

# CRITICAL EVALUATION OF IN VITRO METHODS FOR ESTIMATING DIGESTIBILITY IN SIMPLE-STOMACH ANIMALS

S. BOISEN AND B. O. EGGUM

National Institute of Animal Science, Foulum, DK-8830 Tjele, Denmark

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## INTRODUCTION

When predicting the nutritional quality of foods, information on the digestibility of the various nutrients is of utmost importance. However, *in vivo* determinations are time-consuming and costly, which is why much effort has been devoted to the development of *in vitro* procedures. To simulate the *in vivo* situation *in vitro*, one has to consider not only host enzyme digestion but also the microbial activity in the digestive tract, which often can have a great impact on overall digestibility. Due to the hind-gut fermentation in simple-stomach animals, many workers in digestive physiology (e.g. Tanksley & Knabe, 1984; Just *et al.* 1985; Sauer & Ozimek, 1986) have recommended that digestibility, particularly of amino acids, be measured at the terminal ileum. The present review will not deal with this discussion, but rather will present comparative ileal and faecal digestibility values, and relate these to *in vitro* values. The various approaches described in the literature will be discussed and evaluated. Particular emphasis will be given to feeds for simple-stomach animals such as pigs and poultry. However, some aspects of human nutrition will also be discussed briefly.

When reviewing the various techniques, special attention will be given to simple, rapid and reproducible *in vitro* procedures. Furthermore, the validation of the *in vitro* methods will be based on the degree of relationship between *in vitro* and *in vivo* results from identical materials.

## IN VIVO DIGESTION IN SIMPLE-STOMACH ANIMALS

When developing *in vitro* digestibility techniques it is logical to simulate one or more steps of the *in vivo* conditions. Therefore, some aspects of the digestive physiology in the stomach, small intestine and hind-gut of simple-stomach animals will be briefly outlined.

## STOMACH

In the stomach, the hydrolysis of proteins is initiated by the action of pepsin (*EC* 3.4.23.1) and hydrochloric acid. At least half the proteins leaving the stomach are usually in the form of peptides, with a large proportion being soluble in trichloroacetic acid (TCA), i.e. peptides having ten or fewer amino acids (Low, 1990).

The hydrolysis of starch and fat is also initiated in the stomach by salivary amylase (*EC* 3.2.1.1) and gastric lipase (*EC* 3.1.1.3) respectively. Gastric lipolysis seems quantitatively important for the release of short- and medium-chain fatty acids from triacylglycerols, the main reaction products being diacylglycerols and fatty acids. Thus, gastric lipolysis may be important in the digestion of some dietary fats, but only hydrolysis is effected. Emulsification does not occur in the stomach (Friedman & Nylund, 1980).

## SMALL INTESTINE

Proteins and peptides passing from the stomach are further hydrolysed in the small intestine by the pancreatic proteinases trypsin (*EC* 3.4.21.4), chymotrypsin (*EC* 3.4.21.1) and elastase (*EC* 3.2.21.36) and the peptidases, carboxypeptidases A (*EC* 3.4.17.1) and B (*EC* 3.4.17.2). Dissolved brush-border peptidases may finally contribute to the hydrolysis before absorption of free amino acids and di- and tripeptides. Although the ileum has considerable digestive and absorptive capacity, the absorption of dietary protein occurs mainly in the proximal jejunum (Grimble & Silk, 1989).

The hydrolysis of starch polymers, already started by salivary amylase in the mouth and stomach, is continued by pancreatic amylase in the lumen of the small intestine. The end-products maltose, maltotriose and  $\alpha$ -limit dextrins are finally hydrolysed to glucose by maltase (*EC* 3.2.1.20), sucrase (*EC* 3.2.1.48), and isomaltase (*EC* 3.2.1.10) (Roehrig, 1984). Dietary disaccharides like lactose and sucrose are hydrolysed to monosaccharides by lactase (*EC* 3.2.1.108) and sucrase. All hydrolase enzymes involved in the hydrolysis to monosaccharides are bound to the apical membrane of the enterocytes with their active sites projecting into the intestinal lumen.

The hydrolysis of fats in the small intestine is catalysed by at least three different enzymes and one coenzyme (colipase) from pancreatic juice. These enzymes are (1) pancreatic lipase, which is rather non-specific and splits triacylglycerols into monoacylglycerols and fatty acids, (2) carboxylic ester hydrolase (*EC* 3.1.1.1), which splits carboxylic esters, and (3) phospholipase A<sub>2</sub> (*EC* 3.1.1.4), which hydrolyses fatty acids in the 2-position of glycerophospholipid. The activity of lipase is enhanced by the presence of bile salts, while the two latter enzymes have an absolute requirement for bile salts. Bile salts also promote absorption of long-chain fatty acids and monoacylglycerols, due to their solubilizing effect, which increases the transport across the unstirred water layer between gut lumen and brush border (Friedman & Nylund, 1980). Fat absorption occurs mainly in the jejunum. As digestion progresses, the lipid phase decreases in volume and the products of digestion pass into mixed micelles which are large molecular aggregates consisting of monoacylglycerols, long-chain fatty acids, bile salts and phosphoacylglycerols. The mixed micelles are able to draw into the hydrophobic core the less-water-soluble molecules such as cholesterol, carotenoids, tocopherols, and some undigested triacylglycerols (Gurr *et al.* 1989).

## HIND-GUT

The major site of assimilation of endogenously derived protein (intestinal secretions, secreted plasma proteins and desquamated cells) is assumed to be the hind-gut. The N endogenous losses from the small intestine are about 10–20% of N intake (Grimble & Silk,

1989). However, the microflora in the large intestine are capable of digesting endogenous proteins (e.g. mucin) as well as exogenous proteins. Luminal hydrolysis of protein continues by the action of undegraded pancreatic enzymes as well as secreted and cell-wall-bound bacterial peptidases. The released amino acids may be incorporated into microbial protein or deaminated and fermented to short-chain fatty acids.

The carbohydrate residues in the hind-gut consist of undigested starches, oligosaccharides and non-starch polysaccharides (NSP). In this region there are no endogenous enzymes capable of degrading these residues. However, the gut microflora are able to hydrolyse most of the bonds in those compounds which are partly available such as pectins and  $\beta$ -glucans. The released monosaccharides are fermented by the microflora. The end-products are primarily short-chain fatty acids which can be absorbed and act as an energy source for the host animal.

Fatty acids cannot be fermented, which is why the amount of lipid is only marginally influenced during passage through the hind-gut. On the other hand, unsaturated fatty acids may be hydrogenated, while lipids can be synthesized in this part of the digestive tract. Both factors complicate the measurements of digestibility of individual fatty acids (e.g. Eggum *et al.* 1982).

#### FACTORS INFLUENCING DIGESTIBILITY IN VIVO

The digestive capacity can be modified by numerous stimuli, e.g. the food itself. Thus, the enzymes which are involved with protein, carbohydrate and lipid digestion adapt to any changes in substrate intake (Corring 1980; Corring *et al.* 1989; Eggum *et al.* 1989; Savoie *et al.* 1989). This means primarily that an increase in dietary protein induces an increased secretion of pancreatic proteolytic enzymes, while an increase in starch or lipid intake induces increased secretions of amylase and lipase respectively. Also, antinutritional factors (ANF) and dietary fibre affect enzyme secretions.

