model for protein digestion in humans (Moughan *et al.*, 1994; Darragh & Hodgkinson, 2000; Moughan, 2005). To date, however, only a very few studies have compared protein digestibility directly between pigs and humans (Forsum *et al.*, 1981; Rowan *et al.*, 1994; Darragh & Moughan, 1995), and only one of these (Rowan *et al.*, 1994) has assessed digestibility at the ileal level.

This study aimed to extend the work of Rowan *et al.* (1994) to further evaluate the suitability of the growing pig as an animal model for predicting dietary ileal protein digestibility in humans. A direct species comparison was made using two highly digestible animal protein sources (casein, hydrolysed casein) and a vegetable protein source (rapeseed isolate) expected to have a lower digestibility. Ileal digesta were collected via a post-valve T-caecum (PVTC) cannula in pigs and by naso-ileal intubation in human subjects. The protein sources were  $^{15}\text{N}$ -labelled to allow determination of the ileal endogenous N and AA losses, thus allowing for the correction of apparent to true protein digestibility.

## MATERIALS AND METHODS

### *Test meals*

Similar semi-synthetic test meals (**Table 1**) were prepared for pigs and humans. The test meals contained as the sole source of N a uniformly  $^{15}\text{N}$ -labelled native phosphocaseinate (meal C), a  $^{15}\text{N}$ -labelled casein hydrolysate (derived from the former

native phosphocaseinate, meal HC) or a  $^{15}\text{N}$ -labelled rapeseed protein isolate (meal R). The N content ranged from 29.3 to 32.6 g/kg diet. The  $^{15}\text{N}$ -casein was extracted by microfiltration followed by diafiltration (INRA, France) of  $^{15}\text{N}$ -labelled milk (Mahé *et al.*, 1994). The resulting casein was freeze-dried. Its isotopic enrichment was 0.54 atom%. An aliquot of  $^{15}\text{N}$ -casein was hydrolysed with pig pancreatin as previously detailed (Deglaire *et al.*, 2007). The molecular weight profile, determined by high-performance liquid chromatography (HPLC) gel filtration (Deglaire *et al.*, 2007), indicated that 21% of the peptides were between 1 and 5 kDa in size, and 79% were less than 1 kDa.  $^{15}\text{N}$ -labelled rapeseed protein isolate was obtained from winter rapeseed fertilised with  $^{15}\text{N}$ -ammonium nitrate (CETIOM, France). A rapeseed flour was produced from dehulled seeds by extraction with hexane to remove the oil (CREOL, France). The solvent was eliminated at a low temperature and under vacuum to protect protein functionality. The protein isolate was then purified by solubilisation and ultrafiltration of the rapeseed flour (Bos *et al.*, 2007). Its isotopic enrichment reached 1.16 atom%.

**Table 1.** Ingredient compositions of the test meals fed to growing pigs and adult humans

Ingredient	Diet		
	C	HC	R
	<i>g/100g air dry weight</i>		
Maltodextrin	47 <sup>1,2</sup>	46 <sup>2</sup>	48 <sup>2</sup>
Sucrose	16	16	16
Oil <sup>3</sup>	15	15	15
$^{15}\text{N}$ -casein	22	-	-
$^{15}\text{N}$ -hydrolyzed casein	-	23	-
$^{15}\text{N}$ - rapeseed protein isolate	-	-	21

<sup>1</sup> 1.8 g/100g diet of maltodextrin was replaced by sodium bicarbonate in the pig diets only to equalise the dietary electrolyte balance between C and HC diets. This was not done in the human diets as the sodium bicarbonate rendered the meal unpalatable.

<sup>2</sup> 0.3 g/100g diet of maltodextrin was replaced by titanium dioxide in pig diets only as a dietary marker.

<sup>3</sup> Soyabean oil was used in the C and HC diets. Rapeseed oil was used in the R diet.

## ***Experimental procedure***

### ***Pigs***

Eight 10-week-old Large White x Duroc entire male pigs were housed individually in steel metabolism crates in a room maintained at  $24 \pm 1^\circ\text{C}$ . Ethics approval was received from the Massey University Animal Ethics Committee (protocol 05/29).

On day 0, a PVTC cannula was surgically inserted into the caecum of each pig for the collection of ileal digesta (van Leeuwen *et al.*, 1991). The cannulae were made of medical grade silastic tubing (internal diameter: 24 mm; external diameter: 32 mm). The pigs were not fed for 12 h before surgery. Anaesthesia was induced with an intramuscular injection of Midazolam [Roche products Ltd.; 1 mg/kg bodyweight (BW)] and Ketamine (Parnell Laboratories Ltd.; 10 mg/kg BW) followed by an intravenous injection of Propofol (Gensia Laboratories Ltd.; 2 mg/kg BW). The anaesthesia was maintained via inhalation of isoflurane (Merial Ltd.; 1.5 to 2%) in O<sub>2</sub>. Intravenous crystalloids were infused throughout the anaesthesia period (5-10 mL/kg BW/h) to maintain hydration. Prior to the start of surgery, the pigs were given analgesics: Carprofen (Pfizer Laboratories Ltd.; 3 mg/kg BW) administered by intravenous injection, and Methadone (David Bull Laboratories; 0.2 mg/kg BW) administered by deep intramuscular injection. Immediately after surgery, the pigs received an intramuscular injection of antibiotic (Duplocillin LA, Intervet international B.V.; 2 mL). For the following 4 days, antibiotic powder (Mamyzim, Boehringer Ingelheim Ltd.) was dusted on the wound site daily. The site where the cannula was exteriorised was washed with water, and zinc ointment was applied daily throughout the experiment. The pigs regained consciousness within 1 h of surgery and were standing 7-8 h after surgery. There was a 14-day recovery period before the start of the experiment. At day 14, the mean ( $\pm$  SD) BW of the pigs was  $39.8 \pm 2.6$  kg. During the experimental period (days 14 to 37), pigs were fed at a daily level of 0.08 metabolic BW ( $\text{BW}^{0.75}$ ). Except on the digesta collection day, the pigs received 3 meals daily (0800, 1200 and 1600 h) in equal portions. The meals were mixed with water (1:1, w/w) and water was freely available between meals. On the day of digesta collection, the pigs received (0800 h) the test meal (1/3 of the daily portion) mixed with water (2.3:1, w/w) and 200 mL water was given every 30 min thereafter. The pigs received the rest of their daily portion at 1800 h. During the study, the pigs were weighed every sixth day and the level of food intake was adjusted accordingly.

