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Amino acid availability: aspects of chemical analysis and bioassay methodology

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It is important to be able to characterise foods and feedstuffs according to their available amino acid contents. This involves being able to determine amino acids chemically and the conduct of bioassays to determine amino acid digestibility and availability. The chemical analysis of amino acids is not straightforward and meticulousness is required to achieve consistent results. In particular and for accuracy, the effect of hydrolysis time needs to be accounted for. Some amino acids (for example, lysine) can undergo chemical modification during the processing and storage of foods, which interferes with amino acid analysis. Furthermore, the modified amino acids may also interfere with the determination of digestibility. A new approach to the determination of available lysine using a modified *in vivo* digestibility assay is discussed. Research is required into other amino acids susceptible to structural damage. There is recent compelling scientific evidence that bacterial activity in the small intestine of animals and man leads to the synthesis and uptake of dietary essential amino acids. This has implications for the accuracy of the ileal-based amino acid digestibility assay and further research is required to determine the extent of this synthesis, the source of nitrogenous material used for the synthesis and the degree of synthesis net of amino acid catabolism. Although there may be potential shortcomings in digestibility assays based on the determination of amino acids remaining undigested at the terminal ileum, there is abundant evidence in simple-stomached animals and growing evidence in human subjects that faecal-based amino acid digestibility coefficients are misleading. Hindgut microbial metabolism significantly alters the undigested dietary amino acid profile. The ileal amino acid digestibility bioassay is expected to be more accurate than its faecal-based counterpart, but correction of the ileal amino acid flow for amino acids of endogenous origin is necessary. Approaches to correcting for the endogenous component are discussed.

Protein: Amino acid availability: Lysine: Digestibility: Bioassays

Introduction

Determination of the availability of dietary amino acids is a central concept in the study of nutrition. In human nutrition and in specific situations where dietary amino acid supply may potentially limit protein metabolism (for example, infancy, the immune compromised, the elderly, the malnourished) it is important to be able to accurately monitor the dietary supply of 'available' amino acids in relation to the dietary requirement. The ability to score proteins in terms of their 'quality' (for example, protein digestibility-corrected amino acid score) is also of importance in the international trading of proteinaceous foods for human consumption. For farm and companion animals, amino acids are a relatively costly component of the dietary formulation, and consequently there is a direct economic imperative to match the dietary available amino acids with the designated estimate of requirement to minimise wastage of the dietary amino acids. The term 'availability' has been one that has caused considerable confusion. Sometimes the term is used synonymously with 'digestibility' as a measure of the release and disappearance from the gut lumen of a dietary amino acid during digestion, but more commonly has been used to describe the release, uptake and subsequent post-absorptive utilisation of a dietary amino acid. The latter definition immediately raises the question: Utilisation for what purpose and under what conditions? Absorbed amino acids can be used by the cell for a number of purposes with different metabolic fates. In the present review, the term 'available amino acid' refers to the amount of an amino acid in a diet or food that is absorbed from the lumen of the digestive tract in a chemical form such that the amino acid can potentially be used for body protein synthesis. Following this classical definition (Fuller, 1998), the 'availability' of an amino acid may be quite different from the empirically determined digestibility value or from

Abbreviation: FDNB, 1-fluoro-2, 4-dinitrobenzene.

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the ultimate degree of utilisation, or 'utilisability'. The latter measure is dependent upon a number of dietary and animal factors and is highly variable. It is the very fact that the utilisation of a dietary amino acid for body protein synthesis is affected by numerous dietary (for example, energy supply, vitamins, minerals, dietary amino acid imbalance, anti-nutritional factors) and non-dietary (for example, gender genotype, thermal environment) factors, which are difficult to control experimentally, that explains the inherent shortcomings (Hurrell & Carpenter, 1984) in methods for determining amino acid utilisability such as the slope-ratio-assay (Finney, 1964; Carpenter & Booth, 1973).

Recently an interesting approach has been developed (Bos *et al*. 1999; Mariotti *et al*. 1999; Tomé & Bos, 2000; Fouillet *et al*. 2002) whereby dietary amino acid disappearance to the end of the small intestine and subsequent postprandial amino acid deamination are determined in a subject, following the ingestion of a single meal. The procedure gives a single acute measure of net postprandial protein utilisation, but under well-defined experimental conditions. More generally, however, a reductionist approach has been followed in the determination of amino acid availability, centring on determining amino acid content in foods, describing alterations in the chemical structures of amino acids and determining the amounts of amino acids disappearing from the digestive tract in a form suitable for use as a substrate for body protein synthesis.

The present review addresses aspects of the chemical analysis of amino acids, structural changes to amino acids that can occur during processing and storage, and biological assays for the determination of amino acid digestibility. The review is referenced for the most part to man and the growing pig (a suitable model animal for protein digestion in man; Moughan *et al*. 1992, 1994), but the concepts developed are applicable to simple-stomached mammals and birds in general.

Amino acid analysis

To measure all of the individual amino acids present in a foodstuff or protein, it is necessary to hydrolyse the peptide bonds linking the amino acids, without destroying the amino acids themselves. Because some of the common amino acids are relatively labile to the chemical treatments needed to break the peptide bond, several hydrolytic methods have been developed. It is not possible, to date, to simultaneously analyse for all of the twenty common amino acids in a food matrix.

Modern amino acid analysis is capable of providing data with a within-laboratory repeatability of 5 % or less and a reproducibility between laboratories of around 10 %, but to achieve such results requires careful attention to detail. The main methods of amino acid analysis involve acid or alkaline hydrolysis of the protein followed by separation and quantification of the released amino acids by ion-exchange, gas–liquid or reverse-phase HPLC. Most of the nutritionally important amino acids can be quantified after acid (usually HCl) hydrolysis, but separate hydrolysis procedures need to be used for the quantification of methionine, cysteine and tryptophan. Amino acid analysis is relatively complex and many methods and modifications to methods have been reported in the literature. Comprehensive reviews on the topic have been made by several authors (Finley, 1985; Gehrke *et al*. 1985; Hare *et al*. 1985; Williams, 1988, 1994; Baxter, 1996; Rutherfurd & Moughan, 2000) to which the reader is referred. There is, however, one particular aspect of amino acid analysis, hydrolysis time, that merits special mention.

