Forage Evaluation in Ruminant Nutrition

Edited by D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed

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Preface

Over 3000 million hectares of the land area of the earth (over 25%) is grazing land and another 4000 million hectares of forest and woodland have some grazing potential. World grasslands support approximately 1500 million cattle equivalents (cattle, buffalo, sheep, goats and camels) and forages provide over 90% of the feed energy consumed by these herbivorous animals. The world's forages therefore indirectly provide a very high proportion of the food for its population. This is achieved without seriously reducing the quantity of food available for direct human consumption.

Although forages generally provide nutrients to animals at lower cost than concentrate feeds, they are inherently variable in nutritive value. This depends on many factors such as forage species, climate, degree of maturity, etc. In many parts of the world, forages are conserved by processes such sun curing and ensiling. These processes can fundamentally change the nutritional characteristics of the original forage, sometimes in unpredictable ways.

Given the importance and variability of forages, it is vital that methods exist that can reliably assess their key nutritional attributes including, crucially, their voluntary intake by animals. In recent years a number of important factors have come into play that are changing the ways in which forage characterization in the laboratory is approached. For instance, in some countries characterization of ruminant feeds in general is rapidly moving away from expressions of energy and protein content to an assessment of the nutrients supplied to the animal both directly and indirectly as a result of microbial activity in the rumen. In addition, in some places there is increasingly powerful public pressure to reduce or stop the use of surgically modified animals in nutritional studies. This may rapidly reduce the use of techniques reliant on rumen fluid and alternatives to these will have to be found. There has been a tremendous upsurge in the use of near infrared reflectance spectroscopy for forage characterization in countries where the expensive technology is available. This emphasizes the need for cheaper but still reliable methods for less well-equipped regions of the world.

In this book the current status of forage evaluation is reviewed and discussed. An attempt has been made to establish the key aspects of forage evaluation given the demands of increasing nutritional complexity and the constraints outlined above. An account is given of the new technologies now available, and consideration given to some of the new nutritional characteristics that may be important in forages of the 21st century.

1 Forages and Their Role in Animal Systems

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This introductory chapter will consider the definition of forage, the types of feed encompassed within this definition and their characteristics, the extent to which forages contribute to animal production in different parts of the world and future prospects.

What are Forages?

The terms forage and its equivalents in French (*fourrage*), Spanish (*foraje*) and Italian (*foraggio*) are commonly used by both scientists and farmers, but there is no widely accepted definition and much variation in the breadth of feeds that may be considered within this term. The definition offered by the authoritative *Oxford English Dictionary* of 'food for horses and cattle' does not correspond with either popular or scientific usage, being too wide in terms of 'food' and too narrow in terms of the animals considered. For the purposes of this chapter, I shall follow the definition given by the Forage and Grazing Terminology Committee (1991), a group endorsed by the International Grassland Congress. Forage is defined as 'edible parts of plants, other than separated grain, that can provide feed for grazing animals or that can be harvested for feeding'. This broad definition is also used by Barnes and Baylor (1995) and includes the classes of feed listed in Table 1.1. The narrower term of 'forage crop' is often used to describe crops, generally annual or biennial, which are grown to be utilized by grazing or harvesting as a whole crop (e.g. maize, sorghum, kale).

Thus a wide range of feeds are included as 'forages', but they generally have substantial contents of cell walls (root crops are an exception) and are suited to utilization by herbivores with their substantial capability for microbial digestion of cell-wall constituents. The composition and nutritive value of forages is extremely variable, both overall and within forage types, as illustrated in Table 1.2. This indicates that different forages can make very different contributions to production

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Table 1.1. Feed types included within the definition of forage.

Herbage	Leaves, stems, roots of non-woody species, including sown and permanent grassland and crops that may be grazed or cut.
Hay and silage	
Browse	Buds, leaves and twigs of woody species
Straw	

Table 1.2. Range in nutrient contents of different classes of forages.

