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***In Vitro* and *In Situ* Techniques for Estimating Digestibility**

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Introduction

New feeding systems need to be founded on the mechanisms that govern the response of animals to nutrients, dealing with quantitative aspects of digestion and metabolism in the ruminant animal. Digestibility and rumen degradability have been recognized as the main sources of variation of the energy and protein value of feeds, respectively. For the quantitative description of digestive and metabolic processes, appropriate biological data are required and can be obtained using *in vivo*, *in situ* and *in vitro* methods.

Information obtained *in vivo* is the most reliable and should be the reference to evaluate other methods, because it represents the actual animal response to a dietary treatment. However, *in vivo* digestion trials are expensive, laborious, time-consuming and not readily applicable to large numbers of feeds or when only small quantities of each feedstuff are available. *In vivo* results are restricted to the experimental conditions under which measurements are carried out, such as level of feeding and associative affects between feeds (Kitessa *et al.*, 1999). *In vivo* techniques to determine rumen degradability or intestinal digestibility require animals to be surgically modified, and measurements of digesta flows and of microbial and endogenous contributions of nutrients may be needed, resulting in digestibility and degradability estimates subject to large variability and additional errors associated with use of digesta flow rate markers, microbial markers and inherent animal variation. This variation demands use of sufficient experimental replication to obtain reliable results. Therefore, these trials cannot be considered routine in most laboratories, and cannot be carried out for all the possible feeding situations found in practice. Thus, the prediction of feed digestibility or energy values from *in vitro* or *in situ* information has become a necessity in all the feeding systems.

In vitro and *in situ* techniques represent biological models that simulate the *in vivo* digestion processes with different levels of complexity. These

techniques allow manipulation of parameters defining the state of the animal and, if properly evaluated against *in vivo* observations, can be appropriate to study the response of the animal when one factor is varied and controlled without the interaction of other related factors, which could conceal the main effect. Thus, *in vitro* and *in situ* techniques may be used to study individual processes providing information about their nature and sensitivity to various factors. Also a number of *in vitro* and *in situ* methods have been developed to estimate digestibility and extent of ruminal degradation of feeds, and to study their variation in response to changes in rumen conditions. Such techniques have been used for feed evaluation, to investigate mechanisms of microbial fermentation, and for studying the mode of action of anti-nutritive factors, additives and feed supplements.

This chapter will review recent developments in feed evaluation, with attention given to the role of *in situ* and *in vitro* methods in combination with mathematical modelling, in predicting digestibility and extent of degradation in the rumen of feeds.

***In Vitro* Techniques**

Methods to estimate whole tract digestibility

An overview of methods in use to estimate whole tract digestibility is presented in Table 4.1.

Solubility

The objective of separating soluble and insoluble components by simple extractions is to differentiate fractions that are either readily digestible or potentially indigestible, respectively (Van Soest, 1994). This could explain why with some of these techniques and for some feeds, a significant correlation between solubility and digestibility has been observed (Minson, 1982). Nocek (1988) has reviewed some of the solubility techniques used to predict the digestibility of feeds. Different solvents have been used, but with forages the best results have been obtained with the detergent system of fibre analysis (Van Soest *et al.*, 1991), which separates feeds into a combination of uniform and non-uniform fractions. The uniform fractions are the cell contents (or neutral detergent solubles that are essentially completely digestible), and the lignin that can be considered indigestible. The neutral detergent fibre (NDF) and the acid detergent fibre (ADF) have a variable digestibility that depends on multiple factors, but mainly on the lignification (Van Soest, 1994). The detergent system of fibre analysis has been extensively used to study the chemical composition of forages and also to predict digestibility (Van Soest, 1994).

Methods using rumen fluid

With these methods, digestibility is measured gravimetrically as substrate disappearance when the feed is incubated in the presence of ruminal contents diluted in a buffer solution. According to Hungate (1966), the first reported use

Table 4.1. Methods to estimate whole tract digestibility.

Methods	References
1. Using rumen fluid	
<i>Substrate disappearance</i>	
• Incubation in rumen fluid after 24–48 h	Walker (1959); Smith <i>et al.</i> (1971)
• Incubation in rumen fluid 48 h + incubation in HCl pepsin 48 h	Tilley and Terry (1963)
• Incubation in rumen fluid 48 h + extraction in neutral detergent	Goering and Van Soest (1970)
• <i>In vitro</i> filter bag technique	Ammar <i>et al.</i> (1999)
<i>Fermentation end-products formation</i>	
• Gas production after 24 h incubation in rumen fluid	Menke <i>et al.</i> (1979)
<i>Using faecal instead of ruminal inoculum</i>	El Shaer <i>et al.</i> (1987); Omed <i>et al.</i> (2000)
2. Using cell-free enzymes	
• Cellulase	Jones and Theodorou (2000)
• Acid pepsin + cellulase	Jones and Hayward (1975)
• Amylase + cellulase	Dowman and Collins (1982)
• Neutral detergent extraction + cellulase	Roughan and Holland (1977)
• Acid + cellulase	De Boever <i>et al.</i> (1988)
3. Solubility	
• Neutral detergent extraction	Van Soest <i>et al.</i> (1991)

of these techniques was in 1919, but the key progress in this methodology occurred when buffer solutions able to maintain an appropriate pH were used, thus allowing for longer term *in vitro* incubations. Many early *in vitro* systems consisted of a one-stage digestion in rumen fluid to measure *in vitro* digestibility (Donefer *et al.*, 1960; Smith *et al.*, 1971). One of the first comparisons between *in vitro* and *in vivo* digestibility was reported by Walker (1959).

The two-stage method described by Tilley and Terry (1963) is the most extensively used for *in vitro* digestibility. With this technique, a second stage was introduced after incubation in buffered rumen fluid for 48 h, in which the residue is digested in acid pepsin to simulate the digestion in the abomasum. Using a wide range of forages, Tilley and Terry (1963) confirmed the high correlation between *in vitro* and *in vivo* digestibility, with the *in vitro* values being almost exactly the same as the *in vivo* digestibility determined with sheep. To obtain reliable estimates of *in vivo* digestibility, the *in vitro* technique should be calibrated with samples of known digestibility, and then the conversion of *in vitro* digestibility to estimated *in vivo* results can be achieved by using correction factors (Minson, 1998). The *in vitro* digestibility technique led to the development of the concept of forage D value, defined as the content of digestible organic matter in forage dry matter (DM), used widely to predict digestibility and energy value of forages (Beever and Mould, 2000).

Some methodological modifications of the original technique described by Tilley and Terry have been suggested to facilitate scheduling for routine analysis of large numbers of samples. These include modifications in the acidification of the first stage residue, in the filtering system, in the length of the second stage or in the buffer solution composition (Marten and Barnes, 1980; Weiss, 1994). Goering and Van Soest (1970) proposed the use of neutral detergent solution as an alternative for acid pepsin in the second stage. The extraction with the neutral detergent removes bacterial cell walls and endogenous products in addition to protein, and therefore this modification predicts true digestibility rather than apparent digestibility (Van Soest, 1994). Furthermore, the second stage is substantially shortened allowing for large-scale operation.

One recent and promising alternative is offered by an *in vitro* filter bag technique. Small amounts of sample are weighed into polyester bags, which are incubated within a single fermentation vessel placed in revolving incubators (Ammar *et al.*, 1999; Adesogan, 2002). A large number of samples can be analysed at one time, and determinations of DM, NDF and ADF can be carried out on the residue contained in the bag. The system allows for investigating the effects of changes in the rumen environment on the digestibility of feeds, such as the addition of a substance.

Another *in vitro* method to estimate digestibility that has had wide acceptance is the gas measuring technique proposed by Menke *et al.* (1979), based on the close relationship between rumen fermentation and gas production (Van Soest, 1994). Basically, a small amount of feed is incubated in buffered rumen fluid and then the gas produced by fermentation is measured after 24 h of incubation. The volume of gas accumulated is highly correlated with *in vivo* digestibility, and different empirical equations were developed to predict *in vivo* digestibility from chemical composition and *in vitro* gas production (Menke and Steingäß, 1988). Other methods based on measuring the accumulation of volatile fatty acids (VFA) or heat generation during *in vitro* fermentation have been suggested to estimate digestibility.