Hydrolysis interval

During acid hydrolysis of proteins, some amino acids may be released and destroyed while yet others may be slow to be released from the protein and may require longer than the designated hydrolysis interval (often 24 h in 6 M-HCl in an O_2-free environment at 110°C) for complete hydrolysis and release of the amino acid. In particular, valine, leucine and isoleucine are released only slowly during acid hydrolysis while the labile amino acids, serine and threonine, are continuously destroyed. Thus a standard 24 h hydrolysis interval is a compromise and does not allow a quantitatively accurate determination of amino acid content. For the two different reasons discussed earlier, the results from a 24 h hydrolysis may be underestimates. When absolute accuracy is required, multiple hydrolysis intervals should be employed and amino acid yields obtained by extrapolation. Standard correction factors have been developed (for example, TNO, The Netherlands, 22 h HCl hydrolysis at 110°C: threonine, 1·05; serine, 1·10; valine, 1·07; isoleucine, 1·08), but the response to hydrolysis time is non-linear and varies among types of protein, foods and other biological materials. A more appropriate solution is to derive, using sequential hydrolysis times, curvilinear mathematical relationships describing the simultaneous amino acid release from a protein and amino acid destruction during hydrolysis (Robel & Crane, 1972). Taking into account the estimated rate of release of amino acids and their subsequent rate of destruction during hydrolysis and based on extrapolation, these mathematical models can be used to accurately predict the content of all amino acids from a single 24 h hydrolysis. The model needs to be parameterised for each protein source and as such is costly and time-consuming to develop. Once a model is developed for a particular protein source, however, it can be used routinely to correct standard 24 h hydrolyses. More recently (Darragh *et al*. 1996), an algorithm has been developed based on these principles, to accurately predict the amino acid content of lysozyme. As lysozyme has been sequenced and its amino acid composition is known with certainty, this provides a means of validating the curvilinear correction model (Table 1). The predicted amino acid concentrations were closer to the actual values (sequencing) than values obtained by standard 24 h hydrolysis. The single 24 h hydrolysis often considerably underestimates the amount of an amino acid in a protein. Work in the author's laboratory has led to the development of a modified curvilinear correction model for application to human milk (Darragh & Moughan, 1998) and a model applied to animal hair (Hendriks *et al*. 1998).

It is sometimes considered that the effect of hydrolysis time on amino acid yield is not relevant in the determination of coefficients of amino acid digestibility:

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Table 1. Estimates of the amino acid composition of egg-white lysozyme determined by non-linear least-squares regression using multiple hydrolysis intervals (Ao) compared with the conventional 24 h hydrolysis value and the known amino acid composition (Actual) based on sequencing (from Darragh *et al.* 1996)

	Amino acid composition*		
Amino acid	Ao	24 h	Actual
Glycine	12·1	10·4	12
Serine	9·8	8·4	10
Threonine	6·9	6·3	7
Valine	5·8	5·8	6
Isoleucine	6·0	5·4	6
Leucine	8·0	7·5	8
Lysine	6·1	5·6	6
Arginine	11·0	10·3	11
Cysteine†	8·0	6·5	8

*Expressed as moles amino acid/mole lysozyme.
†Detected as cysteic acid.

$$\left(\begin{matrix}\text{Amino acid}\\ \text{digestibility}\end{matrix} = \frac{\text{amino acid input} - \text{amino acid output}}{\text{amino acid input}}\right),$$

as the errors of determination related to the input and output parts of the equation cancel each other out, and there is no net effect on the coefficient of digestibility. This is only the case, however, if the error of determination is constant across different proteins and materials. The study of Rowan *et al*. (1992*a*) demonstrates that this is not so. Rowan *et al*. (1992*a*) addressed the effect of hydrolysis time during amino acid analysis on individual amino acid yields from samples of a mixed diet, ileal digesta and faeces. Food, digesta and faeces samples were hydrolysed in duplicate in 6 M-HCl in sealed evacuated tubes for 16, 24, 48 and 72 h, and then analysed for their yields of alanine, arginine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine. There was a statistically significant ($P < 0{\cdot}05$) curvilinear effect of hydrolysis time on the yields of all amino acids except tyrosine. The changes in isoleucine, lysine and serine yields with hydrolysis time were parallel for the three types of samples, but for the other amino acids (except tyrosine) there were significant ($P < 0{\cdot}05$) hydrolysis interval × source interactions. Therefore, where a high degree of accuracy in determining the coefficient of amino acid digestibility is required, the differential effect (across types of material) of hydrolysis time on amino acid yield may need to be taken into account. The effects of hydrolysis time on amino acid analysis deserve attention and require further research.

Structural changes to amino acids occurring during processing and storage

Conventional amino acid analysis does not detect changes in amino acid structure that may have come about during processing or during storage of material. Yet such changes in structure may have important effects nutritionally, and have implications for the determination of amino acid availability. When foods are processed or stored at air temperature, chemical reactions occur between the protein fraction and other food components (see Fig. 1). Apart from the destruction of some vitamins, the reactions of food proteins are the main chemical reactions that occur during food processing. Proteins may react with fats and their oxidation products, polyphenols, vitamin B_6, various chemical additives, but perhaps most importantly with reducing sugars. A number of amino acids are involved in these reactions, leading to alterations in the structure of the respective amino acid. One of the most reactive amino acids, and certainly the most studied, is lysine. Because structural changes (damage) to lysine are not detected using conventional amino acid analysis, alternative *in vitro* procedures have been developed to monitor the 'reactive' lysine content of foods. The term 'reactive' is used to describe lysine molecules that have not undergone any form of structural change and are therefore available to the animal nutritionally; the epsilon amino group remains unaltered (i.e. unblocked) and thus is able to link with other compounds, that is, it is 'reactive' (see Fig. 2). The various methods

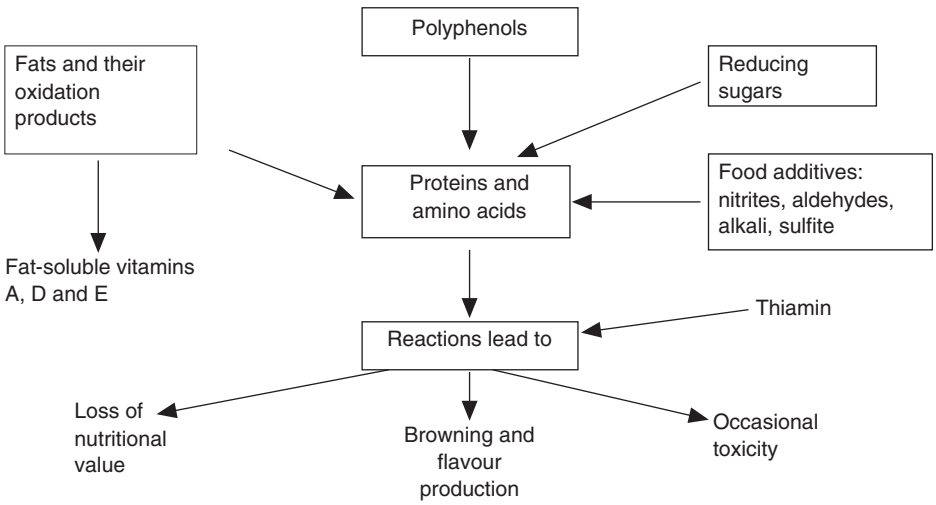

Fig. 1. Some important interactions of food components on processing (from Hurrell & Carpenter, 1984).