The test meals were administered using a duplicated 4x4 Latin Square design such that every test meal followed each other once only. A fourth meal was included in the design but was not part of the species comparison. The pigs were randomly allocated to the Latin Square and were fed their respective test meals every sixth day after having been fed a basal meal (**Table 2**) in the intervening 5-day periods. This was so the feeding of the test meal was acute (one meal) to afford a similar comparison with humans. On the sixth day of each test period, ileal digesta were continuously collected for 10 h after the ingestion of the test meal using polythene bags attached to the cannula. The bung of the cannula was removed 2 h before the collection commenced (van Leeuwen *et al.*, 1991) to allow the ileocaecal valve to protrude into the lumen of the cannula. Digesta collection commenced 30 min prior to the ingestion of the test meal in order to determine the basal <sup>15</sup>N-enrichment in digesta. The plastic bags were removed every 30 min, and digesta were immediately frozen (-20°C) after addition of sodium benzoate (2.3 mol/L) as a bactericide and phenylmethylsulphonyl fluoride (PMSF, 70 mmol/L) as an antiprotease (Salgado *et al.*, 2002). Pig digesta were freeze-dried and finely ground.

#### *Human subjects*

Eighteen subjects (9 female, 9 male; 30 ± 8 years) of mean BW 67 ± 12 kg and of mean body mass index 22.5 ± 3.5 kg/m<sup>2</sup> were included in the study after a thorough medical examination and the conduct of routine blood tests. All subjects received detailed information on the protocol and gave their written informed consent to participate in the study. The protocol was approved by the Institutional Review Board for St-Germain-en-Laye Hospital, France. The volunteers were admitted to hospital for 2 days. In the morning of day 1, a 3-m PVC triple-lumen tube was inserted via the nose under local anaesthesia and then swallowed by the subject so as to progress down the gastrointestinal tract. The progression of the tube was facilitated by inflating a balloon, located at the terminal tip of the tube, after it had passed the pyloric valve. The subjects were then given a standard easily digested hospital meal and received a second meal at 1900 h before fasting overnight. The tube was restrained from further movement once it had reached the terminal ileum, as verified under X-ray. On day 2, the protocol commenced at 0930 h, when a saline solution (NaCl 130 mmol/L, KCl 5 mmol/L, D-mannitol 30 mmol/L) containing 20 g/L polyethylene glycol (PEG)-4000 as a liquid phase marker, was infused continuously through one of the tube's lumen at a constant rate of 1 mL/min. The test meal was given at 1000 h as a liquid drink. The study was

performed while the subjects were resting in a semi-recumbent position and no food other than the test meal was ingested until the end of the study period. Water was given hourly. Digesta collection commenced 30 min prior to the ingestion of the test meal in order to determine the basal  $^{15}\text{N}$ -enrichment. Digesta were collected continuously on ice and pooled over 30-min periods. Digesta were immediately frozen after addition of the antiprotease diisopropylfluorophosphate (DFP, 1mmol/L). For each pooled sample, a 4 mL sample was kept frozen ( $-20^{\circ}\text{C}$ ) for PEG-4000 determination and the remainder was freeze-dried. Subjects were given the rapeseed isolate meal in a separate part of the study which was previously undertaken using exactly the same design as described above (Bos *et al.*, 2007).

**Table 2.** Ingredient composition of the basal meal fed to growing pigs between meals of the test meal.

<b>Ingredient</b>	<b>Basal meal</b>
	<i>g/100 g air dry weight</i>
Cooked wheat	48.43
Sucrose	17
Casein <sup>1</sup>	16
Skim milk powder <sup>1</sup>	8
Soyabean oil	8
Dicalcium phosphate	2
Vitamin - mineral mix <sup>3</sup>	0.30
Sodium chloride	0.16
Synthetic methionine	0.07
Calcium carbonate	0.02
Antioxidant	0.02

<sup>1</sup> New Zealand Milk Products, Palmerston North, NZ.

<sup>2</sup> Vitalean, Vitec Nutrition Ltd., Auckland, NZ. Vitamins provided: (g/kg of meal) vitamin A 3; (mg/kg meal) cholecalciferol 500.0, choline 83.3, niacin 12.5, panthotenic acid 8.3, riboflavin 2.1, vitamin B6 1.7, vitamin E 41.7, vitamin K 1.7; ( $\mu\text{g}/\text{kg}$  of meal) biotin 8.3, folic acid 417, thiamin 833, vitamin B-12 8.3. Minerals provided: (mg/kg meal) Cu 104, Fe 83, Mn 38, Zn 100; ( $\mu\text{g}/\text{kg}$  meal) I 833, Co 417, Se 250.

<sup>3</sup> Ethoxyquin, Unitech industries, Auckland, NZ

### ***Chemical analysis***

Pig digesta were pooled for each pig and meal type between 4 and 10 h after meal ingestion as previously described (Leterme *et al.*, 1996; Hess *et al.*, 2000). Pooled digesta were analysed for TiO<sub>2</sub>, total N, AA and for <sup>15</sup>N-enrichment of total N and single AA. Diets were analysed for TiO<sub>2</sub>.

For humans, each 30-min digesta sample was analysed on an “as is” basis for PEG-4000 content or on a freeze-dried basis for N content and <sup>15</sup>N-enrichment. The 30-min samples were pooled over 8 h for each meal type and subjected to the analysis of AA and their individual <sup>15</sup>N-enrichments.

Protein sources were analysed for AA, total N and for <sup>15</sup>N-enrichment of total N and single AA.