	Metabolizable energy (MJ kg^{-1} DM)	Crude protein (g kg^{-1} DM)
Temperate grasses, hays and silages	7.0–13.0	60–250
Tropical grasses	5.0–11.0	20–200
Maize silage	10.0–12.0	60–120
Cereal straw	5.0–8.0	20–40
Root crops	11.0–14.0	40–130
Kale and rape	9.0–12.0	140–220

systems, varying from feeds not capable of supporting animal maintenance to those with digestibility and energy concentrations as high as in cereal grains.

Forage Production

Grassland

Ruminant production systems throughout the world are based on forages, with grassland feeds being predominant. Food and Agriculture Organization (FAO, 1996) statistics indicate a total area of grassland of some 3500×10^6 ha, some 72% of the total agricultural land and 27% of the total land area. Table 1.3 shows large variation between regions in the proportion of land used for permanent grassland, ranging from 33% of total agricultural land in Europe to 89% in Oceania.

This wide extent of permanent grassland is a feature largely of the adaptation and perennial nature of grasses, making grassland either the climax vegetation in much of the world or a vegetation type that may be established following forest clearance and maintained with low levels of management input by grazing and, in some areas, by burning. The current area of permanent grassland is about twice that of natural climax grasslands. Most permanent grassland is on land with substantial limits to arable cropping, because of topographic or climatic factors.

Whilst much permanent grassland is extensively managed with low levels of external inputs, permanent grasslands in Europe and New Zealand may receive high inputs and maintain high stocking rates (see Wilkins, 1995). Green (1982) noted that 40% of grassland over 20 years of age in England and Wales had no

Table 1.3. Permanent grassland in different regions, 1994 (from FAO, 1996).

	Permanent grassland		
	10^6 ha	As % agricultural area	As % total land area
Africa	884	84	30
North and Central America	362	57	17
South America	495	82	28
Oceania	429	89	51
Asia*	1036	67	34
Europe*	92	33	16
Russian Federation	87	40	5
World Total	3385	70	26

* Excluding Russian Federation.

major impediments to cultivation. Much of this grassland will, however, have been reseeded at some time, either following cultivation or by oversowing.

Global figures on the area of 'temporary' grassland (i.e. sown grassland in an arable rotation) are not available, but I estimate that such temporary grassland covers 10% of the total area of arable crops – about 146×10^6 ha. Again, there are wide variations between different countries, with Fig. 1.1 showing for a range of European countries that temporary grassland as a percentage of total grassland ranged from 88% in Finland to 3% in Ireland. The high figure in Finland reflects both the small extent of moorland and wetland areas in the country (these are commonly occupied by permanent grassland), widespread production of grass leys in rotation with cereal crops and poor persistence of grassland over winter. Sown or

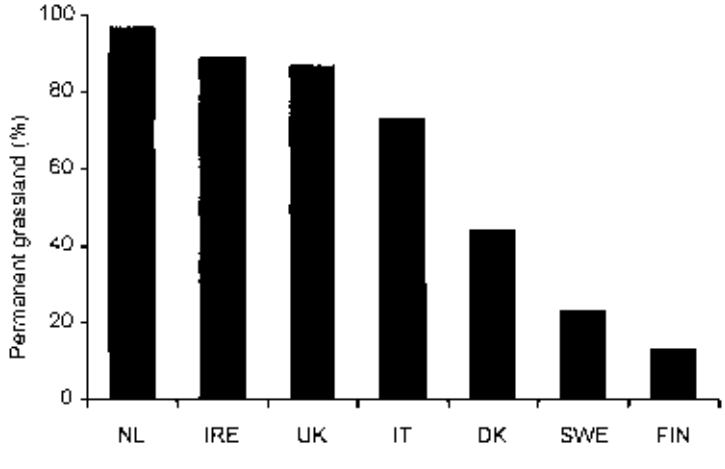

Fig. 1.1. Permanent grassland as a % of total grassland in different European countries (from R.J. Wilkins, unpublished, updated from Lee, 1988). NL, Netherlands; IRE, Ireland; UK, United Kingdom; IT, Italy; DK, Denmark; SWE, Sweden; FIN, Finland.

temporary grassland tends to be managed at a higher level of intensity than permanent grassland, particularly in relation to inputs of fertilizers and other technical chemicals.