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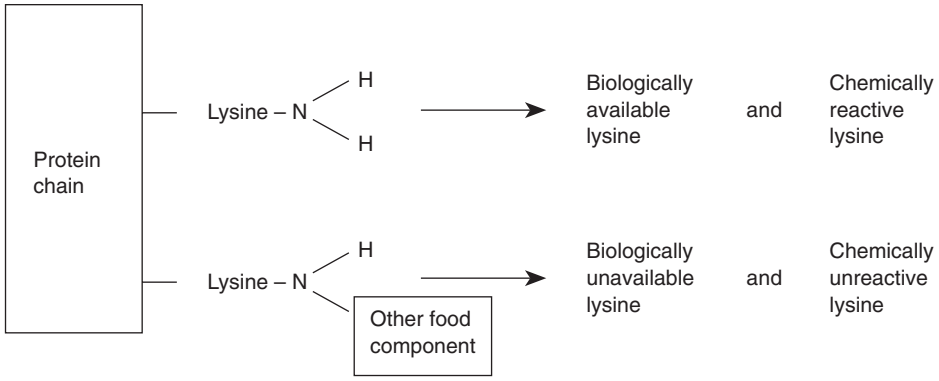

Fig. 2. Theory of the reactive lysine estimation (from Hurrell & Carpenter, 1984).

developed to determine reactive lysine in foods appear to give similar results in general, though it seems that after early Maillard reactions (reactions between lysine and reducing sugars), some procedures can seriously underestimate the degree of nutritional damage. The best methods for monitoring reactive lysine after early Maillard reactions (the reactions most affecting the determination of lysine availability) appear to be the direct 1-fluoro-2, 4-dinitrobenzene (FDNB) method, the guanidination method, the borohydride method and, specifically for milk-based products, the furosine technique (Hurrell & Carpenter, 1981, 1984).

The Maillard reaction has been studied intensively and is known to be a potentially significant cause of loss of nutritional value in foods and feedstuffs. In the Maillard reaction, lysine reacts with reducing sugars to form early or late Maillard components. In the advanced stages of the Maillard reaction (brown pigment formation) the amino acid will have been completely destroyed and is not recoverable following acid hydrolysis during amino acid analysis. However, in the early stages of the reaction, which occur under normal conditions of food processing and storage, the deoxyketosyl derivative (Amadori compound) formed is hydrolysed back to lysine in the presence of strong acids. Such reversion does not occur in the mammalian digestive tract and the deoxyketosyl derivative, although being partly absorbed, has no nutritional value. Thus for foods that have undergone the early Maillard reaction during processing, conventional acid hydrolysis will lead to an overestimation of the lysine content. At least for the amino acid lysine, and for foods whereby a significant degree of structural alteration to lysine may have occurred, a measure of reactive lysine is preferable to conventional lysine analysis. The degree of overestimation of lysine using conventional procedures can be significant even for mildly processed material (Table 2). Other amino acids (particularly arginine, methionine, cysteine and tryptophan) are undoubtedly also susceptible to processing and especially heat treatment, but the chemistry of the reactions is not as well characterised as for lysine, and the degree of error introduced by using conventional amino acid analysis is not clearly established.

During food processing and quite apart from direct reactions involving specific amino acids, the availability of all amino acids can be reduced (Erbersdobler, 1976) due to cross-linkages being formed between protein chains. Such cross-linking can give rise to 'limit peptides' during digestion. In this manner amino acids and lysine units with free reactive epsilon amino groups (i.e. reactive lysine) may be unavailable, passing unabsorbed into the large intestine and faeces (Hurrell *et al*. 1976). The latter crucial observation has implications for developing digestibility assays for lysine, as not all of the 'reactive' lysine as determined by assays such as FDNB-lysine may be absorbed and thus available. This matter will be discussed later (p. 135) in the present review in the context of digestibility assays.

Biological assays for the determination of amino acid digestibility

The digestion of food proteins in the gastrointestinal tract is a complex set of processes, involving interaction among the food, a series of endogenous enzymes and other materials secreted by the animal and the microbial flora resident throughout the gut. It is necessary to understand these processes in order to develop sound bioassays for the determination of dietary amino acid digestibility.

Table 2. The effect of heat treatment (extrusion) on the 1-fluoro-2, 4-dinitrobenzene (FDNB)-available and total lysine contents of soyabean meal and peas (from Hendriks *et al.* 1994)

	Conditions		Lysine content (mmol/100 g DM)*	
	Temperature (°C)	Moisture (%)	FDNB-lysine	Total lysine
Soyabean	Untreated	Untreated	22·7	22·8
	113	25	22·3	22·8
	139	30	20·4	22·3
	135	40	19·6	22·1
Peas	Untreated	Untreated	11·6	11·9
	106	30	11·6	11·9
	140	30	11·1	11·9
	140	15	9·7	11·8

*After 22 h acid hydrolysis.

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Digestion: endogenous enzymes

Protein digestion and the uptake of amino acids occur throughout the digestive tract. Ingested food is partly broken down by mastication in the mouth. Gastric digestion of protein is then carried out by the pepsins secreted by the chief cells. The precursors of pepsins, the pepsinogens, are activated by HCl also secreted in the stomach, and by the autocatalytic activity of pepsin. Estimates of the degree of the digestion of protein in the stomach vary from a low of 10–15 % of protein ingested (Borgstrom *et al*. 1957) to around 50 % of protein ingested being broken down to peptides having ten or fewer amino acids (Low, 1990). It appears that there is no absorption of amino acids in the stomach (Zebrowska, 1980; Zebrowska *et al*. 1983). Proteins and peptides passing from the stomach are further hydrolysed in the small intestine by enzymes secreted by the pancreas, and by intestinal enzymes that are either secreted into the lumen of the gut, attached to the brush border of the intestine, or are active within the mucosal cells. The pancreatic proteinases, secreted as their inactive precursors (zymogens), comprise trypsin, chymotrypsin and elastase while the pancreatic peptidases comprise carboxypeptidases A and B. The important function of the brush-border oligopeptidases and the intracellular peptidases of the mucosal cells in integrated digestion and absorption has been discussed by Das & Radhakrishnan (1976), and transport systems involved in the absorption of amino acids have been reviewed by Davenport (1977) and Webb (1990). It is now considered that in addition to free amino acids, small peptides arising from protein digestion may enter the portal circulation directly (see Reeds & Beckett, 1996).