Several specific responses of ANF on digestibility are known. Thus, trypsin inhibitors increase the pancreatic secretion of trypsin and other enzymes (Liener & Kakade, 1980), while tannins may induce a large increase in specific proline-rich proteins in the saliva. These proteins have a very high affinity for tannins and are therefore assumed to play a role in reducing the adverse effect of tannins on protein digestibility (Mehansho *et al.* 1987). Lectins, however, interfere with the digestion and absorption processes in quite a different way as they bind to surface receptors of the enterocytes and thus induce changes in epithelial cell metabolism, thereby increasing cellular turnover and protein secretion (Pusztai, 1989). The main effect of ANF on digestibility is probably an increased loss of endogenous protein rather than a negative effect on digestibility of exogenous protein. Normally, there is a great surplus of digestive capacity in the small intestine (Low, 1980).

Dietary fibre will influence *in vivo* digestion in several ways, depending on the nature of the fibre. Dietary fibre can thus reduce enzyme activity in the lumen, but probably also protects the enzymes against degradation (Boisen *et al.* 1985). Furthermore, anatomical changes of certain parts of the digestive tract by fibre can be seen (Eggum, 1991). Fibre will in general stimulate microbial activity in the digestive tract significantly and reduce transit time of the digesta.

Although most of the bacterial metabolism takes place in the hind-gut, digestion can be influenced by the microflora throughout the whole gastrointestinal tract. Thus, monosaccharides may be fermented in the upper part of the gastrointestinal tract to volatile fatty acids and various gases. The energy in the absorbed volatile fatty acids will be of value to the host animal, but the net utilization will be much lower than that if the monosaccharide had been absorbed without modification. Furthermore, the bacterial flora can synthesize as

well as degrade the individual amino acids (Mason, 1984). Thus, due to the microbial activity in the upper tract, the ileal apparent digestibility values are not necessarily exact measures of net absorbed amino acids.

Finally, it should also be mentioned that food utilization is influenced by several other factors such as management, environment, disease and genetic capability. In this regard, Elbers *et al.* (1989) found organic-matter digestibility of the same diet on seventeen pig farms to vary from 75 to 85% for a grower diet, and from 78 to 86% for a finisher diet.

## IN VITRO METHODS FOR ESTIMATING DIGESTIBILITY

From the previous discussion it is apparent that many factors are involved in *in vivo* digestibility. Therefore, the *in vivo* conditions can never be completely simulated under *in vitro* conditions. The *in vitro* technique can be designed to use specific enzymes either to give maximal digestibility values or to measure the initial rate of hydrolysis. In both cases the enzymes used should have specificities similar to those which are present in the digestive tract. The applicability of the results depends on a high correlation with *in vivo* values obtained under standardized conditions using identical material. In the following discussion, the different methods are described according to their objectives, principles, use of specific enzymes, and demands for special equipment. Evaluation of the methods is based on the reported results.

### DIALYSIS CELL METHOD

#### *Objective*

To study the influence of various factors on the digestion of protein (and other nutrients) when end-product inhibition is prevented.

#### *Principle*

Enzymic digestion of proteins with continuous removal of low-molecular-weight products by dialysis.

#### *Methods*

Samples containing 40 mg N are suspended in diluted hydrochloric acid at pH 1.9 and digested with pepsin for 30 min. After adjusting the pH to 7.5 with sodium hydroxide, the mixture is poured into the dialysis tube (molecular weight cut-off 1000) of the digestion cell with a pancreatin solution and digested with continuous stirring for 6 h. Digestion products are collected from the external compartment by sodium phosphate buffer circulation. The collected products are analysed for nitrogen and for amino acids (Gauthier *et al.* 1986; Savoie & Gauthier, 1986). This method has been developed from previously described methods (Mauron *et al.* 1955; Steinhardt & Kirchgessner, 1973; Gauthier *et al.* 1982).

#### *Results*

Gauthier *et al.* (1982) observed that the degree of digestion of casein, soya-bean and rapeseed protein was markedly increased by buffer replacements, indicating inhibition by end-products as suggested by Robbins (1978). By this procedure the deleterious effect of heat and alkali treatment on protein digestion was clearly demonstrated (Vachon *et al.* 1983).

The method can also be used for direct measurement of peptide susceptibility to hydrolysis by proteolytic enzymes. Thus, Brulé & Savoie (1988) found that basic and

aromatic amino acids were generally the most readily liberated, while cystine and proline were the least digested in six common protein sources comprising casein, field peas (*Pisum sativum*), peanut meal, rapeseed, soya bean and wheat flour. However, the kinetics of hydrolysis of peptide bonds and release of amino acids and peptides depends on the different specificities of the enzymes involved. Savoie & Charbonneau (1990) demonstrated by adding trypsin and chymotrypsin to pancreatin that the optimal combination of enzymes differed from one substrate to another.

The influence of the time factor was investigated by Galibois *et al.* (1989) by comparing the essential amino acid (EAA) profiles in the dialysate collected at 3 h intervals during 24 h of *in vitro* enzymic proteolysis of casein and rapeseed protein with the pattern of appearance of dietary EAA in the portal vein of pigs fed on the same proteins. The results demonstrated a close relationship between the *in vitro* release of amino acids and the appearance of dietary EAA in the portal vein.

### Discussion

The dialysis cell method appears to be very valuable for studying luminal protein degradation in the small intestine and also seems able to predict the availability of amino acids. Furthermore, this technique can be used for studying starch degradation, as recently reported by Drake (1991), and may be used to measure enzymic release of any soluble low-molecular-weight product from a high-molecular-weight substrate.

## pH-DROP AND pH-STAT METHOD

### Objective

To predict protein digestibility in foods.

### Principle

During proteolysis, protons are released from the cleaved peptide bonds, resulting in a decrease in pH in a suspension. Assuming a correlation between the initial rate of peptide release and protein digestibility, the latter can be predicted by (1) recording the decrease in pH (pH-drop method) or (2) keeping the pH constant with continuous addition of NaOH and measuring NaOH consumption (pH-stat method),

### Methods

Samples containing 10 mg N are suspended in 10 ml distilled water, adjusted to pH 8.00 and equilibrated at 37°. A solution of trypsin, chymotrypsin and peptidase (1 ml) is added to the protein suspension, and after exactly 10 min of incubation, pH is recorded (Hsu *et al.* 1977). A modified method incubating additionally with a proteinase from *Streptomyces griseus* has been described by Satterlee *et al.* (1981). In the pH-stat method, pH is kept constant by automatic titration with 0.1 M-NaOH. At the end of the incubation period the amount of alkali added is recorded (Pedersen & Eggum, 1983).