Total N and <sup>15</sup>N-enrichment were measured on an isotopic ratio mass spectrometer (Optima, Fisons Instruments, Manchester, UK) coupled to an elemental N analyser (NA 1500 series 2, Fisons Instruments, Manchester, UK) (Gausserès *et al.*, 1997). AA were determined after acid hydrolysis (HCl, 6 mol/L containing 0.1% phenol) using a Waters ion exchange HPLC (AOAC, 2003). AA were not determined on digesta from animals or humans receiving the rapeseed protein. Cysteine, methionine and tryptophan, being destroyed during acid hydrolysis, were not determined. The <sup>15</sup>N-enrichments of individual AA were determined by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, Finnigan Delta S; Thermo Fisher Scientific Inc., Bremen, Germany) as described previously (Metges *et al.*, 1996; Petzke *et al.*, 2006; Deglaire *et al.*, 2007). TiO<sub>2</sub> was determined using a colorimetric assay after ashing the sample and digestion of the minerals (Short *et al.*, 1996). PEG-4000 was measured using a turbidimetric method (Hyden, 1955).

### ***Data analysis***

#### ***Pig data***

Ileal total N flows (TNFL, g/100 g dry matter intake, DMI) were determined for the pooled digesta samples with reference to the dietary marker as follows:

$$\text{TNFL}_{\text{pig}} = \frac{\text{N in digesta} \times \text{TiO}_2 \text{ in meal}}{\text{TiO}_2 \text{ in digesta}}$$



Ileal dietary nitrogen N flows (DNFL, g/100 g DMI) were determined according to the isotope dilution as follows (Hess *et al.*, 2000):

$$\text{DNFL}_{\text{pig}} = \text{TNFL}_{\text{pig}} \times (E_s - E_0) / (E_{\text{meal}} - E_0)$$

where  $E_s$  is the  $^{15}\text{N}$ -enrichment in the digesta sample,  $E_{\text{meal}}$  the  $^{15}\text{N}$ -enrichment in the meal, and  $E_0$  the baseline  $^{15}\text{N}$ -enrichment in digesta.

Similar equations were used to determine total and dietary AA flows.

Ileal N digestibility (%) was calculated as follows:

$$\text{Apparent ileal digestibility} = \frac{(\text{Dietary N intake} - \text{TNFL}) \times 100}{\text{Dietary N intake}}$$

$$\text{True ileal digestibility} = \frac{(\text{Dietary N intake} - \text{DNFL}) \times 100}{\text{Dietary N intake}}$$

Similar equations were used to determine ileal AA digestibility coefficients.

#### *Human data*

Ileal TNFL (mg/8 h) was determined from the cumulative recovery of total N the 8-h postprandial period using the following equation:

$$\text{TNFL}_{\text{human}} = \sum_{T=1}^{16} (N_{\text{tot-digesta-(T)}} \times \text{DM}_{\text{S-(T)}} \times F_{\text{(T)}})$$

where  $N_{\text{tot-digesta-(T)}}$  is the N content of the digesta sample for the T 30-min period (mg/g),  $\text{DM}_{\text{S-(T)}}$  is the dry matter of the sample (g/mL) and  $F_{\text{tot-(T)}}$  (mL/30 min) is the total ileal liquid flow rate.  $F_{\text{tot-(T)}}$  was determined as follows:

$$F_{\text{tot-(T)}} = (\text{PEG}_i / \text{PEG}_{\text{S-(T)}}) \times F_i \times t$$

where  $\text{PEG}_i$  and  $\text{PEG}_{\text{S-(T)}}$  are the PEG concentrations in the infusion solution and in the digesta sample for the T 30-min period, respectively,  $F_i$  the PEG infusion rate (1 mL/min), and  $t$  the duration of the collection period (30 min).

DNFL (mg/8 h) was calculated from the cumulative recovery of undigested dietary N over the 8-h postprandial period using the following equation:

$$\text{DNFL}_{\text{human}} = \sum_{T=1}^{16} (N_{\text{tot-digesta-(T)}} \times [(E_{\text{S-(T)}} - E_0) / (E_{\text{meal}} - E_0)] \times F_{\text{tot-(T)}})$$

where  $E_{\text{S-(T)}}$  is the  $^{15}\text{N}$ -enrichment in the digesta sample for the 30-min period.

Total and dietary AA flows (TAAFL, DAAFL; mg/8 h) were determined in pooled digesta samples, reconstituted so as to be representative of the total collection:

$$\text{TAAFL} = \text{AA} \times \text{F} \times \text{DM}$$

where AA is the AA content in the pooled digesta sample (mg/g), DM the dry matter of the pooled digesta sample (g/mL) and F is the total estimated flow rate over 8 h (mL/8 h).

$$\text{DAAFL} = \text{TAAFL} \times (\text{E}_s - \text{E}_0) / (\text{E}_{\text{meal}} - \text{E}_0)$$

where  $\text{E}_s$  is the  $^{15}\text{N}$ -enrichment of individual AA in the pooled digesta sample.

Ileal apparent and true digestibilities of N and AA were determined as described above for the growing pig.

### *Statistical analysis*

The data set was firstly subjected to an outlier test (Dixon, 1950, 1953) with  $P < 0.05$ . Statistical analyses were performed using SAS (version 9.1, SAS Institute Inc., Cary, NC). The data were analysed using the following general linear model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ij}$$

where  $Y_{ij}$  is the dependent variable,  $\mu$  is the general mean,  $\alpha_i$  is the fixed effect of the meal and  $\beta_j$  is the fixed effect of the species and  $\epsilon_{ij}$  the random residual error. Significance was considered to be reached at  $P < 0.05$ . Results are means  $\pm$  SD.

## **RESULTS**

The pigs remained healthy and grew normally throughout the study, except for one pig that was removed from the study because of internalisation of the cannula. This animal was replaced by a spare cannulated pig of a similar age and BW. Minimal leakage from the PVTC cannula occurred during digesta collections. At post-mortem dissection, no signs of adverse effects due to the cannulation were observed. Mean pig live weight at the completion of the trial was  $61.3 \pm 4.3$  kg.

All human subjects completed the trial without complication, and complied with the experimental protocol.

For meals C and HC fed to the pigs, one observation each for apparent and true AA and N digestibility was removed from the data set, as detected by application of the outlier test. Similarly, for meal R fed to humans, one observation for apparent and true N digestibility was removed from the data set. The removal of these data resulted in a decrease in the standard deviation of the corresponding data set by between 35 to 52%.