Digestion: microbial enzymes

In addition to the action of endogenous enzymes in protein digestion, the proteolytic, deaminative and decarboxylative activities of the intestinal microflora must be considered. Micro-organisms are found throughout the alimentary canal, and these undoubtedly make a contribution to the digestive process. Bacterial activity is considered to be mainly concentrated in the large intestine, this being in accordance with the hindgut having the most dense bacterial population (Rerat, 1978). However, although the upper digestive tract has a lower population of micro-organisms, the possible effect that this may have on digestion cannot be discounted especially in view of the rapid turnover of bacterial cells (Boorman, 1980). Bacterial proteases may enhance the digestion of dietary protein in the small intestine (Coates, 1976), though also microbial fermentation may result in the degradation of amino acids. Furthermore, microbial metabolism may result in structural alterations to amino acids and to their synthesis. Urea secreted into the gut in saliva, gastric juice, bile and other secretions or entering the gut by simple diffusion is hydrolysed by the gut bacteria to NH_3 and CO_2. The NH_3-N, in turn, can be reabsorbed or can become fixed in bacterial protein, with possible subsequent reabsorption after breakdown of the bacterial body. Such urea-N cycling has long been recognised as important in ruminants. It is now being recognised that such cycling may also be quantitatively important in non-ruminants, and may be regulated (Jackson *et al*. 1990; Danielsen & Jackson, 1992).

The significance of the microflora inhabiting the large intestine to protein digestion has been appreciated for some time. Nitrogenous material entering the large intestine consists of undigested dietary protein, peptides and free amino acids as well as products of endogenous origin such as digestive enzymes, mucoproteins, desquamated cells, urea, amino acids and proteins such as serum albumen. These nitrogenous materials may be acted upon by the hindgut bacteria leading to a net appearance or disappearance of amino acids between the ileo-caecal valve and the rectum (Rerat, 1981). In certain cases (Holmes *et al*. 1974; Mason *et al*. 1976; Low, 1979) faecal amounts of some amino acids have been higher than the amounts measured at the terminal ileum, indicating that net bacterial synthesis has occurred. Such synthesis may be particularly significant for methionine (Just, 1980). The assimilation of nitrogenous materials into microbial cell components is based principally on NH_3 (Mason, 1980), although peptides and amino acids can be utilised directly by some bacterial species (Payne, 1975). The fact that microbes resident in the gut can assist to break down proteins (both dietary and endogenous), can transform amino acids and degrade them and urea to NH_3 and can synthesise amino acids from simple (for example, NH_3) and more complex precursors, has important implications for the determination of amino acid digestibility. Recently, compelling evidence has been published, from studies using stable isotopes, that essential amino acids are synthesised by gut microbes and are absorbed. This work has been the subject of recent review (Fuller & Reeds, 1998; Metges, 2000). Of particular note in the studies reported is the detection of labelled lysine in the body. As lysine is not subject to transamination, any labelled lysine appearing in the portal blood or body tissues must originate from microbial synthesis and, discounting coprophagy, must have been absorbed from the digestive tract. How can this occur?

Clearly, given the intense microbial metabolic activity that occurs in the hindgut of simple-stomached mammals, if amino acids are absorbed by the hindgut, then potentially, bacterial amino acid synthesis could make an important contribution to the host.

Potential for amino acid absorption from the large intestine

The ability of large-intestinal tissue to absorb amino acids has been investigated in a number of studies. Several studies point to the potential for a limited degree of absorption for some amino acids. Fordtran *et al*. (1964) infused tryptophan *per rectum* into normal subjects and found that the tryptophan metabolites indican and indole-3-acetic acid rapidly increased in the urine, suggesting that at least the C skeleton of tryptophan can be absorbed in the large intestine. Further, Niiyama *et al*. (1979) reported the appearance of ^{15}N-labelled amino acids in the colic branch of the ileocolic vein 3 h after infusing labelled material into the caecum of the pig. James & Smith (1976) demonstrated that the proximal colon of the newborn piglet has the ability to actively transport methionine and Olszewski & Buraczewski (1978) provided evidence that asparagine, serine, threonine, tyrosine, arginine, histidine, lysine and aspartic acid are absorbed to varying degrees from isolated

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pig caecum examined *in situ*, whereas the remaining ten amino acids investigated were not absorbed. More recently (Ugawa *et al*. 2001) a colonic amino acid transport system has been characterised in the mouse and evidence has been recorded (Doring *et al*. 1998) of colonic peptide transport. Binder (1970), however, found that mammalian colonic mucosa does not have significant amino acid absorptive capacity and Wrong *et al*. (1981), in giving a critical overview on the subject, concluded that the true active transport of amino acids across the large bowel has not been convincingly demonstrated in any adult animal. McNeil (1988) who also reviewed the topic concluded that evidence for amino acid absorption by the human large intestine under normal conditions is lacking.

A number of experiments have been conducted to evaluate the effects on body N balance of infusions of protein, hydrolysed protein or amino acids into the caecum or colon (Zebrowska, 1973, 1975; Sauer, 1976; Hodgson *et al*. 1977; Gargallo & Zimmerman, 1981; Just *et al*. 1981). These studies demonstrated that the infused N, although being well absorbed by the large intestine, was almost entirely excreted in the urine. Fuller & Reeds (1998), however, in reviewing these infusion experiments have pointed out that there is a tendency in most of the studies toward improved N balance, which suggests that with greater experimental precision, statistically significant differences may have been found. A more specific, and perhaps discerning, approach has been afforded by well-controlled studies involving large-intestinal infusions of single dietary essential amino acids, for animals given a diet clearly deficient in the infused amino acid (Wunsche *et al*. 1982; Darragh *et al*. 1994). There was no significant improvement in N balance thus indicating zero or negligible amino acid (lysine, isoleucine, methionine) absorption from the large intestine. Furthermore, in investigations whereby homoarginine (an analogue of lysine) has been infused into the large intestine of the growing pig, homoarginine has not been detected in the blood, post-infusion (Schmitz *et al*. 1991).