The *in vitro* rumen fermentation methods are subject to multiple sources of variation, such as the type of fermentation vessels, the composition of the buffer-nutrient solution, the conditions of incubation (anaerobiosis, pH, temperature, stirring), the sample size or the sample preparation (drying, grinding, particle size) (Marten and Barnes, 1980; Weiss, 1994). However, the most important factors are the length of incubation and the inoculum source, processing and amount used. As to the length of incubation, a 48-h incubation period has been suggested for the gravimetric techniques as the overall optimal time for better accuracy of the digestibility estimates, whereas for the gas production method, the best results were observed with incubation times of 24 h. The length of the *in vitro* fermentation, however, can be altered depending upon the objectives of the trial.

The inoculum represents the greatest source of uncontrolled variation in these techniques. The activity and microbial numbers in the inoculum can show significant differences for different animal species, breeds, individuals, and within the same animal from time to time, as well as for the diet of donor animals (Marten and Barnes, 1980; Weiss, 1994). To overcome the

requirement for fistulated donor animals to provide the liquor, the use of faecal samples as an alternative source of fibrolytic microorganisms has been considered (El Shaer *et al.*, 1987; Omed *et al.*, 2000). The inoculum activity is affected by dietary effects to a lesser extent when faecal liquor is used, and the technique seems to be more suitable for free-ranging animals, although the values obtained are somewhat different from those observed with ruminal inoculum (Omed *et al.*, 2000).

Enzymatic methods

The use of enzymes as alternatives to rumen fluid has the advantages of overcoming the need for fistulated animals and anaerobic procedures, simplifying analytical methodology and eliminating the variability in activity of the inoculum (Nocek, 1988; Jones and Theodorou, 2000). The enzyme activities must reflect the digestive process in the ruminant. Cell-wall-degrading enzymes able to digest the structural carbohydrates have been used to estimate digestibility of forages. In most cases these enzymes are commercial and have been obtained from aerobic fungi. In particular, crude cellulases from *Trichoderma* species have generally been found to be the most reliable sources of fibrolytic enzymes (Jones and Theodorou, 2000). Although the main activity of these enzymes is cellulolytic, they can hydrolyse other structural carbohydrates.

Initially, one-stage methods consisting of incubating feed samples for some time in a buffer solution containing the cellulase were used. However, the low substrate disappearance values observed suggested that the enzymes could not remove readily all the soluble constituents of the feed. Hence, different treatments of the samples prior to the incubation in cellulase were suggested, such as incubation in acid pepsin (Jones and Hayward, 1975) or in amylase (Dowman and Collins, 1982), neutral detergent extraction (Roughan and Holland, 1977) or treatment with hot acid (De Boever *et al.*, 1988). The potential of these techniques in feed evaluation depends on the reliability and robustness of the predictive equations derived for *in vivo* digestibility. Results reported seem to indicate that enzymatic solubility can be considered a good estimator of digestibility, with small prediction errors (De Boever *et al.*, 1988; Jones and Theodorou, 2000; Carro *et al.*, 2002). But the values observed with these enzymatic techniques differ to some extent from the actual digestibility coefficients, and the regression equations are affected by forage species, methods of pre-treatment and source of enzyme (Weiss, 1994; Jones and Theodorou, 2000). Nevertheless, when a simple relative ranking of digestibility is the objective, enzymatic digestion is clearly an attractive prospect.

Methods for rumen studies

In vitro systems to investigate rumen fermentation

The direct study of rumen fermentation is difficult, and different systems have been designed to allow rumen contents to continue fermenting under controlled laboratory conditions to follow fermentation patterns (Table 4.2). Several systems have been developed with the aim of attaining conditions

Table 4.2. Methods to investigate rumen fermentation.

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1. Batch cultures or bulk incubations
 - Short- or medium-term experiments
 - Non-steady-state conditions
 2. Continuous cultures
 - Medium- or long-term experiments
 - Quasi-steady-state conditions
 - Types:
 - 2a. The semi-permeable or dialysis type
 - 2b. The continuous flow type
 - (a) The dual-flow system
 - (b) The single outflow system
 - 2c. The semi-continuous flow type: the Rusitec
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approaching those observed within the rumen *in vivo*, with the system design being prompted, to some extent, by the particular objectives of the research. The system will also be different, depending on the type of microbial population to be cultured: isolated pure cultures of either one single species or a group of microorganisms or incubation of mixed rumen contents. Czerkawski (1991) considered some obligatory (temperature and redox-anaerobiosis control, provision for replication, ease of use) and optional (efficiency of stirring, pH control, removal of end-products, provision for gaseous exchanges, sterile conditions) criteria for successful *in vitro* rumen fermentation work. *In vitro* systems have been classified into two main types: bulk incubations (also called batch cultures) and continuous cultures. Within each type it is possible to have open (accumulated fermentation gas is released or gas is circulating through the reaction mixture) or closed (the mixture is incubated under a given volume of gas and the gas produced is somehow collected to be measured) systems (Czerkawski, 1986).

BATCH CULTURES. Batch cultures are the simplest and most commonly used *in vitro* fermentation systems, and are very useful for experiments in which a large number of samples or experimental treatments are to be tested ('screening trials'), or when the amount of sample available is very small (Tamminga and Williams, 1998). The main application of these systems is to estimate digestibility or the extent of degradation in the rumen, either by single end-point or kinetic measurements of either gravimetric substrate disappearance or end-products accumulation (Weiss, 1994). VFA production can be measured easily *in vitro* as the accumulation of VFA when the substrate is incubated. Internal (purines) or external (^{15}N , ^{14}C , ^{32}P) markers are required to measure microbial synthesis (Hristov and Broderick, 1994; Blümmel *et al.*, 1997a; Ranilla *et al.*, 2001). The main drawback of using batch cultures to study rumen fermentation is that only short- (hours) and medium-term (days) experiments are possible and steady-state conditions cannot be reached owing to the microbial growth pattern. After reaching an asymptote, the

microbial population tends to decrease due to the shortening of substrate and the accumulation of waste products, resulting in lysis and death of microbial cells.

CONTINUOUS CULTURES. In continuous culture systems or chemostats, there is a regular addition of buffer and nutrients and a continual removal of fermentation products, reaching steady-state conditions, which allow for the establishment of a stable microbial population that can be maintained for long periods of time. The systems allow measurement of fermentation parameters, extent of DM degradation, output of end-products and microbial protein synthesis (Czerkawski, 1986). Thus, these systems simulate the rumen environment closer than batch cultures, and enable the study of long-term (weeks) effects of factors affecting the microbial population and the digestion of nutrients under controlled conditions of pH, turnover rate and nutrient intake (Michalet-Doreau and Ould-Bah, 1992; Stern *et al.*, 1997). However, some time is required after inoculating the culture before steady-state conditions are achieved. Czerkawski (1991) defined three types of *in vitro* rumen continuous cultures or fermenters:

- The *semi-permeable type*, a continuous dialysis system in which the microbial culture is enclosed inside a semi-permeable membrane. This system is very complex, not suitable for routine use, and cannot be fed with solid substrates.
- Continuous cultures in which the fermenter contents are completely mixed up, a liquid buffer-solution containing nutrients is infused continuously, the feed (particulate matter) is dispensed regularly into the vessel, and some of the reaction mixture, containing particles in suspension, is either pumped out or simply allowed to overflow. As the input and output of both liquid solutions and solid feed are continuous, these systems are regarded as *continuous flow type systems* (Czerkawski, 1991). Several fermenters of this type have been described in the literature (Stern *et al.*, 1997). The dual-flow systems (Hoover *et al.*, 1976) incorporate a dual effluent removal system, simulating the differential flows for both liquids and solids. In the single outflow systems a specially designed overflow device is fitted, so the feed particles stratify in the vessel according to density, providing the basis for differential liquid and solid turnover rates as in the rumen (Teather and Sauer, 1988).
- The *Rusitec* (Rumen Simulation Technique), a fermenter (Czerkawski and Breckenridge, 1977) with just a single outflow to control dilution. Both the infusion of the buffer solution into the vessel and the removal of the liquid effluent by overflowing are continuous. However, there are no provisions for continuous feed supply and solid particles outflow from the vessel, so the Rusitec is considered a *semi-continuous flow system*. Despite its limitations, the Rusitec represents a simple and elegant system to simulate the compartmentation occurring in the rumen (Czerkawski, 1986), and kinetic studies are facilitated in comparison with continuous flow systems where the use of markers is required.