The output of dry matter (DM) from sown grassland in Europe is generally in the range of 5–12 t DM ha^{-1} $year^{-1}$, although potential production has been suggested by Leafe (1978) to be some 20 t DM ha^{-1}. Hopkins *et al.* (1990) demonstrated that the production potential of permanent grassland in England and Wales closely approached that of reseeded grassland. Much higher yields may be obtained from tropical grasses, with figures of over 80 t DM ha^{-1} having been recorded in sown swards with adequate water and high fertilizer inputs (Snaydon, 1991). High radiation receipts, linked with the efficient C-4 photosynthetic pathway, contribute to these outputs, which are much higher than those for grain crops, because of continuous crop cover and a high harvest index.

Coupland (1992) reviewed data on the net primary production of above-ground biomass from, mainly, natural grassland ecosystems. The range in values was from 2.4 to 34 t DM ha^{-1}, with the highest values from tropical systems. Applying a figure of 8 t DM ha^{-1} to the world's grassland area of *c.* 3600×10^6 ha gives net primary above-ground production of some $29{,}800 \times 10^6$ t. Coupland (1993), however, estimated that only 7% of this primary production was consumed by domesticated herbivores. Jones *et al.* (1992) drew attention to underestimates in net primary production in much earlier research, because of inadequate allowance for turnover of biomass, and suggested that net primary production may be underestimated by two- to five-fold. Whilst this is highly relevant to arguments about the grassland contribution to carbon sequestration, this extra turnover of biomass would have little effect on quantities harvested by domesticated herbivores. Despite the acknowledged high potential of sown tropical grasses, their impact on world agriculture is relatively small. The resources of water and plant nutrients required to sustain high yields are not readily available in many areas and, particularly in countries with a high human population density, the limited resources of land, water and fertilizers are likely to be directed towards food or cash crops, rather than grassland (and other forage crops) for animal feeds, despite their high production potential.

In addition to producing DM (and energy), permanent and temporary grassland make a major contribution to protein production and supply of mineral elements. Grasses are extremely effective in taking up soil nitrogen and many grasslands include legumes capable of biological nitrogen fixation. Annual yields of crude protein (CP) from grasses and forage legumes are, as for DM, characteristically higher than those for grain crops.

Other forage crops

A wide range of crops in addition to perennial grasses and legumes can be used as forage crops. In temperate conditions, the major forage crops are maize, the leafy brassica crops, such as kale (*Brassica oleracea* var. *acephala*) and rape (*Brassica napus* var. *napus*), and root crops, such as fodder beet (*Beta vulgaris* ssp. *vulgaris*) and swedes (*B. napus* var. *naprobrassica*). Global statistics are not available, but, with the exception of maize, there are few situations in which annual or biennial forage

crops provide the basis for ruminant production systems. When used as main crops, they are not only in competition with cash crops and food for direct human consumption, but also with low-cost production of DM and nutrients from perennial grasses and legumes.

Forage crops must have particular features in order to warrant their inclusion in production systems. These relate generally to yield, seasonality of yield, usability or quality, as discussed by Wheeler (1986). Higher yield potential for maize than grass provides a major motivation for growing maize in many areas of North America and Europe. Likewise, high annual yields contribute to the use of sorghum and small-grain cereals (e.g. wheat, barley and oats) as forages. Mitchell (1960) suggested that in New Zealand the potential production of maize and sorghum was 47 t DM ha^{-1}, compared with 29 t DM ha^{-1} for kale and only 13 t DM ha^{-1} for continuously grazed perennial ryegrass pastures, with differences in potential arising from differences in plant canopy characteristics and rooting systems.