### Results

The *in vitro* digestibility obtained with the pH-drop method has been found to be highly correlated with apparent faecal digestibility of protein in rats in investigations on a variety of plant proteins (Hsu *et al.* 1977; Satterlee *et al.* 1981). However, both the pH-drop and pH-stat methods have given poor agreement with *in vivo* values for a great variety of foods and feedstuffs (Pedersen & Eggum, 1981; Wolzak *et al.* 1981). These authors found it necessary to use different regression equations for each class of food to obtain a reliable estimate of digestibility. Some of the problems were obviously related to different buffer

capacities in the incubated materials. This was demonstrated by Moughan *et al.* (1989) investigating twenty meat-and-bone samples. The authors compared the results obtained with the four-enzyme method with *in vivo* true ileal protein digestibility obtained with rats. A significant relationship was found between the pH-drop and *in vivo* digestibility. However, meat-and-bone meal samples which were highly digestible *in vivo* caused a low initial drop in pH, which is opposite to what was expected. The explanation for this was a negative correlation between pH-drop and ash content in the samples, suggesting a strong buffering capacity of meat-and-bone meal due to the high mineral content (mean ash 275 g/kg).

The *in vitro* digestibility obtained with the pH-stat method has been correlated with true protein digestibility values determined in rats. Compared with the pH-drop method, the pH-stat method improved the prediction of protein digestibility, and the same regression equation could be used for a variety of materials. In general a good agreement has been obtained with faecal true digestibility of plant protein ( $n$  31,  $r$  0.85, residual standard deviation (RSD) 2.4) as well as with the proteins of animal origin ( $n$  11,  $r$  0.92, RSD 2.2), and also in samples having high buffer capacities. However, a predigestion step with pepsin has been suggested for samples containing proteinase inhibitors (Pedersen & Eggum, 1983).

The pH-stat method was found to be highly reproducible in an interlaboratory investigation by six laboratories (McDonough *et al.* 1990).

Results obtained in a recent comparative study of *in vitro* protein digestibility of nineteen food products by the pH-stat method and the dialysis-cell method (Savoie *et al.* 1989) were compared with *in vivo* protein digestibility (Eggum *et al.* 1989) using the rat balance method of Eggum (1973). The *in vivo* determinations took place in two different laboratories. In general, good agreements were obtained between results from all four measurements (Savoie *et al.* 1989).

### Discussion

The pH-stat method seems to be superior to the pH-drop method, probably mainly due to a lower influence of varying buffer capacity of the test material. This method has also been proved to be very reproducible between different laboratories. The method seems therefore to be very suitable for predicting digestibility in many protein sources. However, the proteins investigated hitherto have generally been highly digestible and investigations on a number of feedstuffs with lower protein digestibility have not been reported. Therefore, a general correlation between the initial rate of proton release and protein digestibility has not yet been proved, and the method cannot yet be considered to be applicable to all types of food.

## COLORIMETRIC METHODS

### Objective

To predict starch digestibility or availability, mainly in processed foods.

### Principle

Colorimetric measurements of enzymically liberated glucose or maltose from starch after the specific formation of a light-absorbing dye in the visible spectrum.

### Methods

Starch is generally hydrolysed in two steps either with porcine pancreatic amylase (O'Dea *et al.* 1981) or with the thermostable microbial amylase, Termamyl (Holm *et al.* 1988) in the first step and with microbial amyloglucosidase (EC 3.2.1.3) in the second step.

However, pre-incubation with pepsin improves the digestibility of some materials, e.g. bread made from sour dough (Siljeström *et al.* 1988), probably because of a more rigid protein matrix enclosing the starch. White *et al.* (1988) supplied  $\alpha$ -amylase (from porcine pancreas) with microbial amyloglucosidase and pullulanase (*EC* 3.2.1.41) as substitutions for unavailable mammalian sucrase, maltase, and isomaltase.

Liberated glucose or maltose is measured after reaction with *p*-hydroxybenzoic acid hydrazide (O'Dea *et al.* 1981), dinitrosalicylic acid (Holm *et al.* 1988), glucose oxidase (*EC* 1.1.3.4)-peroxidase (*EC* 1.11.1.7) (Siljeström *et al.* 1988), anthrone (Öste *et al.* 1985) or other reagents that form a light-absorbing product which is measured spectrophotometrically.

### Results

The formation of resistant starch during bread baking was studied by Björck *et al.* (1986). Although the *in vitro* digestibility values of low-energy dextrose bulking agents (for use in the treatment of obesity) were found to be lower than *in vivo* values, the results reported by White *et al.* (1988) were highly correlated ( $r$  0.98).

Several other investigations have shown a significant correlation between carbohydrate availability *in vitro* and *in vivo*, e.g. O'Dea *et al.* (1981). For further references, see Le François (1989).

### Discussion

Some of the variants of this method seem to be very suitable for investigating the effect of processing on starch availability. However, the use of microbial enzymes may lead to overestimations when used for prediction of starch availability at the level of the small intestine which has not been hydrolysed anterior to the ileo-caecal valve. Undigested starch, including resistant starch, is generally easily fermented by the microflora in the large intestine (Björck *et al.* 1986).

## 'FILTRATION' METHODS

### Objective

To predict nutrient digestibility of food at the ileal and/or the faecal level.

### Principle

Enzyme incubations in a closed system followed by measurements of dissolved nutrients. This will usually be based on measurements of the unsolubilized material collected after filtration. However, it may also be based on measurements on the filtrate, or alternatively on one of the separated fractions after centrifugation.

The digestibility is then predicted from the relationship between *in vitro* solubilizability and *in vivo* digestibility.

These methods can be divided into single-, two- or multi-enzyme systems performed in one, two, or three incubation steps (Eggum & Boisen, 1991).

### Methods

Single-enzyme systems include incubations with pepsin (Sheffner *et al.* 1956), papain (*EC* 3.4.22.2) (Buchanan, 1969), trypsin (Maga *et al.* 1973), pronase (Taverner & Farrell, 1981) and rennin (*EC* 3.4.23.4) (Bhatty, 1982). Two-enzyme systems include incubation in two steps with pepsin and trypsin (Saunders *et al.* 1973), and pepsin and pronase (Dierick *et al.* 1985). Multi-enzyme systems include one- and two-step systems for simulating the



Table 1. *Multi-enzyme systems used for in vitro incubations*

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One-step systems:
Intestinal fluids
Two-step systems:
Pepsin (EC 3.4.23.1) – jejunal fluid
Pepsin – pancreatin
Three-step systems:
Pepsin – pancreatin – rumen fluid
Pepsin – pancreatin – fibre-degrading enzymes

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precaecal digestion and three-step systems for simulating the overall digestion. An overview of the different groups of multi-enzyme systems is given in Table 1.

The methods for the five different groups of multi-enzyme systems are briefly described.

*Intestinal fluids.* A simple one-step incubation with three different inocula from duodenal fluid incubated for 12 h, or ileal fluid or faeces extract incubated for 48 h, was recently described and investigated in detail by Löwgren *et al.* (1989). The method was used for measuring in vitro digestibility (disappearance) of various nutrients. Other systems had been described previously (Goering & Van Soest, 1970; Ehle *et al.* 1982; Holzgraefe *et al.* 1985).