Modelling the production and passage of substances in continuous culture systems is simpler than in the rumen because conditions are stable, without confounding effects of endogenous matter, absorption and passage are a single process (removal or outflow), and feed input and outflow rates are constant, regulated and measured directly. Nevertheless, similar to *in vivo* studies, reliable techniques are required for differentiation of microbial and dietary fractions by the use of markers (^{15}N , purines).

Rusitec and dual-flow continuous cultures seem to simulate rumen conditions to an acceptable extent (Hannah *et al.*, 1986; Mansfield *et al.*, 1995) and are excellent biological models for studying ruminal microbial fermentation.

Estimation of degradability of feeds in the rumen

A number of *in vitro* techniques have been described to estimate the degradability of feeds in the rumen (Table 4.3). Specific *in vitro* techniques have been developed to estimate protein degradability.

METHODS USING RUMEN FLUID. The *in vitro* technique of Goering and Van Soest (1970) has been used to estimate degradability in the rumen. Substrate disappearance after incubation in buffered rumen fluid followed by neutral detergent extraction is measured at several incubation times, and the degradation curve fitted to various mathematical models to estimate the fractional rate of degradation. This parameter is used with the passage rate to

Table 4.3. Methods to estimate the extent of degradation of feeds in the rumen.

Methods	References
1. Organic matter fermentation	
• Kinetics of substrate disappearance after incubation in rumen fluid	Smith <i>et al.</i> (1971)
• Kinetics of gas production after incubation in rumen fluid: the gas production techniques	Reviewed by Schofield (2000) and Williams (2000)
• Kinetics of substrate disappearance or end-products formation after incubation in cell-free enzymes (amylases, cellulases, etc.)	Nocek (1988); López <i>et al.</i> (1998)
2. Protein degradability	
• Kinetics of ammonia production after incubation in rumen fluid: the inhibitor <i>in vitro</i> method	Broderick (1987)
• Kinetics of ammonia and gas production after incubation in rumen fluid	Raab <i>et al.</i> (1983)
• Use of microbial markers <i>in vitro</i>	Hristov and Broderick (1994); Ranilla <i>et al.</i> (2001)
• Kinetics of nitrogen loss after incubation in cell-free enzymes (proteases)	Krishnamoorthy <i>et al.</i> (1983); Aufrère <i>et al.</i> (1991)
• Nitrogen solubility	Nocek (1988); White and Ashes (1999)

estimate the extent of degradation in the rumen (Waldo *et al.*, 1972). The fermentation kinetic parameters may also be derived from the cumulative gas production profile, obtained after measuring gas production at different incubation times, and using non-linear models to estimate the fermentation rate. The cumulative gas produced at different incubation times can be measured on a single, small sample (Williams, 2000).

To measure gas production from batch cultures of buffered rumen fluid at several time intervals, different devices and apparatus have been designed, based on essentially two different approaches: measuring directly the increase in volume when the capacity of the container can be expanded so the gas is accumulated at atmospheric pressure, or measuring changes in pressure in the headspace when the gas accumulates in a fixed volume container (Getachew *et al.*, 1998). Using the first approach, Menke *et al.* (1979) incubated the samples in calibrated syringes so the volume of gas produced could be measured from the plunger displacement. In other similar techniques gas volumes are measured by liquid displacement or by a manometric device.

Theodorou *et al.* (1994) used a pressure transducer to measure the volume of gas accumulated in the headspace of sealed serum bottles. This system has been adapted for computer recording to allow for large-scale operation (Mauricio *et al.*, 1999). Some automated systems have been developed to obtain more frequent readings and a large number of data points (Schofield, 2000; Williams, 2000). Basically the systems consist of computer-linked electronic sensors used to monitor gas production. Some of the systems (closed) record the changes in pressure in the fermentation vessel as gas accumulates in the headspace (Pell and Schofield, 1993), whereas in others (open) the accumulated gas is released by opening a valve when the sensor registers a pre-set gas pressure, so that the number of vents and the time of each one are recorded by a computer (Davies *et al.*, 2000).

The gas production technique can be affected by a number of factors, such as sample size and physical form (particle size), the inoculum source as influenced by animal, diet and time effects, inoculum size, manipulation of the rumen fluid, composition and buffering capacity of the incubation medium, anaerobiosis, pH and temperature control, shaking and stirring, correction for a blank, reading intervals when pressure is increased, etc. (Getachew *et al.*, 1998; Schofield, 2000; Williams, 2000). Some uniformity in the methodology is required to compare results from different laboratories. The gas technique also needs to be validated against comprehensive *in vivo* data to develop suitable predictive procedures (Beever and Mould, 2000).

It is important to understand that the technique assumes that the gas produced in batch cultures is just the consequence of the fermentation of a given amount of substrate, and the major assumption in gas production equations is that the rate at which gas is produced is directly proportional to the rate at which substrate is degraded (France *et al.*, 2000). However, there are some questions relating to this assumption that need further consideration: (i) some gas can be derived from the incubation medium, as CO₂ is released from the bicarbonate when the VFA are buffered in the culture (Theodorou *et al.*, 1998); (ii) some gas production is caused by microbial turnover, especially for

prolonged incubation times (Cone, 1998); and (iii) the partitioning of the fermentable substrate into gas, VFA and microbial mass can be different for each substrate (Blümmel *et al.*, 1997b). Gas production is basically the result of the fermentation of carbohydrates, and the amount of gas produced per unit of fermentable substrate is significantly smaller with protein-rich feeds (López *et al.*, 1998), and almost negligible when fat is fermented (Getachew *et al.*, 1998). Furthermore, the amount of gas produced per unit of fermentable substrate is affected by the molar proportions of the VFA, because a net yield of CO₂ and CH₄ is generated when acetate and butyrate are produced, but not when the end-product is propionate (Blümmel *et al.*, 1997b). Molar proportions of acetate and butyrate are greater when fibrous feeds are degraded, and more propionate is obtained when starchy feeds are fermented, giving rise to a significant variability in the fermentable substrate to gas production ratio. This ratio, also called partitioning factor (Blümmel *et al.*, 1997b), is also affected by the efficiency of microbial synthesis, as the partitioning of ruminally available substrate between fermentation (producing gas) and direct incorporation into microbial biomass may vary depending upon, amongst others, the size of the microbial inoculum and the balance of energy and nitrogen-containing substrates (Pirt, 1975). Therefore, across different feedstuffs there is an inverse relationship between the amount of microbial mass per unit of fermentable substrate and the amount of either gas or VFA produced (Blümmel *et al.*, 1997b). Based on this relationship and the stoichiometry of gas and VFA production, it has been suggested that if the amount of substrate truly degraded is known, gas production may be used to predict *in vitro* microbial biomass (Blümmel *et al.*, 1997b).

In vitro techniques to estimate protein degradability by incubating feed samples in rumen fluid are based on measuring ammonia production. However, ammonia concentration in batch cultures will reflect the balance between protein degradation and the uptake of ammonia for the synthesis of microbial protein. The amount and nature of fermentable substrates also affect ammonia concentrations, as uptake by microbes is stimulated to a greater extent than ammonia release in the presence of readily fermented carbohydrates. In order to measure net ammonia release as the main end-product of protein degradation, Broderick (1987) described an *in vitro* procedure using inhibitors of uptake of protein degradation products and amino acid deamination by ruminal microbes (hydrazine sulphate and chloramphenicol), and measuring NH₃ and amino acid concentration in the incubation medium before any uptake by microbes can occur. This procedure has been called the inhibitor *in vitro* method (Broderick and Cochran, 2000) and it gives acceptable estimates of kinetic parameters for protein degradation, as the inhibitors do not affect the proteolytic activity of the microorganisms. However, in the absence of nitrogenous precursors for protein synthesis, microbial growth will be reduced after a few hours of incubation; hence this procedure involves only short-term *in vitro* incubations. Raab *et al.* (1983) proposed an alternative procedure, measuring ammonia concentration and gas production at 24 h when feeds were incubated in rumen fluid with graded amounts of starch or other carbohydrates.