The apparent and true ileal digestibilities of N for meals C, HC and R for pigs and humans are given in **Table 3**. There was a significant ( $P < 0.05$ ) effect of species with both apparent and true digestibilities being lower in humans compared with pigs. The species differences were marked for the apparent digestibility of N (14-16% lower in humans) but were relatively small for true N digestibility (3-5% lower in humans).

**Table 3.** Apparent and true ileal nitrogen (N) digestibility (%) in adult humans and growing pigs for three dietary protein sources [casein (C), hydrolysed casein (HC) and rapeseed protein isolate (R)].

Ileal N digestibility	Meal C		Meal HC		Meal R		RMSE <sup>1</sup>	Meal	Species	Meal x Species
	pig (n = 7)	human (n = 6)	pig (n = 7)	human (n = 5)	pig (n = 8)	human (n = 6)				
<b>Apparent</b>	89.8	76.0	88.4	75.9	81.1	70.4	5.0	**	***	NS
<b>True</b>	97.6	94.1	95.0	92.3	91.4	87.1 <sup>2</sup>	2.0	***	***	NS

Statistical significance: \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; NS, not significant ( $P > 0.05$ ).

<sup>1</sup>RMSE: root mean square error.

<sup>2</sup>Data originally published by Bos *et al.* (2007).

The apparent ileal AA digestibilities for meals C and HC for pigs and humans are given in **Table 4**. For each AA, apparent digestibility was significantly lower ( $P < 0.05$ ) in humans compared to pigs, in both meals C and HC, except for the amino acid Thr for which the difference did not reach statistical significance.

The true ileal AA digestibility values for meals C and HC are given in **Table 5**. Pig and human true ileal digestibilities were not significantly different ( $P > 0.05$ ) over both meals (C and HC) for most AA except for phenylalanine, tyrosine, lysine, histidine and aspartic acid for which digestibilities were significantly lower ( $P < 0.05$ ) for humans compared to pigs.

**Table 4.** Apparent ileal amino acid digestibility in growing pigs and adult humans for two dietary protein sources [casein (C) and hydrolysed casein (HC)]

	meal C		meal HC		RMSE <sup>1</sup>	Meal	Species	Meal x Species
	pig	human	pig	human				
	(n = 7)	(n = 6)	(n = 7)	(n = 5)				
Threonine	78.8	75.7	79.1	70.8	6.8	NS	NS	NS
Valine	90.9	84.6	87.7	81.0	4.5	NS	**	NS
Isoleucine	92.2	83.8	88.0	81.1	4.4	NS	***	NS
Leucine	94.3	90.0	92.8	88.3	2.8	NS	***	NS
Phenylalanine	95.7	88.9	95.0	86.9	3.2	NS	***	NS
Tyrosine	96.0	88.7	95.1	86.0	3.2	NS	***	NS
Lysine	95.9	91.8	95.2	90.6	2.7	NS	***	NS
Histidine	90.8	80.8	84.8	69.1	5.8	**	***	NS
Glutamic acid	94.2	89.7	89.2	86.6	3.5	**	*	NS
Proline	94.4	91.0	92.3	89.1	2.8	NS	**	NS
Alanine	88.0	84.2	84.7	78.9	5.1	NS	*	NS
Aspartic acid	88.3	75.9	84.4	70.1	6.2	NS	***	NS
Serine	84.7	72.9	76.5	66.6	7.8	NS	**	NS

Statistical significance: \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; NS, not significant (P > 0.05)

<sup>1</sup>Root mean square error.

**Table 5.** True ileal amino acid digestibility in growing pigs and adult humans for two dietary protein sources [casein (C) and hydrolysed casein (HC)] and corrected for endogenous amino acid flows determined using the isotope dilution method.

	meal C		meal HC		RMSE <sup>1</sup>	Meal	Species	Meal x Species
	pig (n = 7)	human (n = 6)	pig (n = 7)	human (n = 5)				
Threonine	94.5	93.3	92.2	92.5	2.7	NS	NS	NS
Valine	96.6	93.7	92.2	92.4	2.8	*	NS	NS
Isoleucine	97.2	94.1	92.3	92.9	3.1	*	NS	NS
Leucine	98.9	97.2	97.2	97.0	1.2	NS	NS	NS
Phenylalanine	99.2	96.3	98.4	96.6	1.1	NS	***	NS
Tyrosine	99.4	97.2	98.8	97.1	1.3	NS	**	NS
Lysine	99.3	97.4	98.2	97.6	1.1	NS	*	NS
Histidine	99.0	94.7	96.3	92.9	2.5	*	**	NS
Glutamic acid	97.5	94.0	92.0	91.4	2.9	**	NS	NS
Proline	97.9	96.2	95.6	95.4	1.7	*	NS	NS
Alanine	96.2	95.1	92.6	93.6	2.6	*	NS	NS
Aspartic acid	97.3	91.6	92.3	89.6	3.1	*	**	NS
Serine	93.1	87.0	83.5	82.6	6.0	**	NS	NS

Statistical significance: \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; NS, not significant ( $P > 0.05$ )

<sup>1</sup>Root mean square error.

## DISCUSSION

The objective of the study was to assess the suitability of the growing pig as an animal model for determining the ileal digestibility of dietary protein in adult humans. Overall, close agreement was found between species for the true ileal digestibility of total N and individual AA.

The study used methods of digesta collection optimised for each species and for this reason method of collection was not common across pigs and humans although it was maintained as close as possible and based in both cases on a direct access to ileal fluid as well as the use of  $^{15}\text{N}$ -labelled protein sources. In humans, ileal digesta were sampled through a naso-ileal triple-lumen tube, allowing digesta collection from subjects with an entire digestive tract, unlike that from ileostomized subjects. The intubation method, used for determination of ileal digestibility in humans (Gausserès *et al.*, 1996; Gaudichon *et al.*, 1999; Mariotti *et al.*, 1999; Bos *et al.*, 2005), has been demonstrated to allow a representative sampling, with more than 50% of the total digesta being sampled through the tube, and overall to allow an accurate estimation of ileal N flows (see Chapter V). Although it remains unknown whether the oro-ileal transit time is altered by the presence of the tube, previous studies have reported that digesta transit time, and especially gastric emptying rate, do not influence overall ileal protein digestibility (Weber & Ehrlein, 1998; Gaudichon *et al.*, 1999; Mariotti *et al.*, 2000). In the present study, ileal digesta were collected from pigs through a PVTC-cannula, which, above other cannulation techniques, offers the advantage of leaving the small intestine intact, thus minimizing effects on ileal muscle function. A significant proportion ( $42 \pm 15\%$ , data not shown) of the indigestible marker that had been ingested with the meal was recovered in the present digesta samples, thus indicating that a representative quantity of the total digesta was sampled. The calculations used for determining ileal N flows were those normally employed in each species with the digesta collection methods used and aimed to determine the same final parameter, the proportion of ingested N that had been absorbed by the end of the terminal ileum.