It appears, therefore, that while some limited uptake of amino acids in the large intestine may occur, intact amino acids are not absorbed in amounts that are nutritionally significant. It is clear that N is absorbed (mainly as NH_3) from the large intestine but the overall evidence for more than minor absorption of intact amino acids and peptides is less than convincing. The only other possible route for the absorption of amino acids from the large intestine of non-coprophagic species is that large-intestinal contents are refluxed to the terminal ileum, whereby absorption occurs. Although reflux cannot be entirely discounted (Cuche & Malbert, 1998; Hess & Sève, 1999), it seems unlikely that this would be a quantitatively significant source of material.

If microbially synthesised amino acids are not absorbed from the large intestine, is it possible that they originate from microbial activity in the upper digestive tract?

Potential for microbial amino acid synthesis and absorption in the upper digestive tract

The stomach and small intestine, particularly of man, have traditionally been considered to be virtually sterile (Hill, 1982). Also, the upper-gut has a rapid rate of passage of digesta (Clemens *et al*. 1975), giving limited time for microbial metabolism. Thus the traditional view has been one whereby contributions of microbes resident in the upper digestive tract of simple-stomached mammals have been largely ignored.

Results from different studies are conflicting, but overall it appears that the upper digestive tract of man is colonised with micro-organisms, although they appear to be present in low numbers. Lactobacilli and yeasts have been found in the stomach and small intestine but in relatively low numbers, while there is little evidence of substantial colonisation by *Escherichia coli*, clostridia, streptococci, bacteroides and veillonellae. Concentrations of microbes tend to increase distally, with relatively high numbers of *E. coli*, lactobacilli and bacteroides being found in the lower to terminal ileum (Williams Smith, 1965; Drasar *et al*. 1969; Drasar & Hill, 1974; Moore *et al*. 1978; Drasar & Barrow, 1985). In contrast to man, the pig has a well-established upper-tract microflora with *E. coli*, clostridia, streptococci, lactobacilli, yeasts and veillonellae being reported to be present in relatively high numbers from the stomach to the lower small intestine, though bacteroides have not been reported as being present (Horvath *et al*. 1958; Williams Smith, 1965; Cranwell, 1968). Overall, it would seem that micro-organisms are found throughout the gut of man and pigs, and the types of micro-organisms present are similar. The pig's upper tract, however, appears to host a greater microbial population than that of man.

Other indirect evidence for microbial activity in the upper digestive tract comes from studies of the ileal digestibility of plant NSP. There is evidence in both human subjects and the growing pig that often quite substantial amounts of plant fibre can be degraded in the upper digestive tract (Cranwell, 1968; Keys & DeBarthe, 1974; Holloway *et al*. 1978, 1980, 1983; Kass *et al*. 1980; Sandberg *et al*. 1981; Millard & Chesson, 1984; Rowan *et al*. 1992*b*, 1994). Furthermore, Jensen (1988), measuring ATP concentration and adenylate energy charge in the gut of pigs, has shown that microbial metabolic activity is as high in the distal ileum as it is in the caecum. These latter studies suggest that there is an active microflora in the upper digestive tract of both human subjects (at least ileostomised subjects) and pigs, though the results obtained in these studies may in part reflect the surgical preparations and sampling techniques used. Moreover, the fermentation of carbohydrates does not necessarily imply an accompanying high rate of microbial breakdown of protein (Drasar & Hill, 1974; Hungate, 1978).

There is contrary evidence pointing towards minimal upper-tract microbial activity. For example, Cohen *et al*. (1983) and Englyst & Cummings (1985) found virtually no breakdown of NSP in human ileostomates. Moreover, there are reports of only relatively low amounts of short-chain fatty acids being recovered in jejunal (Chernov *et al*. 1972) and terminal ileal (Dawson *et al*. 1964) contents in human subjects. Also, Macfarlane *et al*. (1986) reported low amounts of NH_3 and volatile fatty acids in small-intestinal digesta taken from sudden-death victims.

Taking all the evidence together it seems that, certainly in the pig and probably in man as well, there is the potential for considerable microbial breakdown of protein and amino

acid catabolism and synthesis in the upper digestive tract. The net effect of such activity and its practical importance in a nutritional context, however, is quite another matter. In relation to nutrition, the net result of amino acid synthesis and catabolism is what is of importance. Dietary essential amino acids may be synthesised by microbes in the small intestine from dietary and endogenous proteins and other nitrogenous materials, but these amino acids may also be catabolised by microbes.

There is no clear-cut information on the extent of net microbial amino acid synthesis in the stomach and small intestine (Fuller & Reeds, 1998) and further definitive studies are required.

In contrast to the current debate concerning the possible effects of upper-gut microbes and the effect these may have on amino acid digestibility determination, there is general agreement concerning the effects on dietary amino acid digestibility determination consequent upon the intense proteolytic activity of the hindgut microflora.

Ileal v. *faecal measures of digestibility*

An indication of the significance of the hindgut microbial metabolism is that around 80 % of faecal N is present in microbial bodies (Low & Zebrowska, 1989). Mason *et al*. (1976) reported that in excess of half of the N in the faeces of pigs fed various diets was contained in microbial cells, while Stephen & Cummings (1980) found that approximately half of the DM and two-thirds of the N in human faeces were of microbial origin. The extent of the microbial synthesis is affected not only by the amount of nitrogenous material entering the large intestine, but also the nature of the carbohydrates in the diet and consequently by the amount of undigested fermentable carbohydrate entering the hindgut. The preponderance of faecal microbial protein means that only a low proportion of faecal amino acid excretion is directly related to the flow of undigested dietary amino acids entering the large intestine. The faecal microbial amino acid composition may bear little resemblance to the undigested dietary and endogenous amino acid composition. With regard to the determination of the digestibility of dietary amino acids, important discrepancies may arise resultant from microbial fermentation in the hindgut (Just, 1980), and measurement of amino acid digestibility at the end of the ileum (Payne *et al*. 1968) is regarded as being more appropriate. The effect of hindgut microbial metabolism on protein digestion appears to be a rather general phenomenon across mammals and birds (Hodgkinson & Moughan, 2000), with the extent of microbial activity and thus the difference between ileal and faecal digestibility coefficients depending on the type and numbers of micro-organisms present, the nature of the food and the time of residence of material in the hindgut. It is thus a function of both species and diet.