A different approach described by Hristov and Broderick (1994) uses a marker (^{15}N) to distinguish newly formed microbial protein from feed protein remaining undegraded. Similarly, differential centrifugation procedures and markers such as ^{15}N and purines have been used to estimate the efficiency of protein synthesis in batch cultures (Blümmel *et al.*, 1997a; Ranilla *et al.*, 2001). Alternative approaches estimate microbial N formation from the incorporation of ^3H - or ^{14}C -labelled amino acids.

ENZYMATIC TECHNIQUES. In these techniques the feed is incubated in buffer solutions containing commercial cell-free enzymes instead of rumen liquor. To estimate the extent of DM or cell wall degradation in the rumen, the techniques used are similar to those already described to predict digestibility. Specific fungal and bacterial enzymes have been used to measure degradation of the different feed carbohydrates, such as amylases (Cone, 1991), cellulases, xylanases, hemicellulases and pectinases (Nocek, 1988). Use of enzymes to simulate ruminal fibre digestion results generally in less DM degradation than with buffered rumen fluid presumably as a result of incomplete enzymatic activity compared with the ruminal environment. Some studies suggest synergism between digesting enzymes, so mixtures of enzymes may be necessary. Enzymatic techniques are usually gravimetric, measuring the disappearance of DM or any other feed component, but the release of any hydrolysis product can be also measured to estimate degradation (López *et al.*, 1998).

A number of different techniques have been reported to predict protein degradability using kinetic or single-point estimates of N loss from feed samples incubated with various proteases (Krishnamoorthy *et al.*, 1983; Aufrère *et al.*, 1991). Enzymes of bacterial, fungal, plant and animal origin have been used, but the reported results seem to indicate that non-ruminal enzymes may be of limited use as they may not have the same activity and specificity (Stern *et al.*, 1997). Protein degradability measurements using enzymatic techniques are affected by factors such as incubation pH, presence of reducing factors, type of protease used and batch-to-batch variability in enzyme activity, pre-incubation with carbohydrate degrading enzymes and the enzyme:substrate ratio. It seems crucial that the enzyme concentration is sufficient to saturate the substrate (Stern *et al.*, 1997). Although with these techniques feeds are ranked roughly in the same order as with other methods, it seems that enzymatic techniques do not provide accurate predictions of protein degradability across all feed types (White and Ashes, 1999).

SOLUBILITY. Nitrogen solubility in buffer or in different solvents varying in complexity has been used to predict protein degradability for some feed types (Nocek, 1988; White and Ashes, 1999). Although some results indicate a significant correlation between solubility and degradability, N solubility can be considered a useful indicator of protein degradation when comparing different samples of the same feedstuff, but of limited use for ranking different feedstuffs (Stern *et al.*, 1997). In fact, soluble proteins can be degraded at different rates or even be of low degradability, in contrast with some insoluble proteins that are readily degraded in the rumen (Mahadevan *et al.*, 1980).

The *In Situ* Technique

In this case, digestion studies are conducted in the rumen of a living animal instead of simulating rumen conditions in the laboratory, hence the term *in situ*. The disappearance of substrate is measured when an undegradable porous bag containing a small amount of the feedstuff is suspended in the rumen of a cannulated animal and incubated for a particular time interval (Ørskov *et al.*, 1980).

The technique is based on the assumption that disappearance of substrate from the bags represents actual substrate degradation by the rumen microbes and their enzymes. However, a number of questions cannot be resolved completely, as not all the matter leaving the bag has been previously degraded, and some of the residue remaining in the bag is not really undegradable matter of feed origin. Furthermore, the bag can be considered an independent compartment in the rumen, with the cloth representing a 'barrier' that on one side allows for the degradation of the feed to be assessed without mixing with the rumen contents, but on the other side implies an obstacle for simulating actual rumen conditions inside the bag. Finally, some methodological aspects require standardization for the technique to be considered precise and reproducible. Many of these questions have been investigated extensively and reviewed in the last 20 years, and a number of technical and methodological recommendations have been made (Ørskov *et al.*, 1980; Setälä, 1983; Lindberg, 1985; Nocek, 1988; Michalet-Doreau and Ould-Bah, 1992; Huntington and Givens, 1995; Vanzant *et al.*, 1998; Broderick and Cochran, 2000; Nozière and Michalet-Doreau, 2000; Ørskov, 2000) (see Table 4.4 for overview of factors).

In situ methodology

Loss of matter from the bag

Matter contained in the bag has to be degraded to pass through the pores out of the bag. However, complete fermentation is not required, and the particles can be lost once their size is smaller than the pore size. It has been suggested that the particles escaping consist of material potentially degradable during short incubation times (Setälä, 1983). Nevertheless, the particulate matter lost from the bag includes particles that have not been previously degraded, which results in overestimation of both the immediately soluble fraction and the extent of degradation, and likely underestimation of the rate of degradation (Huntington and Givens, 1995).

Loss of particles from the bag can be attributed mainly to the interaction between bag pore size and sample particle size. A standard and appropriate particle size to pore size ratio is desirable to minimize the impact of such loss on the estimate of the extent of degradation. As expected, large pore sizes lead to greater loss of particles and undegraded material. Aperture size of the bag affects significantly the initial rate of degradation, but the extent of degradation is affected to a lesser extent (Huntington and Givens, 1995).

Table 4.4. Factors affecting the *in situ* technique.

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1. Loss of matter from the bag
 - a. Bag pore size
 - b. Sample particle size
 - c. Degradation rate of the soluble fraction
 2. Recovery of matter of non-feed origin in the incubation residue
 - a. Post-incubation washing procedure
 - b. Microbial colonization of the residue
 3. Confining conditions inside the bag
 - a. Textile fibre, weave structure of the cloth
 - b. Bag porosity (pore size, open surface area)
 - c. Sample size
 - d. Bag position within the rumen
 - e. Basal diet (forage to concentrate ratio, forage type, level of feeding, long fibre)
 - f. Diurnal changes in ruminal activity (frequency of feeding, time to start incubation)
 4. Other procedural considerations
 - a. Animal effects
 - b. Replication (number of animals, bags, repetitions)
 - c. Sample preparation (high-moisture feeds)
 - d. Routine for introducing and withdrawing bags
 - e. Sampling scheme and mathematical modelling
 5. Multiple interactions amongst factors of variation
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Prior to incubation, feed samples are usually ground to facilitate handling, to provide more homogeneous and representative material for incubation, and to reduce particle size to simulate the comminution occurring normally by mastication and rumination. In the bag, the reduction in particle size is due to microbial fermentation and rubbing forces driven by the movements of the rumen wall and its contents. Milling also increases the area accessible for microbial attachment and degradation, as damaged and cut surfaces are the primary sites for microbial colonization. Different recommendations have been made about the most appropriate particle size for the *in situ* technique, as coarser particles result in lower and more variable disappearance rates, whereas too small particles are associated with greater mechanical losses of material from the bags (Weakley *et al.*, 1983; Udén and Van Soest, 1984).

Intermediate screen apertures (1.5–3 mm) for grinding have been suggested as the most adequate for the *in situ* technique (Huntington and Givens, 1995; Broderick and Cochran, 2000). Forages should be ground using a larger screen than those used for concentrates to reproduce the effect of chewing. However, simple recommendations cannot deal with other complex questions arising, because the particle size distribution after milling using a standard screen size is different depending upon the proportion of different plant parts (stems and leaves) and the physical properties (brittleness) of the feedstuff, with a significant interaction between milling screen size and feedstuff type (Emanuele and Staples, 1988; Michalet-Doreau and Ould-Bah, 1992). Furthermore, the chemical composition is variable for particles of different sizes

(Emanuele and Staples, 1988). As a mean particle size would be preferable to a grinding screen aperture, the best way to overcome this problem in part would be to establish some degree of uniformity in particle size within major feedstuff categories (Nocek, 1988; Michalet-Doreau and Ould-Bah, 1992), but standards based on particle size distribution seem to be impractical (Vanzant *et al.*, 1998).