*Pepsin-jejunal fluid.* A two-step incubation with pepsin digestion for 4 h followed by a further 4-h digestion with pig jejunal fluid was developed by Furuya *et al.* (1979). The method was used for measuring in vitro digestibility of protein and dry matter. Several improvements of this method were suggested by Clunies & Leeson (1984) for use in routine analysis in quality control of feeds and feed ingredients for poultry.

*Pepsin-pancreatin.* A two-step incubation with pepsin digestion for 6 h followed by a pancreatin digestion for 18 h in a bacteriostatic borate buffer was developed by Büchmann (1979). In vitro protein digestibility was measured after TCA precipitation of solubilized, but not digested proteins.

Another method for measuring protein digestibility after pepsin and pancreatin digestion for 1 h each was described by Babinszky *et al.* (1990). In this method starch degradation was improved by addition of amylase to pancreatin while lipids were removed by pre-extraction with light petroleum (b.p. 40–60°). An alternative method with addition of bile salt and extra lipase to the pancreatin solution was also described.

A third method based on pepsin and pancreatin digestion was described by Boisen & Fernández (1991 *a*). The enzymic incubation conditions in this method were modified from those described by Asp *et al.* (1983). The material (1 g) was incubated with pepsin at pH 2 for 6 h followed by an incubation with pancreatin at pH 6.8 for 18 h. Chloramphenicol was added to prevent bacterial growth. Solubilized but not fully degraded proteins were precipitated with sulphosalicylic acid and undigested materials were collected using the standardized filtration equipment of Tecator for measuring dietary fibre. Fat was removed from the fibre by washing with ethanol and acetone, and in vitro digestibility of dry matter and N was measured from analysis of the residue. This method has recently been modified slightly by the introduction of continuous magnetic stirring during the enzymic incubations in order to secure effective starch degradation (Boisen & Fernández, 1991 *b*).

*Pepsin-pancreatin-rumen fluid.* To have a complete in vitro evaluation system the potential-energy value of fibre capable of being degraded by microbial enzymes should be included. As the bacterial population in the hind-gut of pigs seems to be rather similar to that in the rumen (Fonty & Gouet, 1989), the method of Tilley & Terry (1963) described

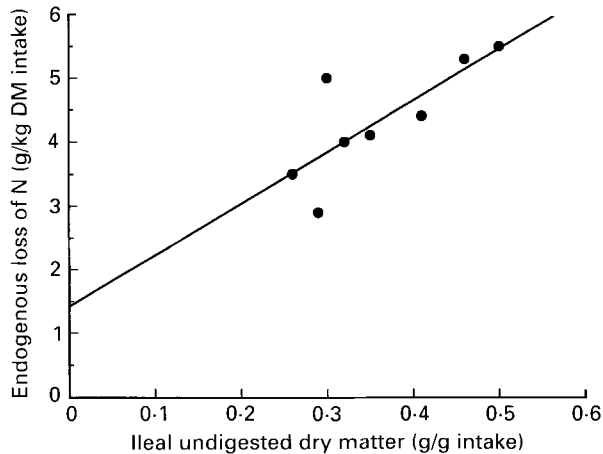


Fig. 1. Relationship between the endogenous loss of nitrogen (calculated from the difference between in vivo and in vitro digested N) and ileal undigested dry matter (DM) in pigs fed on eight common feedstuffs. From Boisen & Fernández (1991 *a*). Regression equation:  $y = 1.41 + 8.1x$ .

for ruminants using rumen fluids as inoculum was applied by Vervaeke *et al.* (1979) to study hind-gut fermentation of organic matter in pigs. The incubation was performed after pre-incubating the diets with pepsin and pancreatin.

*Pepsin-pancreatin-fibre-degrading enzymes.* Metz & Van der Meer (1985) described an in vitro method for predicting faecal digestibility of organic matter after consecutive incubations with pepsin (at pH 1), pancreatin (supplied with lipase, bile salt, and the microbial amylase, Termamyl) and cellulase (*EC* 3.2.1.4). A slightly modified method was described by Van der Meer & Perez (1990). In this method pepsin incubation was performed at pH 1.5 and no additions to pancreatin were made.

Boisen & Fernández (1991 *a*) developed a method for predicting faecal digestibility of dry matter by supplementing the pepsin-pancreatin method described previously with a third incubation step with the microbial enzyme preparation by Novo, Viscozyme. This preparation is produced from a selected strain of the *Aspergillus* group and is a multi-enzyme complex containing a wide range of carbohydrases, including cellulase, hemicellulase, arabinase, xylanase,  $\beta$ -glucanase and pectinase. In a modified method (Boisen & Fernández, 1991 *b*) the degradation of fibre, especially in cereals, was improved with another Novo enzyme, Cellulast, produced from a selected strain of *Trichoderma reesei*, and having a high cellulase activity (Fig. 1).

### Results

The only widely used single-enzyme method is the pepsin method, which is also recognized officially (Association of Official Analytical Chemists, 1984). Recently, Parsons (1991) reported that by using the AOAC (1984) method a good correlation between in vivo and in vitro values was obtained for samples of poultry by-products, but the method was not sensitive enough to test variations in quality of feather meal and of meat meal. However, by reducing the pepsin concentration from 2.0 to 0.02 g/l good estimates of protein quality were obtained for these products.

None of the proposed two-enzyme methods has been used widely. However, several multi-enzyme methods have been investigated in detail. Graham *et al.* (1989) compared the in vitro digestibility (in vitro disappearance), obtained with the method of Löwgren *et al.* (1989), of dry matter, energy, ash, crude protein ( $N \times 6.25$ ), starch, dietary fibre, NSP and

monosaccharides in five diets with in vivo ileal and faecal apparent digestibility in pigs. These workers obtained good correlations between in vitro values and values for digestibility of starch and crude protein in the small intestine, as well as values for the fermentability of dietary fibre.

The results with the pepsin–jejunal fluid method were found to be closely correlated to faecal digestibility of protein and dry matter in pigs (Furuya *et al.* 1979) and in poultry (Sakamoto *et al.* 1980). Dierick *et al.* (1985) compared results obtained with the pepsin–jejunal method with results from two other two-step in vitro methods, both including pepsin in the first step, but one using pancreatin and the other pronase in the second step. The results from this study indicated that jejunal fluid could replace an appropriate pancreatin solution without reducing accuracy. The in vitro results of protein digestibility obtained with the pepsin–pancreatin method and measured in thirty pig feeds and ingredients were highly correlated with values of apparent faecal digestibility ( $r$  0.87), but a much lower correlation ( $r$  0.56) was found with apparent ileal digestibility.

The in vitro digestibility of protein obtained from the pepsin–pancreatin method described by Büchmann (1979) was in good agreement with true digestibility values determined on rats for thirty barley samples, but unsatisfactory results were obtained with other cereals (Büchmann, 1979).

Babinszky *et al.* (1990) investigated seven single feeds and sixteen mixed feeds for pigs by their pepsin–pancreatin method and found an improved correlation to faecal digestible crude protein in fattening pigs by the pre-extraction of lipids with solvent rather than the addition of lipase and bile salt to the incubation mixture with pancreatin.