Of interest in the present study was that the apparent ileal digestibility of N was markedly lower (14 to 16%) for humans compared with pigs across the test meals containing different protein sources. For apparent AA digestibility, the species difference was smaller but still substantial with values being on average 8% lower for

humans. In the present work, an acute (no adaptation to the test meal) feeding procedure was used. This did not appear to influence the results of the study as the present pig digestibility coefficients were very similar to apparent ileal N and AA digestibility coefficients for the same diets but given to adapted (5 days) pigs (see Chapter V) such as has been observed previously (Moughan *et al.*, 2005). The presently reported values for the apparent ileal digestibility of N and AA in casein and hydrolysed casein agree closely with other published values (Chung & Baker, 1992; Nyachoti *et al.*, 1997; Yin *et al.*, 2004). Roos *et al.* (1994) reported apparent ileal digestibility values of casein for pigs (76-80%) which were lower than those found in the present study but closer to the values observed here for humans. Apparent ileal digestibility values for casein and hydrolysed casein in humans have not been reported in the literature. The mean apparent ileal N digestibility value for rapeseed isolate was in line with that determined in pigs fed dehulled and untoasted rapeseed (76%) (Grala *et al.*, 1998). The present differences observed for apparent ileal digestibility between species are likely to be due to a higher proportion of endogenous protein to total ileal protein in humans compared with pigs, as true ileal digestibility was much closer between pigs and humans (less than 5% difference for true ileal digestibility of N). Whether the different amount of endogenous protein in digesta is an actual effect of the species or is due to methodological differences is unknown, and should be the topic of further investigation.

It is more accurate to compare true ileal protein digestibility between species as this measure represents the specific fate of dietary N and AA in the digestive tract (Fuller & Tome, 2005). The present study shows a similar ranking among the protein sources between humans and pigs and that for most AA true ileal digestibility was the same between the species. Although the true ileal digestibility of N and of 5 AA was significantly different ( $P < 0.05$ ) between the species, the coefficients were on average only 4% lower in humans compared with pigs. This inter-species difference might result from physiological and/or methodological considerations. The digestive and/or absorptive capacity of the gastrointestinal tract may be more efficient in pigs than in humans, and this may be particularly important for more poorly digested proteins such as rapeseed. Forsum *et al.* (1981) reported a higher (6%) true faecal digestibility of vegetable proteins in pigs than in humans but similar digestibility of a combination of vegetable and animal proteins between the species. It should be noted, that although we previously observed a higher recycling of the  $^{15}\text{N}$  tracer for meal HC in pigs than in

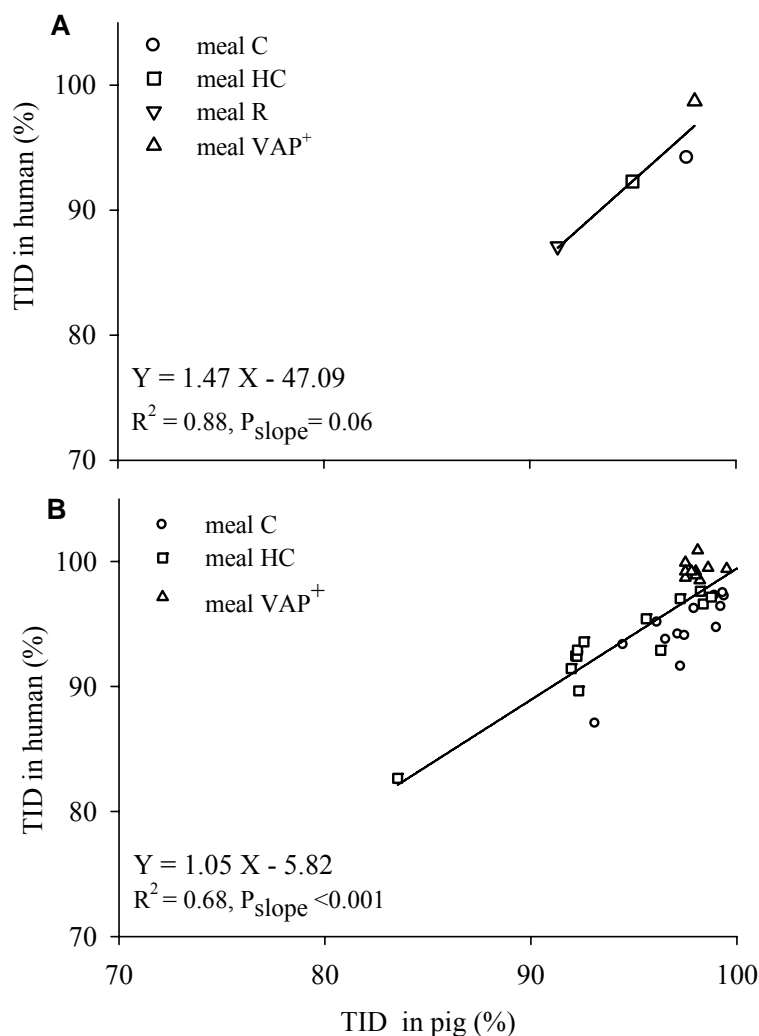
humans (see Chapter V and VII), this had little impact on the relative difference between the species for the true ileal N digestibility of HC with values after correction for recycling being  $96.5 \pm 2.0$  and  $93.4 \pm 1.0\%$  in pigs and in humans, respectively (data not shown).