Particulate matter loss can be quantified as the difference between the total washout from the bag prior to incubation (disappearance of material attributed to mechanical loss and washing) and the soluble fraction measured by filtration. Using the estimated particulate matter loss, some mathematical approaches have been suggested to correct the disappearance rates, the degradation parameters and the estimates of the extent of degradation (López *et al.*, 1994; France *et al.*, 1997).

Most water-soluble materials disappear from the bag unfermented, just by soaking in an aqueous solution. The assumption that this soluble fraction is instantaneous and completely degraded may not be true since some highly soluble compounds show small ruminal degradability (Messman *et al.*, 1994). This problem cannot be easily tackled by the technique. Some mathematical approximations have been suggested to account for this factor in estimating the extent of degradation (Dhanoa *et al.*, 1999), providing estimates of the degradation rate of the soluble fraction are available.

Recovery of matter of non-feed origin in the incubation residue

After withdrawal from the rumen, the bags are washed to stop microbial activity and to remove any rumen digesta and microbial matter in the incubation residue or in the bag. A considerable diversity of post-incubation washing procedures have been used, although a significant influence of the rinsing methodology on degradability estimates has been reported (Cherney *et al.*, 1990; Huntington and Givens, 1995). In the first *in situ* experiments, bags were just soaked and rinsed by hand under cold water until the water appeared to be clear. The main flaw of manual washing is that it is highly subjective, introducing a high and undesirable variability to the measurements. Thus, the use of washing machines was investigated as a means to standardize the procedure, offering better repeatability (Cherney *et al.*, 1990). The duration and number of rinses with cold water in the washing machine and the suitability of agitation and spinning have been tested (Madsen and Hvelplund, 1994).

Some influx of small fine particles into the bags allows faster inoculation of the samples. This ruminal matter that has infiltrated the bag is usually removed after mild rinsing (Udén and Van Soest, 1984), but complete removal of the microbial mass attached to the feed particles is far more difficult to achieve. Microbial colonization of the feed is required for degradation, but its presence in the residue can lead to substantial underestimation of the extent of degradation. The degree of microbial contamination of the residues is variable among different substrates. Contamination can have a large impact on the estimates of protein degradability of low-protein forages (Michalet-Doreau and Ould-Bah, 1992), but its influence using other feeds seems to be almost negligible. A number of procedures to facilitate microbial detachment minimizing

contamination of the residues have been suggested (Michalet-Doreau and Ould-Bah, 1992; Huntington and Givens, 1995), and the proportion of microbial matter in the incubation residue can be determined using markers (Michalet-Doreau and Ould-Bah, 1992). The correction for microbial contamination may give variable estimations of protein degradability depending upon the marker used (purines, ^{15}N) and the microbial pellet isolated (solid- or liquid-associated bacteria).

Confining conditions inside the bag

Despite the physical separation of bag contents from ruminal digesta, conditions inside the bag should be as similar to those in the surrounding rumen contents as possible, so the choice of an appropriate cloth seems crucial. Although silk was the first material used, bags are made from artificial or synthetic textile fibres such as polyester, dacron and nylon. The material should be entirely resistant to microbial degradation. The weave structure of the cloth determines the uniformity of the pore size, with the monofilamentous weave showing a more precisely defined pore size and being less distorted during incubation (Marinucci *et al.*, 1992). Due to the changes in that structure during incubation, repetitive use of bags should be prevented.

If the bags are overfilled with sample, the mixing and soaking of bag contents with rumen fluid can be incomplete (Nocek, 1988; Vanzant *et al.*, 1998). Recommended sample size is expressed in terms of optimal sample weight to bag surface area ratio, and values suggested are in the range of 15–20 mg/cm² (Huntington and Givens, 1995). Some materials (e.g. gluten) tend to clump when wet, which may impede particle movement and proper mixing with rumen fluid within the bag.

However, the main bag characteristic to be considered is pore size. If the pore is too small the exchange of fluids and microorganisms is restricted. Small pores may be clogged, mainly when viscous substrates are incubated. Inhibited removal of fermentation end-products from bags with small pores that become blocked during incubation can lead to accumulation of gas and acidification of the medium inside the bags (Nozière and Michalet-Doreau, 2000). The exchange of fluids between bag and rumen contents is also determined by open surface area of the bag material (proportion of the total surface area of the bag accounted for by the pores) (Weakley *et al.*, 1983; Vanzant *et al.*, 1998). With bags of small pore size, the microbial population reaching the sample may be significantly different from that present in rumen contents. A minimal aperture size of 30–40 μm is necessary to favour entry of rumen bacteria, anaerobic fungi and some protozoa into the bag (Lindberg, 1985). Therefore, intermediate bag pore sizes (35–55 μm) have been recommended to allow for a minimal microbial activity in the bags without major loss of fine particles from the feed incubated.

More diverse microbial colonization is possible with larger pore sizes, but even so the type and numbers of microorganisms inside the bag are somehow different from those in the surrounding rumen digesta. The differences between bag contents and rumen digesta for the proteolytic and amylolytic activities seem to be slight, whereas those for the cellulolytic population are larger, with

fibrolytic activity of solid-adherent microorganisms being lower in bag residues than in rumen digesta (Nozière and Michalet-Doreau, 2000).

The diet fed to the animals may have pronounced effects on the whole rumen environment, and consequently interactions between the type of feed assayed *in situ* and the basal diet fed to the animal are prevalent (Lindberg, 1985). To obtain the most accurate measurement of ruminal degradation, the same food incubated in the bag should be contained in the diet fed to the animal. However, this approach cannot be followed in all circumstances, and when the objective is to compare feeds or to develop tabular values, it seems satisfactory to use a general purpose basal diet to minimize the dietary effects (Broderick and Cochran, 2000). In theory, this diet should support optimal growth and metabolic activity of the rumen microbial population, meeting the energy, nitrogen and micronutrient requirements of most microorganisms. Probably, forage-to-concentrate ratio, type of forage and level of feeding have been the diet-related features that have received most attention. Increasing the amount of grain fed to the animals is associated with lower estimates of rate and extent of *in situ* disappearance of forages (Nocek, 1988; Weiss, 1994), but these values are significantly less affected by the type of forage included in the diet. Altered or extreme rumen conditions as well as the deficiency or excess of nutrients due to unbalanced diets can cause the undesirable exclusion of some of the microbial species. Finally, a minimum percentage of long fibre in the diet seems to be required because fibrous rumen contents enhance the circulation of fluid through the bag and its blending with the sample incubated (Huntington and Givens, 1995).

There are significant diurnal fluctuations in digestive ruminal activity, especially in animals fed once or twice daily. Frequent feeding using automatic feeders can reduce this source of variation (Lindberg, 1985), but in most cases feeds are evaluated for use in practical conditions where animals receive one or two meals per day. In this case, the time that bags are introduced into the rumen in relation to animal feeding can influence digestion rates inside the bags. Thus, to minimize this variability, all the bags should be introduced at the same time to be exposed to the same rapidly changing rumen conditions occurring after feeding (Nozière and Michalet-Doreau, 2000).

To facilitate flow of rumen liquor into and out of the bags and mixing with the feed sample, the bags should remain immersed in the liquid phase of the rumen contents, move freely and be squeezed during muscular contractions. Aspects such as length of string along which bags are fastened or use of a carrier weight have been investigated, as these devices can determine, to some extent, the position of the bags and the lack of restrictions for bag mobility during incubation (Huntington and Givens, 1995).