By the pepsin–pancreatin method developed in our laboratory (Boisen & Fernández, 1991 *a*) the in vitro protein digestibilities for a variety of feedstuffs were in general very close to values for true faecal digestibility determined with rats and pigs (Boisen, unpublished results). When using the same technique to predict ileal digestibility of individual amino acids in eight common feedstuffs, we found that the in vitro digestibility of the individual amino acids in general was very close to the in vitro digestibility of N (Boisen & Fernández, 1991 *a*). Furthermore, the results indicated a relationship between (1) undigested dry matter at the ileal level and (2) the difference between in vitro and in vivo undigested N (or amino acids) at the ileal level (Fig. 1). However, this relationship was not found in a more detailed study with fifty-two feed mixtures varying greatly in composition (Boisen & Fernández, 1991 *b*). On the other hand, after correction for mean values of the differences between in vitro and in vivo undigested N (or amino acids) at the ileal level, the apparent ileal digestibility of N (or amino acids) could still be predicted with considerable accuracy (Boisen & Fernández, 1991 *b*).

Vervaeke *et al.* (1989) demonstrated that after pre-incubation with pepsin and pancreatin, the hind-gut fermentation of organic matter could be estimated with considerable accuracy after incubation with rumen fluid for 48 h. However, several investigations with ruminant feedstuffs have shown that microbial fermentation can be simulated by the use of microbial enzymes (Aufrere & Michalet-Doreau, 1988). In our laboratory (Boisen, unpublished results) we found a close relationship for a variety of feed mixtures between results of dry-matter digestibility obtained after incubation with pepsin, pancreatin, and rumen fluid for 48 h according to Vervaeke *et al.* (1989) and results obtained when rumen fluid was replaced with a mixture of Viscozyme and incubated for only 18 h.

Further studies in our laboratory (see Boisen & Fernández, 1991 *b*) have demonstrated that a general relationship between in vitro digestibility of organic matter and faecal digestibility of energy in a great variety of single feedstuffs and mixtures is obtained after a three-step incubation, with a sequence of pepsin, pancreatin and a mixture of fibre-degrading enzymes.

Table 2. Correlation analyses of the relationship between *in vivo* (pig) digestibility and results from multi-enzymic *in vitro* methods simulating precaecal digestion

<i>n</i>	Feed*	Equation†	<i>r</i>	RSD‡	Reference
Faecal level					
7	CF	dN = -60.9 + 1.70dNm	0.98	1.2	Furuya <i>et al.</i> (1979)
7	CF	dDM = 8.1 + 1.04dDMm	0.98	1.1	Furuya <i>et al.</i> (1979)
30	SF + CF	dN = 7.3 + 0.85dNm	0.87	6.7	Dierick <i>et al.</i> (1985)
7	SF	CPd = -12.2 + 0.97CPdm	0.99	17.4	Babinszky <i>et al.</i> (1990)
16	CF	CPd = -29.1 + 0.11CPdm	0.95	6.2	Babinszky <i>et al.</i> (1990)
61	SF	dN = 0.7 + 0.97dNp	0.93	6.3	Boisen & Fernández (1991 <i>b</i> )
52	CF	dN = -7.4 + 1.09dNp	0.85	2.7	Boisen & Fernández (1991 <i>b</i> )
Ileal level					
30	SF + CF	dN = 24.8 + 0.56dNm	0.56	6.7	Dierick <i>et al.</i> (1985)
7	SF	dDM = -32.5 + 1.47dDMm	0.97	1.5	Boisen & Fernández (1991 <i>a</i> )
7	SF	dE = -6.5 + 1.11dDMm	0.97	1.5	Boisen & Fernández (1991 <i>a</i> )
52	CF	dE = 1.9 + 0.83dOMm	0.76	3.5	Boisen & Fernández (1991 <i>b</i> )
52	CF	dN = 29.9 + 0.60dNp	0.68	3.2	Boisen & Fernández (1991 <i>b</i> )
52	CF	dLYS = 14.5 + 0.83dLYSp	0.78	2.8	Boisen & Fernández (1991 <i>b</i> )
52	CF	dMET = 15.3 + 0.83dMETp	0.74	2.3	Boisen & Fernández (1991 <i>b</i> )
52	CF	dTHR = 34.6 + 0.54dTHRp	0.61	3.7	Boisen & Fernández (1991 <i>b</i> )

\* CF, compound feeds; SF, single feedstuffs.

† dN, nitrogen digestibility; dDM, dry-matter digestibility; dOM, organic-matter digestibility; dE, energy digestibility; dLYS, lysine digestibility; dMET, methionine digestibility; dTHR, threonine digestibility; m, measured value *in vitro*; p, predicted value from *in vitro* measurements; CPd, crude protein (N × 6.25) digested (g/kg dry matter).

‡ Residual standard deviation.

Table 3. Correlation analyses of the relationship between *in vivo* (pig) digestibility and results from multi-enzymic *in vitro* methods simulating overall digestion

<i>n</i>	Feed*	Equation†	<i>r</i>	RSD‡	Reference
34	SF + CF	dOM = -1.8 + 0.99dOMm	0.91	3.8	Metz & Van der Meer (1985)
16	CF	dOM = -1.0 + 0.98dOMm	0.85	2.7	Metz & Van der Meer (1985)
89	CF	dOM = 12.5 + 0.80dOMm	0.92	2.0	Van der Meer & Perez (1990)
7	SF	dDM = -4.6 + 1.02dDMm	0.97	1.9	Boisen & Fernández (1991 <i>a</i> )
7	SF	dE = -8.1 + 1.07dDMm	0.95	2.5	Boisen & Fernández (1991 <i>a</i> )
51	SF	dE = -19.1 + 1.16dOMm	0.98	3.2	Boisen & Fernández (1991 <i>b</i> )
52	CF	dE = -24.9 + 1.23dOMm	0.92	2.2	Boisen & Fernández (1991 <i>b</i> )

\* CF, compound feeds; SF, single feedstuffs.

† dDM, Dry-matter digestibility; dOM, organic matter digestibility; dE energy digestibility; m, measured value *in vitro*.

‡ Residual standard deviation.

Correlation analyses of *in vitro* and *in vivo* digestibility from the evaluation of multi-enzyme *in vitro* methods are summarized in Tables 2 and 3.

### Discussion

The 'filtration' methods appear to give valuable results for predicting faecal and ileal digestibility in simple-stomach animals. Table 2 shows that the results from *in vitro* methods which simulate precaecal digestion are very closely correlated with digestibility

measured at the faecal level in pigs. However, it has been argued that due to extensive fermentation in the large intestine of pigs such in vitro methods should be validated on the basis of ileal digestibility data from pigs, and may also be useful for predicting faecal digestibility in poultry, which have a much lower fermentation in the hind-gut (Clunies & Leeson, 1984).

Nevertheless, Dierick *et al.* (1985) found a much lower correlation between their in vitro and in vivo data when comparing ileal instead of faecal digestibility of protein. However, this was explained by the authors to be partly due to the lack of identity for many of the investigated samples, and partly due to the large amount of endogenous protein.