Statistical correlations between pig and human true ileal digestibilities were investigated using the mean digestibility coefficients for the present meals C, HC, and where applicable for meal R, as well as those for a vegetable/animal protein-based meal (meal VAP) published in a separate study (Rowan *et al.*, 1994). The correlation between pig and human true ileal digestibility coefficients was high [ $r$  (Pearson's coefficient) = 0.94] and close to significance ( $P = 0.06$ ) for N from meals C, HC, R and VAP and was statistically significant ( $r = 0.83$ ,  $P < 0.001$ ) for AA from meals C, HC and VAP. Linear regression equations were derived to allow predictions of human true ileal digestibility values from determined pig true ileal digestibility values for N ( $R^2 = 0.88$ , **Figure 1.A**) and AA ( $R^2 = 0.68$ , **Figure 1.B**). However, caution needs to be exercised in extrapolating from the predictive equation for true ileal digestibility of N beyond the range of data collected, as this was based on four average values only. For instance, a coefficient of 60% in pigs would predict a coefficient of 41% for humans. The latter value appears to be unrealistic.

In conclusion, the present results support an earlier observation (Rowan *et al.*, 1994) that there is a good agreement between pigs and humans for their true ileal digestibility of dietary protein. The growing pig has been widely promoted as a useful model for human nutrition studies due to a physiologically and anatomically similar digestive tract (Pond & Houpt, 1978; Miller & Ullrey, 1987; Moughan & Rowan, 1989; Moughan *et al.*, 1992). In addition, the pig unlike the rat has a meal eating habit (Miller & Ullrey, 1987) and eats most foods consumed by humans. Another advantage with the pig is the possibility for the collection of large samples of representative digesta (Moughan & Rowan, 1989). For all these reasons, the growing pig has been suggested as being a better model than the growing rat for predicting protein digestibility for the adult human (Rowan *et al.*, 1994; Darragh & Hodgkinson, 2000; Moughan, 2005). This is supported by our recent data showing a similar true ileal digestibility for rapeseed and milk proteins in the growing rat ( $95.5 \pm 1.1$  and  $95.6 \pm 0.8\%$ , respectively; Bos C., unpublished), unlike what was observed here in both the adult human and the growing



pig. Overall, the present findings support the use of the growing pig as an animal model for routine determination of true ileal protein digestibility in adult humans. The pig can be used to predict, using regression equations, true ileal protein digestibility in humans.



**Figure 1.** Linear regression relationship between mean values of pig and human true ileal digestibilities (TID) of nitrogen (A) and amino acids (B) for meals based on casein (C), hydrolysed casein (HC), rapeseed protein isolate (R) and a mixture of vegetable-animal proteins (VAP, Rowan *et al.*, 1994).

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# CHAPTER IX

## General discussion

Knowledge concerning ileal endogenous protein flows is important for dietary protein quality evaluation as well as for the factorial determination of protein and amino acid (AA) requirements. In this dissertation results are presented on the modulation of ileal endogenous protein flows by various dietary factors. The first study (Chapter II) aimed to assess, in a practical context using a protein-free diet, the influence of antinutritional factors, when given at amounts commonly ingested in practice, on gut endogenous protein losses. The results of this study demonstrated the importance of endogenous N and AA losses. The research objective was then directed towards the influence on gut endogenous losses of peptides (derived from casein) used as a nitrogen (N) source within the enzyme-hydrolysed protein/ultrafiltration method, previously proposed as an alternative technique to the use of a protein-free diet. Questions have been raised, as to whether bioactive peptides probably present in the casein hydrolysate may potentiate ileal endogenous protein flows, over and above those found with the corresponding intact casein. A set of studies was thus undertaken (Chapters III, IV, V, VII) to allow for a direct comparison of gut endogenous N and AA losses between dietary peptides and peptides naturally released in the gut during protein digestion in rats, pigs and humans. The method used for ileal digesta sampling in humans needed to be validated (Chapter VI) prior to its practical application. In addition, the pig and human studies were designed so as to enable an assessment of the validity of the growing pig as an animal model for predicting true ileal digestibility of dietary protein in the human (Chapter VIII). The metabolic utilization of absorbed dietary N is also an important consideration for protein quality evaluation. The human study (Chapter VII) aimed to investigate dietary modulation of the postprandial metabolism of dietary AA, in particular the influence of the form of delivery of dietary AA (peptide- or protein-bound).

The influence of antinutritional factors on ileal endogenous protein flows has been largely studied in animals (Grala *et al.*, 1998; Jansman *et al.*, 1998); however, the extrapolation of results to humans needs to be undertaken with caution due to the different levels of intake between species. We aimed in Chapter II to investigate the impact of antinutritional factors from a crude extract of kidney beans (*Phaseolus vulgaris*) consumed by humans as a “starch stopper” product to assist in body weight loss. When fed to growing rats for 14 days



at the higher range of what a human subject would consume, a crude extract of kidney beans increased ileal (and faecal) endogenous N and AA losses (except for proline, glycine, alanine and histidine flows at the ileum). This effect was likely due to the effects of trypsin inhibitor and lectin present in the kidney bean extract. Whereas the “starch stopper” did not reduce starch digestibility in this study, it may have had some effect on overall animal energetics. The potentially harmful effects of the *Phaseolus vulgaris* extract on gut tissue integrity needs to be investigated further. This first study in the series of studies reported here was undertaken to demonstrate in a practical context the importance of gut endogenous protein N losses as a marker of dietary physiological effects.

In this study, the protein-free diet method served as a practical and straightforward means to determine and compare gut endogenous protein flows among dietary treatments. However, protein-free diets are known to somewhat underestimate ileal endogenous protein flows (Darragh *et al.*, 1990; Butts *et al.*, 1993; Moughan *et al.*, 2005). Subsequent studies reported herein focussed on the accuracy of an alternative method for determining gut endogenous N losses, the enzyme hydrolysed-protein/ultrafiltration method.