Figure 1.2 shows the differences in daily growth rates between perennial ryegrass and the forage crops kale and fodder radish (*Raphanus sativa*) grown in southern England. Resource capture in early season is highest for perennial ryegrass, in mid-season for kale and in the autumn for fodder radish. Fodder radish is a short-season crop that may be sown after harvest of a grain crop, thus illustrating the ability of some forage crops (catch crops) to utilize land during only part of the year within a rotation, providing a contrast to grass in seasonality of feed supply. Winter forage crops also have a role in many areas, particularly when C-3 winter crops with potential to grow at low temperatures can complement summer production based on C-4 grasses or crops. Wheeler (1986) notes that, in the cool temperate areas of northern New South Wales, native pastures dominated by C-4 grasses have to be complemented by C-3 pastures or oats if sheep breeding through the winter is

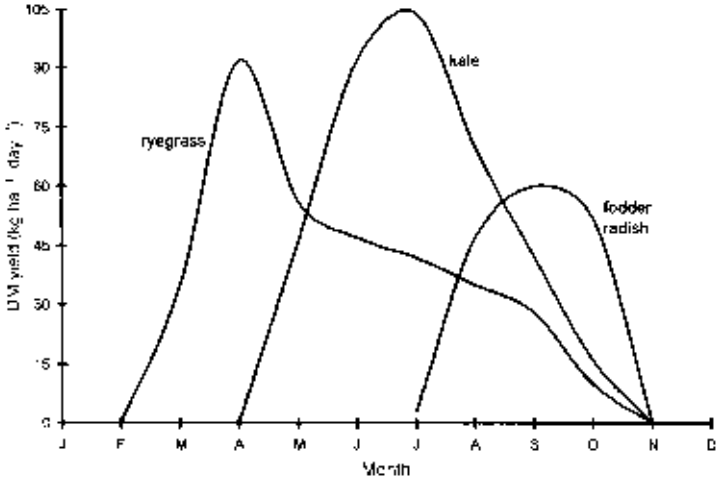

Fig. 1.2. Daily growth rates of contrasting forages grown in southern England (from Wilkins, 1976).

to be successful. Other examples of the impact on system productivity of the inclusion of winter forage crops are given by Dann and Coombe (1987). Oats are commonly used for grazing in the Mediterranean region and in temperate areas of South America and Australia. Berseem (*Trifolium alexandrinum*) is an important winter forage in the Mediterranean basin, the Middle East and India (Knight, 1985; El-Nahrawy *et al.*, 1996; Etman *et al.*, 1998). The high nutritive value of these crops is also relevant, with brassica forages and root crops having organic-matter digestibility up to 90% (Wheeler, 1986). Maraschin and Jacques (1993) discussed the use of winter forage crops in southern Brazil. Natural pastures during the winter period were able to give only 90 kg live-weight gain ha^{-1} $year^{-1}$ from beef cattle. The annual forages oats, ryegrass and rye grown as pure stands increased output to 320–380 kg ha^{-1}, whilst the provision of special-purpose pastures, based on clovers and ryegrass, increased animal production to 600 kg ha^{-1}, associated both with increased stocking rates and improved performance per animal.

When attempts are made to mitigate the effects of seasonality of growth by deferring the grazing of perennial grasses and legumes through to dry or cold periods, there are substantial losses both in quality and in biomass (Wilkins, 1976). These losses may be considerably less with leafy brassica crops and root crops, with their use facilitating grazing over a long season and avoiding the high costs associated with grass conservation as hay or silage. Costs of utilization may also be reduced with maize and other whole-crop cereals grown for silage, because a single cut of high yield may be taken, with lower machinery costs than with perennial grasses and legumes, for which several cuts may be required to achieve the same total yield.