In vitro digestibility of protein and amino acids varies much less between feedstuffs than the in vivo values (Boisen & Fernández, 1991 *a*). Much of the variation found in vivo is due to different protein levels in the diets. Thus, the endogenous losses of protein (and amino acids) result in a lower apparent digestibility when the protein level in the ration is reduced. Therefore, only after correction for the endogenous losses is it possible to compare in vitro digestibility with apparent digestibility at the ileal or the faecal level (Boisen & Fernández, 1991 *b*).

Table 3 shows that the results obtained from in vitro methods which simulate overall digestibility are very closely correlated to faecal digestibility of organic matter and energy. Compared with single feedstuffs, lower correlations between in vivo and in vitro values were generally found for compound feeds due to a lower range of digestibility. On the other hand, the lower variation in the chemical composition also results in lower RSD values for compound feeds.

## GENERAL DISCUSSION

### FACTORS INFLUENCING DIGESTIBILITY IN VITRO

#### *Enzyme specificity*

During digestion in vivo as in vitro, the most potent factors are the enzymes. The specificities of the enzymes determine which bonds are hydrolysed. However, the hydrolysis of a particular bond depends on the access of the enzyme to the substrate. Therefore, the degradation of other bonds by enzymes with other specificities will influence the total degradation. For that reason it is recommended that in vitro incubations include the same enzymes as those occurring in the digestive tract. This should be possible by using intestinal fluids as described by Furuya *et al.* (1979) and Löwgren *et al.* (1989). However, such methods give problems with standardization and, furthermore, these enzyme preparations are not commercially available.

On the other hand, it has been found that the substitution of ileal fluid with a pancreatin solution does not reduce the relationship between in vivo and in vitro values (Dierick *et al.* 1985). In the same study it was also found that incubation with a pancreatin solution gave similar results to those obtained with the mobile nylon-bag technique (Sauer *et al.* 1983, 1989), which can be considered as an intermediate between in vivo and in vitro methods. In fact, many in vitro methods are based on consecutive incubations with pepsin and pancreatin, suggesting that pancreatin contains all the necessary enzymes for solubilizing the potentially digestible nutrients. However, amylase activity in pancreatin can become limiting for starch degradation (Babinszky *et al.* 1990), while fat digestion needs the addition of bile acid. To the knowledge of the authors, no relevant studies on in vitro digestibility of fat have been published. Alternatively, the fat may be pre-extracted (Babinszky *et al.* 1990; Van der Meer & Perez, 1990) or the most easily extractable fat can be washed out from the filter with acetone (Boisen & Fernández, 1991 *a, b*).

Table 4. *Variation in conditions for pepsin (EC 3.4.23.1) incubations*

Sample size (g)	Reaction volume (ml)	Enzyme concentration (g/l)	pH	Temperature (deg)	Reaction time (h)	Reference
Single step procedures						
0.2*	40	0.5	2.2	37	8	Steinhard & Kirschgessner (1973)
2	—	(400 U)		40	48	EEC authorities
0.5	150	2		45	16	Association of Official Analytical Chemists (1984)
0.5	150	0.02		45	16	Parsons (1991)
First step in two- or three-step procedures						
0.5	10	2.0	‡	37	4	Furuya <i>et al.</i> (1979)
1		2.5	‡	37	1	Asp <i>et al.</i> (1983)
0.5	40	2.5	1.0	40	1	Metz & Van der Meer (1985)
0.15*	20	0.5	‡	37	4	Dierick <i>et al.</i> (1985)
0.07†	20	0.87	1.9	37	0.5	Savoie & Gauthier (1986)
0.5	40	2.5	1.5	40	1	Van der Meer & Perez (1990)
1	40	0.25	2.0	39	6	Boisen (1991)

\* Crude protein (nitrogen  $\times$  6.25) content.

† N content.

‡ 0.075 M-hydrochloric acid.

The degradation of dietary fibre, which can only take place by the action of microbial enzymes, can be simulated by rumen fluid (Vervaeke *et al.* 1989), caecal fluid (Ahrens *et al.* 1991), faeces extract (Löwgren *et al.* 1989), or an appropriate enzyme complex of microbial carbohydrases (Boisen & Fernández, 1991 *a, b*).

Despite the complexity of digestion, very simple *in vitro* methods incorporating only one enzyme seem to give valuable information, especially with investigations of such reactions as the influence of processing on protein digestibility in certain feedstuffs (Parsons, 1991). However, for such purposes even simpler methods may be used, such as solubility measurements in potassium hydroxide (2 g/l) (Araba & Dale, 1990; Parsons, 1991).

#### *Enzyme activity*

The activity of the enzyme is also very important, particularly in methods where initial digestibility rates are measured. Several factors, such as concentration, temperature, pH, stability, activators, inhibitors and incubation time, will affect enzyme activities.

Some of the variable conditions of pepsin incubations in published methods are compared in Table 4, and a great variation in most conditions can be seen. Thus, the sample size in some methods depends on the weight of the material and in others on the N content. Other variables are reaction volume, 10–150 ml; pH, 1.0–2.2; temperature, 37–45°; reaction time, 0.5–48 h. Finally, the pepsin concentration varies from 0.02 to 2.5 g/l. However, pepsin concentrations are not directly comparable, as preparations with different activities have been used.

The enzyme activity is also influenced by several factors in the sample itself. In general, fibre and ANF will reduce *in vitro* enzyme activities. Thus, the activity of pancreatic enzymes is inhibited by most purified components of dietary fibre (Schneeman, 1982; Ikeda & Kusano, 1983) and also by the indigestible residues from food in general (Morón *et al.* 1989), while pectin may lead to an elevation of enzyme activity (Schneeman, 1982).

The mechanism of inhibition by most fibres might be due to absorption of enzymes into the fibre matrix (Schneeman, 1978), or unspecific bindings to the fibres (Ikeda & Kusano,

1983). Similarly, phytate can reduce digestibility by formation of protein-phytate complexes (Lathia *et al.* 1987).

The effects of ANF and fibre in vitro are greatly dependent on the experimental conditions. With multi-enzyme assays the reduction in enzyme activity caused by fibre is generally much lower than that found with single-enzyme digestion (Howard & Mahoney, 1989). The pepsin-pancreatin method applied in our laboratory was also found to be influenced only to a slight degree by various fibre types and ANF (Boisen, unpublished results). Similarly, very little effect of ANF has been observed in situ with the mobile nylon-bag technique (Van der Poel, 1990).

#### *Sample and particle size*

At least two important factors are influenced by sample size: representative sampling and relative losses during transferring processes in the procedure. Because of this, the samples should be of constant size and preferably not smaller than 0.5 g.

Normally a smaller particle size is used in in vitro assays than in in vivo tests. This smaller size is necessary in order to ensure representative sampling. It has been shown that an increased particle size requires a longer incubation time in vitro, but final digestibility may not be affected (Löwgren *et al.* 1989).