In the growing pig numerous studies have demonstrated that the ileal digestibilities of most amino acids are lower than corresponding digestibilities determined over the entire digestive tract (Table 3). According to Zebrowska (1978) the amount of amino acids disappearing in the large intestine usually ranges from 5 to 35 % of the total amino acids ingested. It is also apparent that the lower the ileal digestibilities of N and amino acids, the greater is the difference between ileal and faecal digestibilities (Table 4). This is to be expected, as with diets containing highly digestible protein most is absorbed before the digesta enter the large intestine, whereas with protein sources of lower quality there are larger residues available for fermentation. The extent of digestibility overestimation varies with the amino acid, the type of dietary protein and the influence of dietary components. Lenis (1983) has surveyed the world literature from 1964 to 1982 for some thirty-five foodstuffs given to the growing pig. For threonine and tryptophan, the mean overestimations of apparent digestibility by the faecal method (in comparison with ileal values) were 10 and 11 % units, respectively. The ileal–faecal differences tended to be smaller for lysine. The faecal method overestimated (mean overestimation 5·6 % units) lysine digestibility for eleven foods and underestimated it (mean underestimation 4·3 % units) in ten further foods. Faecal values appear to often considerably underestimate the actual digestibility of methionine, though the opposite is found for cysteine. The inability of the faecal method of analysis to account for the effect of hindgut metabolism may explain the frequently reported low statistical correlations between pig growth performance and faecal estimates of amino acid uptake (Crampton & Bell, 1946; Lawrence, 1967; Cole *et al*. 1970). Ileal digestibility coefficients, on the other hand, have been shown to be sensitive in detecting small differences in protein digestibility due to the processing of foods (van Weerden *et al*. 1985; Sauer & Ozimek, 1986) and several studies (Tanksley & Knabe, 1980; Low *et al*. 1982; Just *et al*. 1985; Moughan & Smith, 1985; Dierick *et al*. 1988) have demonstrated that ileal values are accurate in describing the extent of uptake of amino acids from the gut lumen.

There is much less published information on faecal and ileal amino acid digestibility in human subjects. Sammons (1961) determined daily rates of faecal N output from normal human subjects and ileal N output from ileostomates given the same diet, of 1·8 and 2·7 g respectively, which suggests quantitatively important differences in ileal and faecal N digestibility. In contrast, however, Gibson *et al*. (1976) and Bos *et al*. (1999) reported only marginally lower digestibility coefficients determined at the terminal ileum rather than across the whole digestive tract for human subjects receiving highly digestible proteins.

Table 3. Ileal and faecal digesitibilities of essential amino acids in diets fed to the growing pig (*n* 30) (from Sauer & Just, 1979)

	Location		
Amino acid	Ileum	Faeces	Difference
Arginine	0·88	0·92	0·04
Histidine	0·85	0·92	0·07
Isoleucine	0·81	0·87	0·06
Leucine	0·83	0·89	0·06
Lysine	0·85	0·87	0·02
Methionine	0·85	0·85	0·00
Phenylalanine	0·82	0·89	0·07
Threonine	0·73	0·85	0·12
Tryptophan	0·79	0·89	0·10
Valine	0·79	0·87	0·08
Average	0·82	0·88	0·06

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Table 4. Apparent digestibilities of some amino acids in wheat flour and wheat offal measured at the terminal ileum and in faeces of the growing pig (from Sauer *et al.* 1977)

	Wheat flour		Wheat offal	
Amino acid	Ileum	Faeces	Ileum	Faeces
Lysine	0·84	0·86	0·66	0·76
Histidine	0·91	0·94	0·79	0·88
Methionine	0·94	0·94	0·78	0·82
Isoleucine	0·94	0·95	0·73	0·75
Leucine	0·95	0·96	0·75	0·79

Sandstrom *et al*. (1986) gave soya- and meat-based diets to ileostomates and reported true ileal digestibility coefficients for total N in the range of 80 to 85 %. In comparison, in earlier work with human subjects receiving soya-based diets, true faecal digestibility coefficients ranging from 90 to 98 % have been reported (Istfan *et al*. 1983; Scrimshaw *et al*. 1983; Wayler *et al*. 1983; Young *et al*. 1984). Evenepoel *et al*. (1998) fed ^{15}N-labelled egg protein to human ileostomates and recorded true ileal digestibility values for crude protein in cooked and raw egg of 90·9 and 51·3 %, respectively. The latter authors concluded that the ileal digestibility value for cooked egg was lower than the comparable published range for faecal digestibility (92–97 %). A more detailed and structured study with human subjects has been reported by Rowan *et al*. (1994). Five subjects with established ileostomies and six normal subjects consumed a constant diet consisting of meat, vegetables, fruit, bread and dairy products for 7 d with collection of ileostomy contents or faeces, respectively, over the final 4 d of the experimental period. Generally the apparent faecal digestibility coefficients were higher than their ileal counterparts with significant ($P < 0·05$) differences being recorded for arginine, aspartic acid, glycine, phenylalanine, proline, serine, threonine and tryptophan (Table 5). The faecal digestibility of methionine was significantly lower than the ileal value. Some of the differences recorded (Table 5) were quantitatively important, and particularly when viewed against the background of the ileal values being determined using ileostomates. Ileostomates develop a characteristic and quite extensive microflora at the end of the ileum (Vince *et al*. 1973).

Overall, published evidence suggests that ileal amino acid digestibility values are quantitatively different from faecal amino acid digestibility values and should be used for predicting the uptake of amino acids from the gut, in the growing pig. The situation is less certain for man, but experimental observations to date are consistent with findings in the pig and point to ileo–faecal differences that may be of sufficient magnitude to be important in practice. A potentially useful experimental technique has been developed (Tomé & Bos, 2000), which allows the determination of true gastroileal protein digestibility in normal human subjects. The technique involves the ingestion of a single meal containing ^{15}N-labelled protein and the subsequent sampling of ileal digestion via nasointestinal intubation. Application of this technique in a series of studies (Gausserès *et al*. 1997; Bos *et al*. 1999; Mariotti *et al*. 1999, 2002) has provided new information on true ileal protein digestibility in adult human subjects. True ileal protein digestibility values of 89·4, 91·0, 91·0 and 95·5 % were found for peas, sweet lupins, soya-protein isolate and cows' milk, respectively. The method has also been applied to allow determination of true ileal amino acid digestibility values in human subjects (Gaudichon *et al*. 2002). For cows' milk, true ileal digestibility ranged from 91·6 % for glycine to 99·3 % for tyrosine, while for soyabean, digestibility ranged from 89 % for threonine to 96·8 % for tyrosine.