The ability to produce silages with high nutritive value is another reason for the growth of forage crops, particularly maize, which is often of higher digestibility and intake potential than grass silage. High performance of cows fed mixtures of grass silage with maize silage or fodder beet was noted by Phipps *et al.* (1995) and was associated with particularly high levels of intake of the mixed feeds. Winter-grown feeds, such as oats and berseem, are generally of much higher quality than alternatives, such as standing herbage remaining from the previous summer season of growth, as noted by Maraschin and Jacques (1993).

Browse

The consumption of buds, leaves and twigs of woody species, either directly or after harvesting, is an important part of the annual feed supply to ruminants in Mediterranean, tropical and subtropical areas, although good statistics on the magnitude of this feed source are lacking. Browse is characteristically important as a source of feed during dry and winter periods, when either the quantity or quality of available grass is deficient. Dzowela (1993) notes that there are over 200 browse species native to continental Africa which have acceptable nutritional characteristics. Tree leaves may have CP contents up to 250 g kg DM^{-1} and, in addition to their direct contribution to nutrient supply, may increase total DM intake and increase the digestibility of the basal low-CP diet, as discussed by Atta-Krah (1993), with beneficial effects on animal survival and productivity. The yield potential from fodder trees grown alone is high, with Atta-Krah (1993) noting

forage yields of up to 40 t DM ha^{-1} for *Leucaena leucocephala* grown in Nigeria with 12-weekly cutting and 0.5 m spacing. More usually, fodder trees are grown at lower density in grassland. Many fodder trees are legumes, with their associated symbiotic nitrogen fixation contributing to increased soil fertility. The importance and utilization of forage tree legumes in tropical agriculture is reviewed by Gutteridge (1994). The contribution of fodder trees to the total diet may be restricted by high contents of tannins and other antimetabolites.

Straw and crop residues

Estimates of the production of fibrous by-products from cereals and other crops are given for 1981 in Table 1.4. It is probable that, with further increases in global crop production, there has been some increase in by-product availability since that time. Kossila (1984) calculated that the total output of by-products could, in theory, supply 84% of the energy and 74% of the CP required by the world's ruminants. The overall contribution is much less than this, because of losses in harvesting, processing and storage and use as fuel and as a raw material. Much straw is also returned to the field, because of poor nutritive value, absence of livestock or shortage of labour. The substantial availability of crop residues in smallholder systems in the tropics was also highlighted by Smith (1993), with the common crop residues listed in Table 1.5. He noted, though, that only a small fraction of the amount available is used strategically. Cereal stems are normally left on the field for *in situ* grazing, but the residues rapidly deteriorate and a large amount is trampled upon and wasted. Further nutrient imbalances characteristic of such residues are normally not corrected by appropriate supplementation. He stresses the need for appropriate harvesting and storage, treatment to improve digestibility and diet supplementation to improve utilization.

Table 1.4. Quantity of total digestible nutrients (TDN) and crude protein (CP) in by-products produced in different regions (from Kossila, 1984).

	TDN (10^6 t) Fibrous by-products from		CP (10^6 t) Fibrous by-products from		By-product/ livestock unit of 500 kg LW (t DM)
	Cereals	Other crops	Cereals	Other crops	
Africa	124.9	56.3	11.7	9.4	2.1
North and Central America	447.1	182.7	44.2	27.7	5.5
South America	100.8	99.9	10.0	13.3	1.7
Asia	542.0	261.3	53.4	44.7	3.0
Europe	173.4	86.7	16.3	15.8	2.3
Oceania	17.2	8.7	1.5	1.0	1.0
USSR	103.4	53.0	9.3	10.4	2.2
World	1508.8	748.6	146.5	122.3	2.8

Table 1.5. Common crop residues in tropical feeding systems (from Smith, 1993).