#### *Separation technique*

Continuous separation of end-products from undigested material by dialysis or by ultrafiltration techniques generally increases the hydrolysis rate, probably due to removal of end-product inhibition. Cave (1988) found that ultrafiltration techniques with membranes of 500 Dalton pore size were superior to precipitation with TCA acid when in vitro and in vivo estimates of availability of N and amino acids were compared. On the other hand, in most in vitro methods the great surplus of enzymic capacity ensures that end-product inhibition has little or no influence on digestibility (Büchmann, 1979).

## RELATIONSHIP BETWEEN IN VITRO AND IN VIVO RESULTS

#### *Prediction of in vivo digestibility*

A close relationship between in vivo and in vitro results has been obtained after in vitro measurements of (1) partly digested materials, e.g. dialysis cell method, (2) initial velocity of digestion, e.g. pH-drop and pH-stat method, (3) complete digestion of susceptible nutrients, e.g. most colorimetric and 'filtration' methods, (4) complete digestion of susceptible protein corrected for endogenous losses of protein (or amino acids), e.g. for prediction of apparent digestibility of protein and amino acids in some 'filtration' methods.

In some investigations very high correlations have been obtained, particularly when a relatively low number of samples has been included, and when the investigations were performed on (1) single feedstuffs having a relatively high variation in digestibility, (2) compound feeds with a systematic composition, or (3) the same or closely related feedstuffs which have been processed differently.

However, the objective of most of the described in vitro techniques, particularly those from the 'filtration' group, is that they should be able to give a more general prediction of all relevant feedstuffs and mixtures. The results in Tables 2 and 3 strongly indicate that this is possible with multi-enzyme methods, and that these methods are able to predict nutrient digestibility either at the faecal or the ileal level.

The in vivo measurements include variable amounts of endogenous and bacterial matter and are therefore 'apparent' measures of digestibility, while in vitro measurements in most of these methods correspond more to actual or 'true' values of digestibility. In vitro values

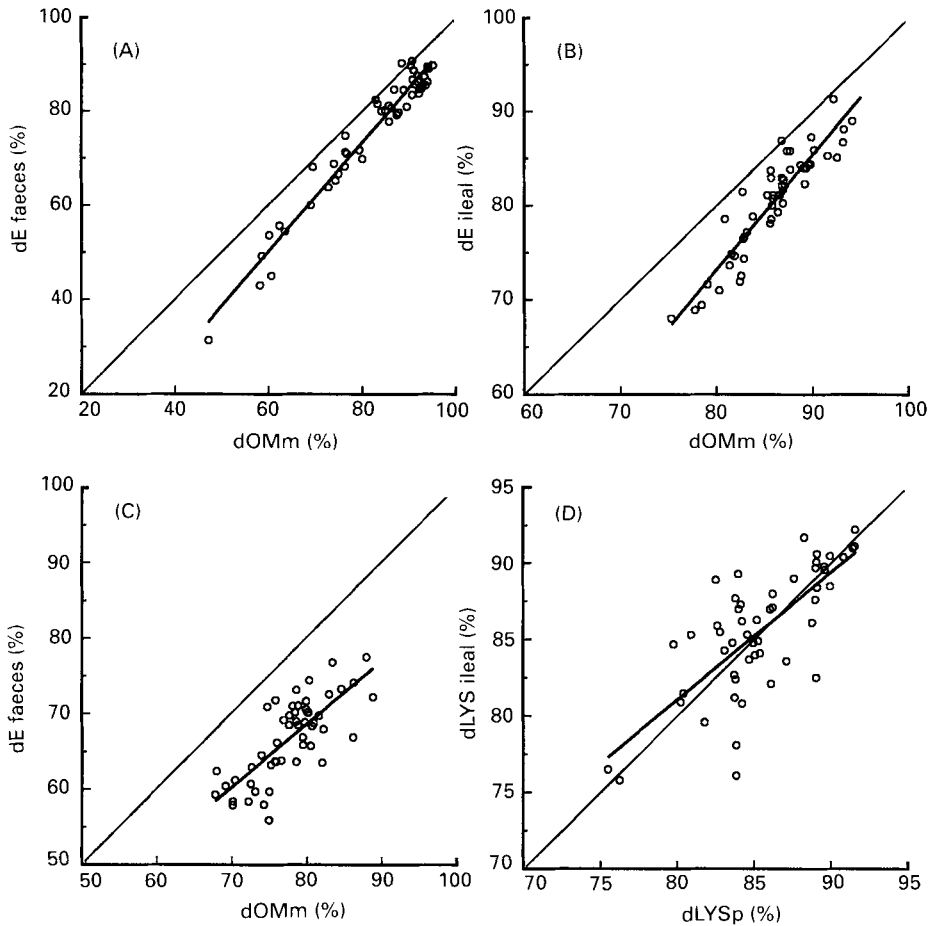


Fig. 2. Relationship between the in vivo digestibility of energy at the faecal level (dE faeces) or the ileal level (dE ileal) and in vitro digestibility of organic matter (dOMm) measured with single feedstuffs (A) or compound feeds (B and C). (D) Relationship between apparent ileal digestibility of lysine (dLYS ileal) and the predicted digestibility of lysine (dLYSp) based on in vitro measurements of N and the endogenous loss of lysine; for details, see Tables 2 and 3. From Boisen & Fernández (1991 *b*).

from these methods are therefore expected to be higher than in vivo values, as is also seen in Fig. 2, which illustrates some of the results reported by Boisen & Fernández (1991 *b*). Many of the differences between in vitro and in vivo digestibility are expected to be due to the bacterial content at the faecal level (Fig. 2(A and B)). However, at the ileal level (Fig. 2(C)) most of these differences are related to endogenous losses, and probably also undigested starch and other potentially digestible nutrients which have been digested (solubilized) in vitro but not digested in vivo. Fig. 2(B and C) shows also that the relationship between in vivo and in vitro digestibility is somehow lower at the ileal level than at the faecal level. This appears to be a general tendency, and is probably due to the lower accuracy of the in vivo measurements and also to higher individual variation between the animals at the ileal level caused by incomplete in vivo digestion of the potentially digestible nutrients.

Fig. 2(D) shows the relationship between apparent ileal digestibility of lysine ( $x$ ) and the predicted digestibility of lysine when corrected for the endogenous loss of lysine ( $y$ ). The



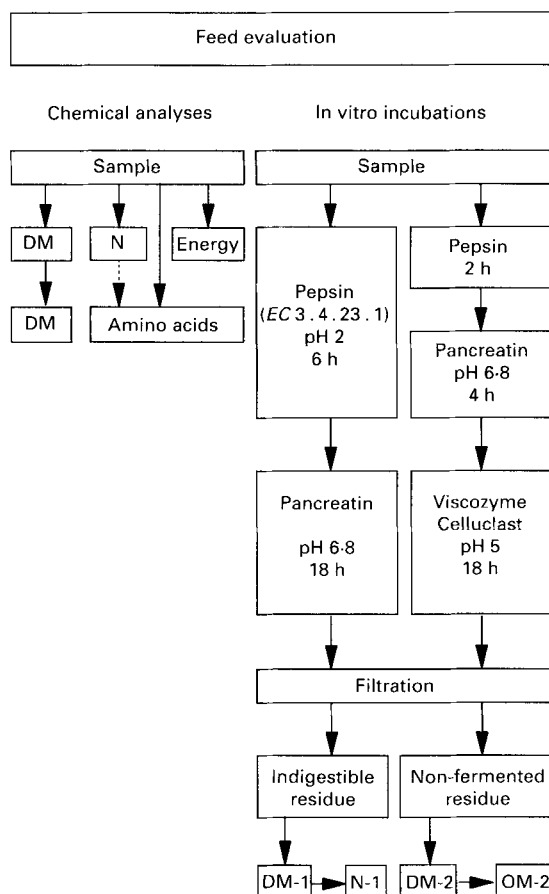


Fig. 3. A complete system for feed evaluation based on chemical analyses supplied with in vitro incubations for predicting precaecal digestibility of amino acids and energy and overall digestibility of energy. Modified from Boisen (1991). DM, dry matter.

correlation obtained is rather low due to a relatively low range of lysine digestibility. However, the RSD value is also low (Table 3).