Other procedural considerations

It is advisable that *in situ* disappearance procedures are standardized to increase precision, as lack of standardization has been reported as the main source of variation in the assay (Madsen and Hvelplund, 1994). As for the animal effects, there may be small but significant differences in the estimates of extent of degradation of feeds if samples are incubated in the rumen of different

ruminant species and breeds (Udén and Van Soest, 1984; López *et al.*, 2001), and ideally the same type of animal for which the information is intended should be used. To improve the precision of measurements, the animal variability needs to be minimized using the same type of animals for each experiment, in the same physiological state and maintained in the same husbandry and environmental conditions (Nocek, 1988; Huntington and Givens, 1995). Provision for adequate replication (number of animals, number of bags per animal, number of incubations to account for day-to-day variation) is also necessary (Weakley *et al.*, 1983; Vanzant *et al.*, 1998). More replicates should be used for short incubation times, when the effects of particle size or host diet are more pronounced. The use of standards has been suggested as a means of accounting for the variation among animals and time periods (Weiss, 1994; Vanzant *et al.*, 1998).

The evaluation of high moisture feeds (fresh herbage and silage) is complicated because grinding is difficult unless the sample is previously dried. Wet grinding or hand-chopping and macerating are probably the best ways to simulate chewing, but these procedures cannot guarantee a uniform particle size distribution, result in some inevitable sewage and it is necessary to incubate the samples immediately after harvesting (Nozière and Michalet-Doreau, 2000). Freeze drying is a better alternative for sample preparation than oven drying (López *et al.*, 1995), but affects the physical properties of the material and thus the particle size distribution after milling.

The routine to be followed for introducing and removing the bags has also been examined. When bags are not machine washed, introducing bags at different times to be removed all at once seems preferable in order to minimize the variation attributed to bag washing technique. Otherwise, it is better to introduce all the bags at the same time and withdraw them at the intended incubation times, so that the samples are subject to the same rumen conditions in all cases. Huntington and Givens (1995) did not detect significant differences between both incubation sequences on DM degradability of feeds.

Finally, the values determined for the soluble, degradable and undegradable fractions, rate, extent and lag time may be also affected by the sampling scheme, the approach (either logarithmic-linear transformation or non-linear fitting) to derive kinetic parameters (Nocek and English, 1986) and the model selected to represent degradation kinetics (Dhanoa *et al.*, 1996; López *et al.*, 1999) (see Chapter 2). Mathematical modelling of degradation kinetics will be discussed in detail later. The incubation times and the number of data points to be recorded for kinetic studies should be established according to the minimum requirement for statistical analysis of the disappearance profiles (Chapter 2) and will depend on the shape of the curve (Michalet-Doreau and Ould-Bah, 1992). More frequent measurements are required in the first 24 h of incubation, the most sensitive part of the curve, to obtain reliable and precise estimates of the lag time and degradation rate. On the other hand, some bags will be incubated for prolonged times, long enough to reach the asymptotic values of disappearance, for the potential extent of digestion to be estimated accurately. These long incubation times vary with type of feed (in general longer for forages and shorter for concentrates).

Maybe the most important feature concerning all these factors of variation is that there are multiple interactions amongst many of them; those standing out involve the feed characteristics (Vanzant *et al.*, 1998). Because of these interactions, not a single standardized procedure seems to be applicable across all feedstuffs, but even so some concordance in the methodology used should be pursued to provide a more reliable, precise and accurate technique. It also seems necessary to assess the relative importance of each methodological factor on the precision and accuracy of degradability estimates, because some of the recommendations for the *in situ* procedures may be not applicable to experimental objectives.

Use of the in situ technique in feed evaluation and rumen studies

Initially, the technique was set out to predict *in vivo* DM digestibility, mainly of forages. In the late 1970s the technique was used to measure the extent of protein degradation in the rumen (Ørskov and McDonald, 1979). Nowadays, the *in situ* technique is a standard method for characterizing the rumen degradability of protein, given the high correlation and concordance between *in vivo* and *in situ* values (Poncet *et al.*, 1995).

Therefore, the technique has been used to study the digestive processes in the rumen and to predict the degree to which nutrients are made available for the rumen microorganisms and for the host animal (Ørskov *et al.*, 1980). The *in situ* technique is suitable for kinetic studies following the time course of disappearance of an individual feedstuff, and has been used widely to evaluate the rate and extent of degradation in the rumen (Ørskov, 2000). More recently, the technique has been used to estimate the extent of starch degradation in the rumen (Cerneau and Michalet-Doreau, 1991). Rumen degradation kinetics of lipids have been also studied *in situ* (Perrier *et al.*, 1992). Rates of fermentable organic matter and protein degradation can be estimated, and then the synchronization between energy and nitrogen availability for microbial synthesis in the rumen can be evaluated (Nozière and Michalet-Doreau, 2000).

The *in situ* technique has also been used for studying animal (species, physiological state, level of intake) or dietary (additives, diet composition, fat supplementation) factors affecting rumen conditions or microbial activity (mainly the fibrolytic activity of ruminal microorganisms) (Nozière and Michalet-Doreau, 2000; Ørskov, 2000). Due to the interaction between the basal diet and the feed evaluated in the bag, the *in situ* technique appears to be a good method for quantifying the associative effects, especially between forage and fermentable carbohydrates. Finally, based on the relationship between degradation rate and rumen fill, rumen degradation parameters estimated with the *in situ* technique have been used to predict voluntary intake of forages (Hovell *et al.*, 1986; Carro *et al.*, 1991).

Despite all its limitations, this technique is one of the best ways to access the rumen environment, it is fairly rapid and reproducible and requires minimal equipment. Therefore it is one of the techniques used most extensively in feed evaluation for ruminants.

Methods to Estimate Post-Ruminal Digestibility

Some *in vitro* techniques have been designed to estimate digestibility (mainly of the feed protein) in the small intestine (Calsamiglia *et al.*, 2000). These techniques are based on the use of enzymes to simulate abomasal and intestinal digestion (Stern *et al.*, 1997). The most commonly used technique is a three-step procedure consisting of a ruminal pre-incubation followed by an incubation in acid pepsin and a phosphate buffer–pancreatin digestion (Calsamiglia and Stern, 1995).

An *in situ* mobile bag technique has been used to determine intestinal protein digestion in ruminants (Hvelplund, 1985). Samples of feed or residues after incubation in the rumen are weighed in small polyester bags that are introduced directly into the abomasum or proximal duodenum and subsequently collected either from the ileum or from the faeces. Endogenous or other contaminating materials are removed by washing, and the indigestible residue is determined. This technique is affected by a number of potential sources of variation such as porosity of bag material, sample weight to surface area ratio, animal and diet effects, ruminal pre-incubation, pepsin HCl pre-digestion, retention time, site of bag recovery and microbial contamination of the residue (Hvelplund, 1985). Although loss from the bag may not necessarily relate to protein absorption, the technique seems to be useful in predicting intestinal protein digestibility (Stern *et al.*, 1997).

Role of Mathematical Modelling in *In Vitro* and *In Situ* Techniques

The goal of most *in vitro* and *in situ* techniques is to estimate total-tract digestibility or rumen degradability. It is very unlikely that values measured *in vitro* are identical to the intended *in vivo* values, and thus mathematical modelling is a useful tool to link the data obtained *in vitro* or *in situ* with the processes occurring *in vivo*. Mathematical models used to estimate digestibility or degradability from *in vitro* measurements can be either empirical or mechanistic.

Empirical modelling

A large number of empirical equations for predicting DM intake, digestibility, DM or protein degradability in the rumen or energy value of forages from *in vitro* and *in situ* measurements is provided in the literature (Minson, 1990; Hvelplund *et al.*, 1995). In most cases, the predictor used is a single end-point measurement determined by one of the *in vitro* techniques described previously. When end-point measurements are used, incubations are usually run for a given time interval, although in the animal the residence time in the rumen depends upon the level of feed intake, type of feedstuff and composition of the diet, and thus no single end-point measurement will be valid for all circumstances.

Using analytical results and actual values determined by feeding trials for a number of standard representative feeds, multiple regression equations can be derived statistically and used to predict the digestibility or degradability of other samples. Most of these equations are based purely on the statistical relationship between the variables and the performance of regression methods facilitated by improved computing facilities, resulting sometimes in equations with little biological meaning. One of the consequences of this empirical approach is that there are a large number of equations available in the literature differing significantly in the predicting variables, in the regression coefficients for the same predictors, and in the estimated prediction error. These empirical prediction equations are a consequence of the specific data sets used for their derivation, and thus have a variable degree of unreliability and are only useful when the situation to be predicted corresponds to the original data set. Despite these criticisms, empirical equations are used widely in feed evaluation systems.