Crop	Primary product	Field residue	Primary processing residue
Cereals			
Maize	Grain	Stovers	Cob
Rice	Grain	Stubbles	Straw
Sorghum	Grain	Stovers	–
Wheat	Grain	Straw	–
Grain legumes/oil-seeds			
Groundnut	Oil	Haulms	Husk
Cowpea	Grain	Vines	Husk
Pulses	Beans	Vines	–
Roots/tubers			
Cassava	Tubers	Tops	Peels/rejects
Sweet potato	Tubers	Tops	Peels/rejects
Fruits			
Banana/plantain	Fruit	Tops Pseudostems	Peels/rejects
Coconut	Copra	–	Husk
Cocoa	Seeds	–	Pods
Others			
Sugar cane	Cane	Tops	Bagasse

Clearly, crop residues represent an underutilized feed source, although physical or chemical treatment may be necessary in order for fibrous by-products to make a major contribution to the energy requirements of productive livestock (Owen and Jayasuriya, 1989). Greatest reliance on straw occurs generally in areas where the number of ruminant animals is high in relation to the area of productive grassland and in which there is substantial production of cereals for human food. Normally, varieties of cereal will be selected on the basis of efficiency of grain production, but, in some situations, varieties may be used because of enhanced yield or quality of straw. The grazing of crop stubbles is discussed by Dann and Coombe (1987).

Contribution of Forages to Animal Systems

In view of the broad definition adopted for forages and their characteristic substantial contents of cell-wall components, it is not surprising that most ruminant systems are based on forages. Grasses and other forage crops may provide nutrients at low cost, because of the high yields of DM and energy that can be obtained (associated, as noted earlier, both with high light interception through the year and a high harvest index compared with grain crops) and the possibility for *in situ* utilization by grazing. Likewise, straw and browse may be available at low cost. Straw is a by-product from grain production, whilst supply of feed as browse may be associated

with tree growth for other products. Fitzhugh *et al.* (1978) estimated that forages provide more than 90% of the feed energy consumed by the herbivorous livestock of the world. It is unlikely that this figure has changed radically in the last 20 years. The contribution of grassland feeds in the dairy cow's ration in the Netherlands, however, decreased from 90% in 1950 to 70% in 1970 and 50% in the late 1980s, associated with a favourable price ratio between animal products and concentrates (Van der Meer and Wedin, 1989). These authors, however, note adverse environmental effects from this intensification in dairy production and suggest a need to reverse this trend.

The major feeds not included within the definition of forages are grain and other seed crops and agroindustrial by-products derived from crop harvesting and processing (e.g. extracted oil-seed meals, bran and molasses, vegetable and fruit waste). Data from Fitzhugh *et al.* (1978) stress the limited use of these feed sources, with grain representing only 7% of the feed energy available in developed regions and a negligible proportion in developing regions (Table 1.6). Fitzhugh *et al.* (1978) and Fitzhugh (1998) draw attention to the discrepancy between the quantity of feed available and that calculated as required by ruminant livestock, with less than half of the feed resources apparently being utilized. The figures in Table 1.6 thus probably overestimate the quantity of forages and crop residues that are consumed by livestock.

Why Other Feeds?

Feeds other than forages, and particularly grain crops, are usually used because forages are not capable of sustaining the required levels of animal production, due to limitations in feeding value. They are widely used in situations in which high product prices (e.g. for milk) encourage systems involving high rates of individual animal production. This will be accentuated if climatic factors lead to relatively

Table 1.6. Feed energy resources available and calculated animal requirements for 1970 (Mcal $\times 10^9$) (based on Fitzhugh *et al.,* 1978).