The enzyme-hydrolysed protein/ultrafiltration method was proposed as an alternative method to the feeding of a protein-free diet to allow for a more physiological measure of ileal endogenous protein losses (Moughan *et al.*, 1990). This technique consists of measuring ileal endogenous N and AA under conditions in which the gut is supplied with dietary AA and peptides (usually from hydrolysed casein, molecular weight < 5 kDa) mimicking the breakdown products of natural digestion. Previous studies have reported an influence of bioactive peptides from casein on gut endogenous protein secretions (Yvon *et al.*, 1994; Pedersen & Boisen, 2001; Claustre *et al.*, 2002; Trompette *et al.*, 2003). Consequently, questions have been raised as to whether bioactive peptides likely present in the hydrolysate may potentiate the loss of AA from the small bowel (Jansman *et al.*, 2002; Yin *et al.*, 2004; Stein *et al.*, 2007) over and above that which may be found with the corresponding intact protein. In the present work, we have clearly shown that peptides *per se*, in comparison with peptides naturally released in the gut during protein digestion, do not modulate ileal endogenous protein losses in rats (Chapter IV), pigs (Chapter V), and humans (Chapter VII) using the same casein in the intact form or after enzyme (pig pancreatin) hydrolysis. The influence of casein-derived peptides on ileal endogenous

protein losses was similar between peptides from pre-digested casein and peptides naturally released in the gut during casein digestion. In contrast, it was previously reported in pigs that enzyme-hydrolysed casein enhanced ileal endogenous protein flows in comparison with normal casein (Yin *et al.*, 2004). However, a study in chickens reported that both forms of casein (hydrolysed or normal) induced similar ileal endogenous protein flows (Ravindran *et al.*, 2004). Overall, the present work supports the use of peptide alimentation (especially casein-derived peptides), combined with digesta ultrafiltration and centrifugation, previously reported as a valid technique to separate endogenous protein from total protein in digesta samples (Hodgkinson & Moughan, 2001), for determining ileal endogenous N and AA losses. The enzyme-hydrolysed protein/ultrafiltration method is of practical interest for routine determination of ileal endogenous protein flows, and thus more generally for evaluation of true protein digestibility. Indeed, this method avoids the shortcomings of the protein-free diet method but is still a straightforward technique allowing direct determination of ileal endogenous flows of N as well as all AA. The same cannot be said for alternative methods such as guanidination, <sup>15</sup>N-isotope dilution, linear regression.

The present results are based on one defined extent of casein hydrolysis, somewhat lower than that used in previously reported studies. The impact of the hydrolysate profile on endogenous protein flow remains unknown.

Body N balance *per se*, after one week of dietary adaptation, was shown to have no influence on ileal endogenous N and AA flows in the growing rat when free AA-based and protein-free diets were compared (Chapter III). Similar conclusions have been drawn in the past (Skilton *et al.*, 1988; de Lange *et al.*, 1989; Darragh *et al.*, 1990; Butts *et al.*, 1992; Leterme *et al.*, 1996a). Similarly, free AA-based diets compared with intact or hydrolysed protein-based diets (with matching AA compositions) induced generally lower ileal endogenous AA flows in the growing pig after one week of dietary adaptation (Chapter IV), which is also in line with previous results (Darragh *et al.*, 1990; Chung & Baker, 1992; Butts *et al.*, 1993). However, this was not observed when these diets (free AA, hydrolysed or intact casein-based) were given to pigs or humans acutely, whereby ileal endogenous flows as determined directly for the omitted AA from the free AA-based diet were similar or somewhat higher than ileal endogenous AA flows after ingestion of peptide- or protein-

based diets (Chapter V and VI). This result was surprising. Adaptation to the different forms of delivery of dietary AA (free, peptide or protein-bound) may induce metabolic/physiological changes in the gut, as has been previously suggested (Poullain *et al.*, 1989; Zaloga *et al.*, 1991; Daniel, 2004; Guay *et al.*, 2006), potentially influencing the amount of endogenous protein recovered at the terminal ileum.

When comparing the isotope dilution and hydrolysed protein/ultrafiltration methods for determining ileal endogenous protein flows, different results were obtained for the rat and pig experiments (chapter IV and V). Compared with the hydrolysed protein/ultrafiltration method, the isotope dilution technique resulted in ileal endogenous AA and N flows that were lower within the rat study (Chapter IV) but similar (although numerically higher) within the pig study (Chapter V). Both methods rely on different assumptions and are thus likely to respond differently to the experimental conditions in which they are applied, which included on the one hand the growing rat fed with a frequent-feeding regimen, and on the other hand, the growing pig subjected to a meal-feeding regimen. The experimental design might have influenced the extent of tracer recycling, which was higher in the rat study than in the pig study (65 and 21% of ileal endogenous proteins were  $^{15}\text{N}$ -labelled in rats and pigs, respectively). In the meal-fed humans, tracer recycling was minimal with only 11% of ileal endogenous protein being  $^{15}\text{N}$ -labelled.

Tracer recycling arises from the incorporation of dietary N into gut endogenous proteins subsequently secreted into the intestinal lumen (Alpers, 1972; Leterme *et al.*, 1996b; Stoll *et al.*, 1998). Some of these  $^{15}\text{N}$ -labelled endogenous proteins escape further digestion and reabsorption and are recovered at the terminal ileum. Thus,  $^{15}\text{N}$  incorporated in these endogenous proteins is falsely accounted as a tracer for unabsorbed dietary N, resulting in an underestimation of endogenous protein flows. It was previously reported in pigs that some dietary  $^{15}\text{N}$  tracer was incorporated into pancreatic enzymes within 50 min, in bile secretion within 90 min and in mucins within 4 h (Leterme *et al.*, 1996b). Additionally, it has recently been reported that microbial proteins, considered as endogenous, although this is not strictly true, may metabolise luminal dietary N (Bartelt *et al.*, 1999; Libao-Mercado *et al.*, 2007), thus contributing to tracer recycling. However, the present findings also demonstrate that tracer recycling induces only a slight underestimation of endogenous N flows in meal-fed pigs and humans (Chapter V and Chapter VII) and hence has a negligible

effect on the calculation of true ileal digestibility. Overall, the present work supports the use of the  $^{15}\text{N}$ -isotope dilution method, especially with a meal-feeding regimen, in pigs and humans to determine endogenous N and AA flows and the true ileal digestibility of dietary protein.