*Prediction of in vivo availability*

The results from most in vitro methods are based on the formation of digestion products that are (1) soluble and eventually not precipitated by precipitating agents or (2) dialysable. The digestion products are those separated from the undigested residue (1) by filtration, ultra-filtration or centrifugation and (2) by dialysing through a membrane with a defined pore size. The in vitro results of amino acid digestibility may be directly correlated with in vivo estimates of availability (Cave, 1988). However, especially in processed foods, the amino acid availability may not always be synonymous with these criteria of digestibility. Therefore, the in vitro results obtained by these methods might be improved by an additional measurement of the availability of amino acids, particularly of lysine which is easily modified by reaction of the ε-amino group with sugars or lipids, and furthermore is most often the first limiting amino acid in cereal-based diets.

The availability of lysine may be predicted by the almost classic, but not very specific, Carpenter (1960) method. Alternatively, available lysine can be predicted from a simple

and specific method with lysine oxidase (*EC* 1.4.3.14) (Assoumani *et al.* 1990). In our laboratory we have just started to investigate the use of a specific microbial method (Tuffnell & Payne, 1985) with mutants of *Escherichia coli* bacteria which are deficient in the ability to synthesize single essential amino acids, e.g. lysine, methionine or tryptophan.

#### *Prediction of feed value*

Detailed investigations have been performed by several research groups in order to improve the *in vitro* technique in such a way that all relevant feedstuffs and their mixtures can be included in a general and close relationship between *in vitro* and *in vivo* results. This is also necessary if the *in vitro* technique is to be generally used (1) for analysing the feed ingredients before production of compound feeds with an optimal composition and (2) for controlling the energy and protein value of compound feed with unknown composition.

The final system suggested for feed evaluation based on *in vitro* measurements includes chemical analyses of dry matter, ash, N, energy and amino acids, combined with two parallel multi-enzyme systems for predicting precaecal digestibility of amino acids and energy and overall digestibility of energy (Fig. 3). From this system the protein value of the food can be calculated from the amino acid composition in the digested protein in relation to ideal protein, while the energy value can be calculated from digested energy, fermented energy and energy loss from the calculated non-utilizable amino acids (Boisen, 1991). The system can be further improved by supplementing with specific analyses of (1) amino acid availability, mainly in processed foods, and (2) ANF and fractions of dietary fibre.

Compared with other chemical methods enzymic degradability provides the most precise estimate of the digestibility of pig feedstuffs (Van der Meer & Perez, 1990). A similar conclusion for ruminant feedstuffs has previously been made by Aufrere & Michalet-Doreau (1988). Furthermore, the prediction of feed value may be improved by supplementing analyses of chemical fractions, like crude fibre, in a multiple regression (Van der Meer & Perez, 1990).

An alternative method using near-infrared reflectance spectrophotometry (NIRS) has the potential to be developed into a reliable method for predicting digestibility (Harrison *et al.* 1991; Van Leeuwen *et al.* 1991). This method is much more rapid than *in vitro* methods, but is also dependent on calibration with a relatively large number of samples with known *in vivo* values, which is the major barrier to further development (Harrison *et al.* 1991). However, for this purpose reliable *in vitro* results can be of great importance.

## CONCLUSIONS

Several enzymic *in vitro* methods for investigating digestibility of nutrients in foods have been developed.

The objectives of the methods are (1) to study kinetics of digestion as influenced by various factors, (2) to predict digestibility of one or more nutrients in processed and closely related foods, or (3) to predict digestibility of nutrients in all relevant foods for simple-stomach animals, e.g. pigs, poultry or man.

The principles of the methods depend to some degree on the objectives. The digestibility values may be based on measurements of (1) initial velocity of digestion with or without removal of digestion products which may inhibit the digestion, (2) partial digestion in single- or multi-enzyme systems, and (3) complete digestion of susceptible nutrients in multi-enzyme systems.

The choice of enzymes and incubation conditions and the need for equipment are also dependent on the objectives. Single-enzyme methods can be useful for predicting the digestibility of single nutrients, e.g. protein by the use of pepsin, or starch by the use of

amylase. However, as the digestion of one nutrient generally is influenced by the digestion of the other nutrients, multi-enzyme methods are believed to give the most reliable values.

For simulating precaecal digestion, satisfactory enzyme mixtures may be prepared from pure enzyme preparations, pancreatin, or intestinal fluids. For simulating post-ileal fermentation, incubations may use live bacteria from intestinal or rumen fluids or from extracts of faeces. Compared with intestinal fluids, pancreatin is (1) easy to obtain (commercially available), (2) more constant and well defined in composition, which increases reproducibility within the laboratory and in particular between laboratories, (3) free from microbial enzymes, which makes it possible to measure digestion with purified endogenous enzymes and microbial enzymes separately and therefore also more correctly to predict the energy value. This suggestion is based on the lower energy value of fermented materials compared with carbohydrates digested in the small intestine. Prediction of fibre degradability by the use of commercially available fibre-degrading enzymes, rather than living bacteria, improves standardization and reproducibility and reduces incubation time.

Fibre and ANF, like trypsin inhibitors, tannins, lectins, glucosinolates and alkaloids, which depress nutrient digestibility *in vivo*, have a much smaller effect on measurements *in vitro* (as well as *in situ* by the mobile nylon-bag technique). This is due to the specific and quantitative action of these substances on *in vivo* conditions.

For kinetic studies, the dialysis-cell method seems to be superior to other methods. The pH-drop and pH-stat methods can be useful for studying variations in protein digestibility within related foods, e.g. processed foods. However, the pH-stat method seems to be superior to the pH-drop method, especially for foods with a high buffer capacity.

For general and routine use in practical feed evaluation, multi-enzyme incubations in two or three steps followed by a collection of undigested materials by filtration have been developed. These methods have been demonstrated to be able to predict precaecal and overall digestibility respectively in a great variety of foods. Specific analyses of available amino acids, ANF and fractions of dietary fibre may improve the prediction of energy and protein value of the foods.

In future, reliable *in vitro* methods may also become the basis for the development of other methods like NIRS, which is an even simpler and more rapid but at present a less precise method of predicting digestibility than enzymic *in vitro* methods.

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