Source of feed energy	Developed regions	Developing regions
Permanent pasture	2.0	2.8
Forage from non-agricultural land	0.3	0.7
Arable land		
Forages	1.7	1.4
Crop residues	1.4	1.6
Grain	0.4	0.0
Agroindustrial by-products	0.0	0.1
Total available	5.8	6.6
Total required	2.3	3.5

high rates of production from grain as opposed to grassland, such as in the Midwest of the USA or where the feeding value of available forages is low. Characteristically, the digestibility and intake levels of tropical and subtropical grasses are lower than those of temperate grasses (Minson, 1990), with the result that, in order to achieve particular levels of animal performance, higher levels of supplementary feeds are required with these grasses. This contributes, for instance, to the substantial development of grain finishing of cattle in parts of northern Australia. Further factors that may increase the use of non-forage feeds are costs of transport and storage. Transport costs are particularly relevant when the location of animal production is remote from that of feed production. Examples are intensive animal production in Japan, largely dependent on feeds imported into the country, and town dairies in India. In such situations, transport costs will be much lower for grains with high bulk density and high nutrient density than for dried forages of low bulk density, such as straw and hay; fresh forage and silages are perishable and not suited to long-distance transport. Grains may also be used as a drought reserve feed for ruminant livestock, because of their high density and stability and, in some circumstances, the existence of grain reserves for market stabilization and for security of human food supplies.

The supply of processed agroindustrial feeds and vegetable wastes will be limited by the output of the main products and, increasingly, by alternative uses for these by-products. Molasses may be used for fermentation and alcohol production, whilst there is increased interest in using vegetable wastes for bioenergy rather than as a feed. Their role as animal feeds will be determined by their energy and protein value, the location of production in relation to that of the ruminant populations, and transport and storage characteristics.

Reliance on Forages

Some of the factors determining the extent of reliance on different classes of forages have already been mentioned. Table 1.7 illustrates the extent to which grassland and other feeds contribute to energy supply to ruminants in a number of European countries. The contribution from grassland ranges from 97% for Ireland down to 34% for Bulgaria. Countries with extremely high grassland usage are broadly those with high rainfall, giving high grassland yield potential (see Lee, 1988) and often difficult conditions for arable crop production. In contrast, countries with low grassland usage have a combination of low grassland yields and large areas of cereal grains and other arable crops producing an abundant supply of straw and other by-products. In countries with intermediate grassland usage (e.g. France), there is often substantial supply of forage from maize. The rapid increase in milk yields and genetic potential of dairy cows in Western Europe has resulted in a need for increased nutrient density in the complete diet, with increases in the required feeding value from forages and increases in the use of grain and concentrate feeds (Van der Meer and Wedin, 1989).

Animal production in extensive rangeland conditions in America, Africa, Australia and northern Asia is almost entirely dependent on the native grassland vegetation, with animals often being exported to other agroclimatic zones for fin-

Table 1.7. Estimated contribution of grassland, other forages and feeds to ruminant feed composition in selected European countries (%, on dry basis) (from Lee, 1988).

	Grassland	Root crops	Maize silage*	Other annual forages	Straw	Sugar-beet leaves and tops	Other feeds
Ireland	97	1	–	–	1	–	1
UK	83	2	1	–	1	–	13
France	71	3	9	2	–	–	15
Netherlands	54	–	4	–	–	–	42
Denmark	47	8	–	–	4	2	39
Hungary	45	2	4	–	5	2	42
Bulgaria	34	1	6	–	4	2	53

* The use of maize silage will have increased in most European countries over the last decade.

ishing. Low effective rainfall and non-availability of water for irrigation are the major factors that restrict more intensive grassland production or arable cropping. In some areas, however, particularly in South America, low human population pressure and market opportunities for ruminant products have led to extensive production in areas of reasonable climatic potential.

Straw is a particularly important feed resource in areas of Asia and Africa with high human population pressure, as noted earlier. Little cultivable land is available for producing specialist forage crops, so that ruminant animals, often required for draught, are dependent for feed supply on forage from uncultivated land, straw and other crop by-products.

Future Prospects

Forages will provide the basis for most ruminant production systems for the foreseeable future. Extensive production from grassland will continue to be a major form of land use, although management strategies, particularly in North America and Europe, will have to increasingly consider management not only in the context of animal production, but also in relation to effects on landscape and resource sustainability.