Correlation between *in vivo* and *in vitro* or *in situ* values and statistical goodness-of-fit are the only criteria considered in evaluating these prediction equations. But the accuracy of these methods relies on a proper evaluation of the techniques and empirical models. The starting point of such evaluation would be the systematic measurement of the variable to be predicted using a reference technique (*in vivo* methods) to create a comprehensive database of the actual values against which the *in vitro* and *in situ* values can be challenged. Then, suitable prediction equations can be developed and evaluated following the stages of initial calibration and subsequent validation. New data becoming available can be incorporated into the original database contributing not only to extending its size, but also to making the prediction stronger and valid for a wider range of situations. This is a long-term approach necessary to achieve a satisfactory degree of accuracy in the estimations of digestibility and degradability.

However, many of the *in vitro* and *in situ* techniques described previously are still at a stage of methodological standardization, and thus cannot be considered sufficiently precise. This current lack of precision precludes any discussion about their potential accuracy.

Mechanistic modelling

Mechanistic mathematical modelling can simulate reality and predict nutrient utilization and availability within the digestive tract by representing quantitatively concepts and mechanisms (Dijkstra and France, 1995). This type of modelling can be used to derive kinetic parameters from data obtained *in vitro* or *in situ*, which can then be incorporated in holistic models to simulate whole system behaviour. It is expected that, in the future, mechanistic models will yield superior predictions of animal performance and will be applicable more generally than empirical models. As feed digestibility is affected to a large extent by rumen degradation and fermentation, mechanistic modelling has focused on representing and quantifying the rate and extent of substrate degradation in the rumen. Modelling of other crucial processes occurring

in the rumen, such as kinetics of VFA production or microbial growth and synthesis are reviewed elsewhere in this book (Chapters 6 and 8, respectively).

Rate and extent of degradation

Kinetic degradation parameters are necessary to predict feed digestibility, and thereby the energy available, and also protein degradability in the rumen. The amount of substrate degraded in the rumen is the result of competition between digestion and passage. Several models have been proposed since that of Blaxter *et al.* (1956), in which kinetic parameters for degradation and passage are integrated to estimate the actual extent of degradation of feed in the rumen. Degradation parameters are usually estimated from degradation profiles (Fig. 4.1) obtained using either gravimetric or gas production techniques. To associate disappearance or gas production curves with digestion in the rumen, models have been developed based on compartmental schemes, which assume that the feed component comprises at least two fractions: a potentially degradable fraction S and an undegradable fraction U . Fraction S will be degraded at a fractional rate μ (per hour), after a discrete lag time L (h). The scheme is shown in Fig. 4.2, and the dynamic behaviour of the fractions is described by the differential equations:

$$dS/dt = 0, \quad 0 \leq t < L \quad (4.1a)$$

$$= -\mu S, \quad t \geq L \quad (4.1b)$$

$$dU/dt = 0, \quad t \geq L \quad (4.2)$$

Therefore, the parameters to be estimated are the initial size of the fraction S , the size of U , the lag time (L) and the fractional degradation rate (μ) (Fig. 4.3).

Fig. 4.1. Examples of sigmoidal and non-sigmoidal cumulative gas production curves *in vitro*.

Fig. 4.2. The two-compartment model of ruminal degradation. Deletion of the dashed arrows gives scheme for disappearance during incubation *in vitro* or *in situ*.

Precise estimation of U is critical to accurate description of degradation kinetics because the degradation rate, by definition, applies only to the fraction that is potentially degradable, with the assumption that each pool is homogeneous in its kinetic properties. Fraction U of protein and fibre components has been measured by long incubations (from 6 days to several weeks) either *in vitro* or *in situ*, or estimated from non-linear fitting of degradation profiles. When degradation profiles are obtained by gravimetric techniques, the non-fibre components are assumed to contain a third fraction that disappears immediately after incubation begins, and is assumed to be degraded instantly in the rumen (called soluble fraction or washout value, W). The loss of undegraded particulate matter from polyester bags leads to an overestimation of W , underestimating the undegradable fraction. Estimation can be improved significantly by measuring the extent of particle loss from the bag and applying mathematical corrections to the parameter estimates (López *et al.*, 1994; France *et al.*, 1997). Using *in vitro* techniques allows degradation profiles with much more data points to be obtained, revealing the existence of multiple pools, which would be degraded at different rates. Some models have been reported that include several degradable pools (Robinson *et al.*, 1986; Groot *et al.*, 1996). Such models contain a considerable number of parameters requiring a large number of data points, complicating satisfactory parameter estimation due to the limitations of the non-linear regression.

The lag phase of the degradation profiles has been described in terms of either a discrete or a kinetic lag (Van Milgen *et al.*, 1993). The initial lag phase is due in part to the inability of the rumen microbial population and its enzymes to degrade the substrate at a significant rate until microbial growth is sufficient for enzymatic production to increase and ultimately to saturate the substrate. Lag may be due to factors other than microbial capacity, such as the rate of hydration of the substrate, microbial attachment to feed particles and nutrient limitations. A discrete lag is not a mechanistic interpretation of the process in the rumen. *In vitro* and *in situ* systems may induce an artificial lag because of experimental procedures, and this parameter is therefore required in the models representing the system from which the degradation profiles are obtained.

The degradation rate of nutrients in the rumen is a key factor in predicting extent of ruminal degradation, because it can have significant effects on both the ruminal microbes and the host. The fractional degradation rate can be considered an intrinsic characteristic of the feed, depending on factors such

Fig. 4.3. Representation of the degradation parameters (L , lag time; S_0 , potentially degradable fraction; YS_0 , asymptotic gas production; W , 'soluble' fraction and U , undegradable fraction) in a gas production profile (a) and in an *in situ* disappearance curve (b), showing the differences in shape attributed to the rate parameter (the higher the rate, the steeper the curve).

as chemical composition of the forage, the proportion of different plant tissues as affected by the stage of maturity, surface area and the cell wall structure. Once feed enters the rumen, the degradation rate may also be affected by factors related to the animal, such as rate of particle size reduction, and ruminal conditions (pH, osmotic pressure, mean retention time of the digesta), that

have a profound effect on microbial degradative activity. Associative effects of feeds in the diet can be very important. For example, the depressive effect of easily degradable non-fibre carbohydrates on the degradation rate of forage DM is generally recognized.

An essential aspect of estimating the rate of degradation concerns the kinetics assumed for the process. The most commonly used model (Ørskov and McDonald, 1979) assumes first-order kinetics, implying that substrate degraded at any time is proportional to the amount of potentially degradable matter remaining at that time, with constant fractional rate μ (Fig. 4.4), and that only characteristics of the substrate limit degradation. This model has been used extensively owing to its simplicity, but it is not capable of describing the large diversity of degradation profiles (Fig. 4.1), which have been observed (Dhanoa *et al.*, 1995), and cannot represent mechanistically the reciprocal influences of substrate degradation and microbial growth.

France *et al.* (2000) postulated that μ may vary with time according to different mathematical functions (Table 4.5). From the various functions used to represent μ , different models can be derived to describe either *in situ* disappearance (López *et al.*, 1999) or *in vitro* gas production profiles (Dhanoa *et al.*, 2000) (Fig. 4.4). Some of these functions are capable of describing both a range of shapes with no inflexion point and a range of sigmoidal shapes in which the inflexion point is variable. Therefore, other models are versatile alternatives to the commonly used simple exponential model for describing degradation profiles. On substituting the function proposed for μ and integrating, Eq. (4.1b) yields an equation for the S fraction remaining during the incubation *in situ* or *in vitro* at any time t , which can be expressed in the general form:

$$S = S_0 \times [1 - \Phi(t)] \quad (4.3)$$

where S_0 is the zero-time quantity of the S fraction, $\Phi(t)$ is a positive monotonically increasing function with an asymptote at unity (Table 4.5) and t is incubation time (h). *In situ* or *in vitro* disappearance (D , g/g incubated) is given by:

$$D = W + S_0 - S = W + S_0 \times \Phi(t) \quad (4.4)$$

Similarly, gas production profiles observed *in vitro* can be represented by:

$$G = YS_0 \times \Phi(t) \quad (4.5)$$

where G (ml) denotes total gas accumulation to time t and Y (ml gas per g degradable DM) is a constant yield factor. For each function, μ could be obtained from Eqs (4.1b) and (4.3) as:

$$\mu = -\frac{1}{S} \frac{dS}{dt} = \frac{1}{(1 - \Phi)} \frac{d\Phi}{dt} \quad (4.6)$$

Fig. 4.4. Change in fractional degradation rate (a) and in gas production rate (b) with time as represented by different mathematical models (EXP, exponential; FRN, France; MMF, Morgan–Mercer–Flodin).