Endogenous protein and amino acids in digesta collected at the terminal ileum

If dietary amino acid digestibility is to be determined at the terminal ileum, and given that the ileal digesta contain copious quantities of endogenous proteins, it becomes necessary to determine the endogenous amino acid component. If coefficients of amino acid digestibility are not corrected for the ileal endogenous amino acids, the resultant digestibility coefficients are referred to as 'apparent' coefficients, whereas if the correction is made the coefficients are termed 'true'. True digestibility is a fundamental property of the food and is not affected by the dietary conditions under which the food is given to the animal or subject. The apparent digestibility measure, however, will be affected by the assay conditions and is, therefore, variable and open to

Table 5. Mean apparent ileal and faecal amino acid digestibility coefficients for adult human subjects (65 kg body weight) receiving a meat, vegetable, cereal, and dairy-product-based diet (from Rowan *et al.* 1994)

	Digestibility coefficients			
Amino acid	Ileal (*n* 5)	Faecal (*n* 6)	Statistical significance	Difference
Arginine	0·90	0·93	*	0·03
Aspartate	0·87	0·90	*	0·03
Serine	0·87	0·92	***	0·05
Threonine	0·85	0·89	**	0·04
Proline	0·90	0·95	**	0·05
Glycine	0·72	0·87	***	0·15
Phenylalanine	0·90	0·91	***	0·01
Methionine	0·93	0·83	***	0·10
Tryptophan	0·77	0·83	*	0·06

*$P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

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error. True digestibility is a superior measure for determining the dietary amino acids that are absorbed from the gut and gives a better representation of protein quality than apparent digestibility (Boisen & Moughan, 1996).

Estimates of the total amount of N of body origin secreted into the digestive tract of the growing pig (30 to 80 kg body weight) vary from 16 to 33 g per 24 h (Souffrant, 1991), with around 75 % of the secreted nutrient being reabsorbed. The remaining 25 % of material secreted enters the large bowel.

The secretion of endogenous proteins into the upper digestive tract is influenced, sometimes quite markedly, by numerous dietary factors such as diet DM, anti-nutritional factors and plant NSP. Recent findings suggest that the amount of dietary protein or peptides released during digestion may also influence the extent of endogenous protein loss from the small bowel (Hodgkinson *et al*. 2000).

Traditionally, endogenous ileal amino acid flow has been determined after feeding animals or human subjects a protein-free diet. This approach, however, may give rise to error. Feeding an animal a protein-free diet may result in a reduction in the amount of gastric and pancreatic enzymes secreted (Fauconneau & Michel, 1970; Schneeman, 1982) and a general decrease in the rate of protein synthesis in the body and gut (Millward *et al*. 1976; Wykes *et al*. 1996). All of these effects would be expected to lead to a lowered endogenous protein loss. Conversely, however, it is possible that the reduced enzyme synthesis and activity accompanying the feeding of a protein-free diet may lead to a lowered digestion and reabsorption of endogenous protein thus leading to an accumulation of endogenous protein at the end of the ileum. Over the last decade a considerable amount of research has been undertaken to develop alternative approaches to the protein-free dietary stratagem and to elucidate the effect of protein nutrition on endogenous ileal amino acid losses. The various methods and experimental approaches that have been developed to allow determination of endogenous amino acid losses at the terminal ileum are listed in Table 6 and have been discussed in detail (Lien *et al*. 1997; Nyachoti *et al*. 1997; Fuller & Reeds, 1998; Moughan *et al*. 1998; Hess *et al*. 1998, 2000; Hodgkinson & Moughan, 2000).

It is now generally accepted that the protein-free method leads to a substantial underestimation of the actual endogenous amino acids present at the terminal ileum when a normal diet is given, at least for most of the amino acids (Leterme *et al*. 1996; Hess *et al*. 2000). Alternative approaches to the determination of endogenous loss need to be adopted.

For practical purposes, standardised true ileal amino acid digestibility coefficients (Boisen & Moughan, 1996) should be used in both human and animal nutrition. It is particularly important when determining the protein digestibility-corrected amino acid score for human foodstuffs that standardised true digestibility coefficients be used, otherwise significant unintended biases will occur (Fenwick *et al*. 1995; Darragh *et al*. 1998). In determining standardised true ileal amino acid digestibility, correction of the ileal total amino acid flow is made for the 'basal' endogenous amino acid component. The basal endogenous flow is defined. One approach that has been taken (Boisen & Moughan, 1996) is to define the basal flow as that flow commensurate with the ingestion of a purified protein (for example, casein) included in a purified maize-starch-based diet. When non-purified proteins or foods are ingested, the endogenous amino acid flow may be higher than the basal flow due to the action of plant NSP and/or anti-nutritional factors. The incremental endogenous amino acid losses are referred to as the 'specific' endogenous losses. These specific endogenous losses are not corrected for in determining 'true' ileal amino acid digestibility and the 'specific' losses effectively lower the value of the true digestibility coefficient and are regarded as a nutritional cost.

Ileal amino acid digestibility for foods that have undergone structural changes during storage or processing

In the section concerning structural changes to amino acids occurring during processing and storage in the present review (see p. 130), it was noted that for foods that have been stored or subjected to processing, chemical reactions may have occurred leading to the formation of cross-linkages between protein chains, which may give rise to 'limit peptides' (de Vrese *et al*. 2000) being produced during digestion in the gut. In this manner chemically reactive amino acids that would otherwise be absorbed and available for anabolism may be unavailable. This is an important emerging research topic.

For the amino acid lysine, structurally unaltered molecules can be accurately determined using chemical methods (for example, FDNB-lysine assay; see earlier, p. 130), but there is evidence (Hurrell & Carpenter, 1981) that the unaltered or chemically available molecules may not be fully

Table 6. Alternative approaches to the protein-free method, for the determination of endogenous ileal amino acid flows in mammals (from Nyachoti *et al.* 1997; Fuller & Reeds, 1998; Moughan *et al.* 1998; Hodgkinson & Moughan, 2000)

Method
Linear regression
Synthetic amino acid based diets
Protein-free diets with intravenous amino acid infusion
Natural proteins devoid of specific amino acids
Guanidination of proteins
Enzymically hydrolysed protein and ultrafiltration
Isotopes (labelling of diet or body)
Mathematical estimation based on endogenous or exogenous amino acid patterns

Table 7. Amounts of acid-hydrolysed lysine, 1-fluoro-2, 4-dinitrobenzene (FDNB)-lysine, reactive lysine and absorbed reactive lysine in a heated casein–glucose mixture (from Moughan *et al.* 1996)

	Acid-hydrolysed*	FDNB	Reactive†	Absorbed reactive‡
Lysine (g/100 g)	2·60	1·91	1·98	1·40

* After conventional amino acid analysis.
† Lysine units remaining chemically reactive after heating, determined from furosine levels.
‡ Reactive lysine absorbed by the end of the small intestine.