The present work demonstrates that both forms of casein (intact or hydrolysed) are highly digestible (above 93%), as determined at the terminal ileum of the rat, pig and human using  $^{15}\text{N}$ -labelled dietary protein (Chapter IV, VIII). The present digestibility values were in close agreement with previously reported values for  $^{15}\text{N}$ -labelled casein or  $^{15}\text{N}$ -labelled milk protein (Bos *et al.*, 1999; Gaudichon *et al.*, 1999; de Vrese *et al.*, 2000). The true ileal N digestibility of hydrolysed casein was close to that of intact casein ( $\pm 3\%$ ) although the relative results differed among species. In rats, the true ileal N digestibility of hydrolysed casein was slightly higher (3%), but statistically significantly so, than that of intact casein (Chapter IV), whereas, in pigs and humans, it was slightly lower (2–3%) and close to statistical significance (Chapter VIII). This discrepancy probably results from the different methodologies used across species.

The pig is of special interest in digestibility studies as its digestive tract is very similar to that of the human, both anatomically and physiologically, and to a greater extent than that of the rat (Pond & Houpt, 1978; Miller & Ullrey, 1987; Moughan & Rowan, 1989; Moughan *et al.*, 1992). In the present work, a controlled comparison of pig and human true ileal protein digestibilities showed a similar ranking of proteins (intact casein, hydrolysed casein and rapeseed protein) between the species, and a statistically significant correlation for true ileal AA digestibilities between the species (Chapter VIII). This agrees with previous less comprehensive findings obtained at the ileal (Rowan *et al.*, 1994) and faecal levels (Forsum *et al.*, 1981; Rowan *et al.*, 1994; Darragh & Moughan, 1995). It is interesting to note that recent data have suggested that rats might be able to digest normally dietary proteins poorly digestible in humans, such as rapeseed protein for which the true ileal digestibility was 84–87% in humans versus 95% in rats (Bos *et al.*, 2007; Boutry *et al.*, 2008). Overall, the present findings support the use of the growing pig as an animal model for predicting true ileal protein digestibility in the adult human.

Within each species, the best technique available was used to access ileal digesta samples, i.e. a post-valve T-caecum cannula (PVTC) in pigs or a naso-ileal tube in humans. The

PVTC cannula was previously reported to allow for representative digesta collection (Kohler *et al.*, 1990; Kohler *et al.*, 1991; Hodgkinson *et al.*, 2000). The intubation method was shown in the present work to allow for accurate sampling of ileal digesta from the conscious adult human (Chapter VI).

The present study confirmed the specific impact of the form of delivery of dietary AA on their rate of appearance in the peripheral blood (Chapter VII) as previously reported (Collin-Vidal *et al.*, 1994; Metges *et al.*, 2000; Dangin *et al.*, 2001; Calbet & Holst, 2004). This was likely due to a faster digestion/absorption rate of dietary AA from hydrolysed casein compared with that from intact casein, probably related to a faster gastric emptying rate (Mahé *et al.*, 1992; Mahé *et al.*, 1996) and/or intestinal transit of hydrolysed casein (Daniel *et al.*, 1990; Allescher *et al.*, 2000; Patten *et al.*, 2001; Mihatsch *et al.*, 2005). Based on these considerations, hydrolysed casein can be considered as a fast-digested protein, and intact casein as a slow-digested protein as previously reported (Boirie *et al.*, 1997). Differences were observed in terms of AA catabolism kinetics between the two forms of casein; these were compensated for, however, over the entire postprandial period, maybe through different urea recycling amplitude and kinetics between meals. Overall, both forms of casein elicited the same nutritional value, which is in agreement with previous N balance studies (Moriarty *et al.*, 1985; Yamamoto *et al.*, 1985; Chan *et al.*, 1993; Sales *et al.*, 1995). The present result contradicts the paradigm, based on the observation of whole body <sup>13</sup>C-leucine kinetics, that slow-digested proteins promote protein retention compared with fast-digested proteins (Collin-Vidal *et al.*, 1994; Boirie *et al.*, 1997; Metges *et al.*, 2000; Daenzer *et al.*, 2001; Dangin *et al.*, 2001). The present results suggest that the correlation between digestion/absorption rates and net protein retention, indicating the protein nutritional value, is more complex than first suggested by the slow/fast protein concept (Boirie *et al.*, 1997).

The result, showing a similar nutritional value between hydrolysed and intact casein when ingested within a mixed meal, is of practical importance as hydrolysed casein may be used in formulas for infants with recognized or suspected milk allergy (Hernell & Lonnerdal, 2003) or for patients with compromised digestive capacities (Meier *et al.*, 2006).

In the absence of a specific labelling, the present study did not provide information

regarding the partitioning of dietary AA from the meal based on free AA (meal A), but this may be of particular interest for future research as dietary free AA are used in isotopic methods for determining dietary AA requirements.

In summary, the following conclusions can be drawn:

- Within a practical context, antinutritional factors (from a crude extract of *Phaseolus vulgaris*) enhanced rat ileal endogenous protein flows when ingested in the higher range of what humans usually consume.
- A casein hydrolysate, compared with the parent intact casein, did not enhance ileal endogenous protein flows as determined in animal models (rats and pigs) as well as in humans.
- The intubation method used for sampling intestinal digesta was shown to allow for an accurate sampling of intestinal digesta from conscious human subjects.
- The form of delivery of dietary AA (peptide- or protein-bound) influenced the postprandial metabolic fate of dietary AA, especially in terms of AA catabolism kinetics, however this did not influence the overall nutritional value of these dietary AA.
- The growing pig appeared to be a valid animal model for predicting true ileal protein digestibility in the adult human.

Based on the present results, the following recommendations for future research can be proposed:

- To assess the influence of the degree of hydrolysis of a casein hydrolysate on ileal endogenous protein flows
- To assess the influence of purified proteins on ileal endogenous protein flows
- To compare ileal AA digestibility between the growing pig and adult human for a wider range of protein sources, including non-purified protein sources and mixed protein sources.
- To explore further the metabolic events associated with the ingestion of hydrolysed casein in comparison with intact casein, especially the regional body distribution of dietary N, the postprandial protein anabolism and the amplitude of urea recycling.

- To investigate further the influence of the delivery form of dietary AA, especially in comparison with free crystalline AA, firstly on the importance of the dietary adaptation and potential modifications in gut metabolism/physiology, and secondly on the postprandial consequences for AA metabolism.

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