This function constitutes the mechanistic interpretation of the degradation processes.

Rates of degradation and passage can be combined to calculate the extent of degradation of the substrate in the rumen (France *et al.*, 1990, 1993). In the rumen, if S is the amount of potentially degradable substrate remaining that is subjected to both passage and degradation, the rate of disappearance of S is given by (Fig. 4.2):

Table 4.5. Alternative functions for Φ in the general equations for the *in situ* disappearance curves and the gas production profiles, with corresponding functions for the fractional degradation rate (μ) of the substrate for each (for the meaning of the constants, which is specific to each model, see France *et al.*, 1990, 2000; López *et al.*, 1999).

	Φ	μ
France	$1 - e^{-c(t-L)-d(\sqrt{t}-\sqrt{L})}$	$c + (d/2\sqrt{t})$
Simple exponential	$1 - e^{-c(t-L)}$	c
Morgan–Mercer–Flodin	$t^c/(t^c + K^c)$	$ct^{(c-1)}/(t^c + K^c)$
Logistic	$(1 - e^{-ct})/(1 + Ke^{-ct})$	$c/(1 + Ke^{-ct})$
Gompertz	$1 - \exp[(b/c)(1 - e^{ct})]$	be^{ct}

$$\frac{dS}{dt} = -kS, \quad t < L \quad (4.7a)$$

$$= -(k + \mu)S, \quad t \geq L \quad (4.7b)$$

where k (per h) is the fractional rate of passage from the rumen, and is assumed constant. To obtain S , the solutions of these differential equations are:

$$S = S_0 e^{-kt}, \quad t < L \quad (4.8a)$$

$$S = S_0 e^{-kt} (1 - \Phi), \quad t \geq L \quad (4.8b)$$

Using these equations, the extent of degradation in the rumen (E , g degraded per g ingested) is given by the equations:

$$E = \frac{W + \int_L^\infty \mu S dt}{W + S_0 + U} = \frac{W + kS_0 \int_L^\infty \Phi e^{-kt} dt}{W + S_0 + U} \quad (4.9)$$

for *in situ* and *in vitro* disappearance profiles (López *et al.*, 1999), and

$$E = \frac{\int_L^\infty \mu S dt}{S_0 + U} = \frac{kS_0 \int_L^\infty \Phi e^{-kt} dt}{S_0 + U} \quad (4.10)$$

for *in vitro* gas production profiles.

Although ranking of and comparisons between feeds according to their *in situ* or *in vitro* E values are similar, the estimates of E values obtained using the *in situ* technique are numerically greater than those obtained using the *in vitro* gas production method (López *et al.*, 1998, 2000). The first explanation for this bias could be the loss of particulate matter from the bag, as part of this material is lost without being degraded. However, the discrepancies persist when the *in situ* values are corrected for particle loss assuming that passage losses for particulate matter escaping from the bag at zero time are according to the fractional passage rate or assuming that there is no instantly degradable fraction (Dhanoa *et al.*, 1999). The calculation for E using *in situ* parameters

assumes that there is a soluble fraction (W) that is degraded completely and instantly in the rumen, whereas in the gas production technique the soluble and the insoluble but potentially degradable fractions are both degraded at the same rate (μ) and subject to passage, so neither substrate fraction can be degraded completely in the rumen.

Furthermore, fractional rates of substrate degradation (μ) in the *in situ* technique are higher than those estimated from *in vitro* profiles. The differences in fractional degradation rates between *in situ* and *in vitro* techniques are larger with feeds having high protein contents (López *et al.*, 1998). Possible differences in gas yield per unit of substrate degraded are not directly important in the calculation of the extent of degradation E , as can be seen from the absence of Y in Eq. (4.10). However, if Y varies during the course of incubation, then the rate of gas production does not properly reflect the rate of substrate degradation. For example, a low yield at the start of the incubation period (coinciding with a high propionic acid production from rapidly degrading fractions, including the soluble fraction), and a high yield towards the end of the incubation period will underestimate the rate of substrate degradation and consequently E . The value of Y might well vary during the course of incubation for a substrate with different chemical entities (e.g. fibre, starch, sugars) because starch and sugars generally have a higher fractional degradation rate than fibre and cause a lower pH in the rumen fluid. Also, during the course of fermentation the amount of substrate becoming available per unit of microbial mass decreases, resulting in an increase in the yield of gas in the later phases of incubation. Other reasons for the discrepancies could be methodological differences between the two techniques. Possibly rumen fluid is less active *in vitro* than *in situ*, and accumulation of end-products may affect long-term fermentation in batch cultures (López *et al.*, 1998).

In conclusion, the equations derived herein provide a general expression for calculating the extent of degradation in the rumen from *in situ* and *in vitro* data, which are applicable to any model expressed in the form of Eqs (4.4) and (4.5). A number of equations have been proposed in the literature to describe the gas production curve without considering the quantitative relationship to extent of degradation in the rumen, thus failing to link the *in vitro* technique to animal performance. Now that expressions for ruminal extent of degradation for various models have been worked out (López *et al.*, 1999; France *et al.*, 2000), testing more flexible models will contribute to enhancing our understanding of degradation and fermentation kinetics, leading to better diet formulation and animal nutrition.

Concluding Remarks

In vitro and *in situ* techniques are used widely to estimate digestibility and rumen degradability, and to study ruminal fermentation. It is difficult to appraise the accuracy of many of these techniques. Only the *in vitro* digestibility technique was developed following calibration and validation of the *in vitro* estimations against the *in vivo* values. Few studies have been conducted to determine

how to obtain more accurate *in vitro* and *in situ* data, mainly because there are few reference data to which comparisons can be made. With this limitation, *in vitro* and *in situ* data are at least useful to detect treatment effects, for relative comparisons of feeds or, in some cases, as intrinsic characteristics of feeds that can be used in diet formulation.

On the other hand, values obtained with most of these techniques are less variable than those measured *in vivo*, although the reproducibility of some techniques needs to be increased substantially by standardizing the experimental procedures. The greatest level of standardization has been attained with the *in vitro* digestibility methods, whereas a large multiplicity of analytical techniques exists for the gas production method. For the *in situ* technique, important agreement has been achieved and a number of recommendations are available in the literature. But not all the variables can be completely standardized, and some flexibility is required for some of them, such as the animal species or the basal diet fed to the animals, to accomplish the research objectives and accommodate the different facilities available in each laboratory. The important point is that results can be interpreted by anyone and, if possible, compared with other reported data.

All the limitations of *in vitro* and *in situ* methods need to be borne in mind when interpreting the results, but there is no point expecting these techniques to give exactly the same values measured *in vivo*. It is possible to design very complex techniques with the aim to improve accuracy, but then many of the inconveniences of the *in vivo* experiments will be prevalent and still there will be discrepancies between estimated and actual values. In this context, mathematical modelling can play an important role, first detecting the bias between estimated and actual values in order to overcome possible methodological weaknesses of the techniques or to introduce mathematical corrections to achieve a better approximation to *in vivo* values. It is important to accept that *in vitro* and *in situ* techniques represent biological models, and hence are just simplifications of reality. The target should be a balance between that simplicity and the accuracy and precision of the values determined. A wide range of techniques is available; each with its advantages and disadvantages, and the final decision should be based on the type of work (number of feeds to be tested and amount of sample), facilities available and research objectives.

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