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absorbed from the damaged proteins. This has been clearly demonstrated in the study of Moughan *et al*. (1996) (see Table 7), in which the ileal absorption of reactive lysine was determined in the growing pig. A casein–glucose mixture was heated to produce early Maillard compounds, and the amount of epsilon-*N*-deoxy-fructosyl-lysine (blocked lysine) and lysine regenerated after acid hydrolysis in the resulting material were calculated from the determined amount of furosine. The amount of unaltered or reactive lysine was found by difference between the total lysine (acid hydrolysis) and regenerated lysine. The study demonstrated that the FDNB method allowed accurate assessment of the amount of chemically reactive lysine and, importantly, that the reactive lysine was incompletely absorbed. The results of Moughan *et al*. (1996) also clearly reinforce the point that, for proteins whereby lysine has undergone structural change, lysine determined by conventional analysis (involving strong acid hydrolysis) is grossly overestimated.

During strong acid hydrolysis used in conventional amino acid analysis, early Maillard compounds are known to partially revert to lysine. Such reversion, however, does not occur in the animal's digestive tract. Consequently, the lysine concentrations in the food and ileal digesta, determined by conventional amino acid analysis, will be misleading and the conventional true ileal digestibility assay will generally overestimate the amount of available lysine present in processed foods. This at least partly explains the observations of Dr E. S. Batterham & colleagues (Batterham, 1992), that not all ileal digestible amino acids are available to the animal and that the available amino acid:ileal digestible amino acid value varies according to the source of protein. Clearly a different approach is needed for the determination of amino acid digestibility in foods that have been altered structurally due to processing or prolonged storage.

Moughan & Rutherfurd (1996) have proposed a new bioassay for lysine bioavailability based on reacting both the diet and ileal digesta with *o*-methylisourea. The food (in its natural state) is fed to the test animal and samples of ileal digesta are collected. The reactive lysine in samples of the food and digesta are then determined, after reaction with *o*-methylisourea under controlled conditions, by analysing the diet and digesta for homoarginine. Reactive lysine molecules will readily react with *o*-methylisourea to form the amino acid homoarginine. The true ileal digestibility of reactive lysine is then calculated. These coefficients can be used to calculate digestible reactive lysine (i.e. available lysine). This new approach places emphasis on determining the uptake of chemically available lysine molecules from the gut, rather than the previous preoccupation of workers describing the uptake and utilisation of chemically altered lysine molecules. It is the former that is required for dietary formulation. For unprocessed foods the digestible reactive lysine content should be equivalent to the digestible lysine content determined using conventional methods. However, for processed foods, the total lysine content (conventionally determined) may be higher than the reactive lysine content due to the conversion of lysine derivatives to lysine during the acid hydrolysis stage of conventional amino acid analysis, and total lysine digestibility will be lower than its reactive lysine counterpart. Overall, for the processed food, the digestible reactive (available) lysine content will be overestimated using conventional procedures. The overestimation of the dietary total lysine content by conventional amino acid analysis is not completely compensated for by the lower determined total lysine digestibility value, the value of which is related to the uptake from the digestive tract of blocked lysine. In severely damaged protein sources, some of the structurally altered lysine derivatives may be acid-stable, and thus may not convert back to lysine during acid hydrolysis. In this case, reactive and total lysine values would be more similar.

The new bioassay has been shown to be more accurate than assays based on conventional amino acid analysis as an indicator of digestible reactive lysine (Rutherfurd & Moughan, 1997; Rutherfurd *et al*. 1997*a*) and the bioassay has now been applied to a range of processed foodstuffs (Table 8). It can be seen from Table 8 that determination of true ileal digestibility using conventional amino acid analysis significantly underestimates lysine digestibility in soyabean meal, dried maize, heated skimmed-milk powder, cottonseed meal and a lucerne-based mix, whereas for the same materials the digestible lysine content was generally overestimated using conventional lysine analysis. For unprocessed foods, the conventional true ileal digestibility assay is appropriate. For processed ingredients, and at least in terms of lysine, conventional true ileal digestible lysine will overestimate the lysine that is available to the animal. This is possibly also so for amino acids other than lysine. Therefore, other methods such as the true ileal reactive (available) lysine digestibility assay are required to give accurate results. Research is needed into the availability of amino acids other than lysine in foods where the proteins of the food may have been damaged.

Table 8. Comparison of mean true ileal lysine digestibility (%) and mean digestible lysine contents (g/kg) determined using conventional amino acid analysis (Total) or based on determined reactive lysine (Reactive) (from Rutherfurd *et al.* (1997*b*)

	Lysine digestibility		Digestible lysine	
	Total	Reactive	Total	Reactive
Blood meal	96·3	96·7	85·9	85·1
Wheat meal	92·6	92·1	3·2	2·9
Meat and bone meal	88·9	91·5	32·5	31·6
Soyabean meal	94·5	96·5*	30·6	31·2*
Dried maize	80·5	84·3*	2·6	1·9***
Heated skimmed milk powder	69·1	94·0***	19·8	16·6***
Cottonseed meal	62·1	71·9**	12·9	10·3***
Lucerne-based mix	74·2	86·3***	14·4	10·8***

* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

Conclusion

Amino acid availability has been and continues to be an active and important research area in both human and animal nutrition. Many studies have been made into technical aspects of the chemical analysis of amino acids though, despite this, analytical techniques have not changed greatly

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over the years, though speed and precision have improved. Where a high degree of accuracy is required, attention should be given to the effect on amino acid yield of hydrolysis time, and recent research has emphasised the importance of hydrolysis interval. There is compelling evidence for determining amino acid digestibility at the terminal ileum rather than over the total digestive tract, and recently developed alternatives to the protein-free method for determining endogenous amino acids should be adopted in practice. It is becoming increasingly clear that microbial activity in the upper digestive tract contributes dietary essential amino acids to the host, and research is now required to quantify the net effect and thus the practical importance of this phenomenon. In the case of foods, the constituent amino acids, some of which may have undergone structural changes, new approaches to dietary amino acid availability are needed. Certainly for the amino acid lysine, and probably for other amino acids as well, conventional procedures may be misleading.

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