There is a major challenge for intensive grassland to maintain its place in production systems, in view of the progress that is being made in improving yields and quality from other forages, such as maize, the high quality required in the diet of high-production animals and the need in some areas to adapt methods of production to restrict environmental pollution, as discussed by Van der Meer and Van der Putten (1995) and Wilkins (1996). However, considerable progress in improving yield potential and nutritive value is now being made by grass breeding (Camlin, 1997) and approaches to restrict pollution risk are being developed (Wilkins, 1996). In some areas, particularly Europe, there will be pressures to reduce intensity of production in order to deliver increased landscape and biodiversity benefits from grassland at the expense of herbage output and quality. The production in both

temperate and tropical conditions of improved forage legumes (Cameron *et al.*, 1993; Rhodes and Ortega, 1997) will be of particular importance in relation both to sustaining plant production with low levels of external inputs and to improving feeding value. In order to constrain costs of animal production, attention should continue to be focused on actions to increase the reliance on grazed rather than conserved forage, as discussed by Wilkins (1995). There are undoubtedly situations, though, in some tropical and subtropical countries, in which some increase in use of conserved forages would improve overall production efficiency.

The contribution of specialist forage crops, particularly maize and sorghum, is likely to increase. The large research effort with maize is likely to lead to further improvement in yield potential and stress tolerance with this species. There are opportunities for breeding to enhance the quality of the whole crop or of the straw, but it is disappointing that varieties with enhanced digestibility, associated with the brown-midrib gene, have not yet met with wide commercial success.

The major challenge with straw and many other by-products is to develop ways for increasing feeding value, particularly digestibility and intake potential, in order to increase animal production in straw-based feeding systems and to increase the proportion of straw in the diet. The possibilities for improvement by plant breeding and physical, chemical and microbial processing have been extensively reviewed (e.g. Sundstøl, 1988; Chesson *et al.*, 1995), but, as yet, the global impact has been small. I consider that the incorporation within breeding programmes of criteria in relation to straw quality has high potential for success and should be vigorously pursued. Variation in relevant nutritive characteristics exists and appropriate techniques are available for use in breeding and evaluation.

The increased use of trees and shrubs for forage as browse has considerable potential, particularly in tropical and subtropical regions, as shown by the substantial impact of *L. leucocephala* in northern Australia (Shelton *et al.*, 1991). There is a need for a more concentrated world effort on trees for forage, including both nutritive and agronomic factors.

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2 Forage Evaluation for Efficient Ruminant Livestock Production

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Background

Forages represent a diverse range of feedstuffs that make a significant contribution to the overall nutritional economy of meat-, wool- and milk-producing ruminants. However, it is this diversity which presents both opportunities and challenges when attempting to exploit these feeds in ruminant diets. Within the UK, forages are derived from a number of distinct sources. Undoubtedly, the principal forage is grass, which may be consumed *in situ* (i.e. grazing) or after conservation as silage or field-cured hay. The area of forage maize grown in the UK has increased considerably over recent years and, depending upon local climatic conditions, will be harvested and ensiled at dry matter (DM) contents between 250 and 375 g kg^{-1} fresh weight. Forage maize is grown principally for its high energy content and high intake characteristics. In addition, depending upon level of cob formation, starch content in more mature crops can reach 350 g kg^{-1} DM. Such is the popularity of maize that serious consideration is being given to the production of maize grain in more favourable growing areas. However, maize cannot be grown successfully in all areas of the UK, and whole-crop wheat silage may be considered as an alternative. According to preference, the wheat crop can be harvested and ensiled at an immature stage of growth (350 g DM kg^{-1} fresh weight) and offered as a fermented feed. Alternatively, it can be harvested later (550 g DM kg^{-1} fresh weight), when starch levels are higher, but, as such crops are unlikely to undergo fermentation after ensiling, urea is added at harvesting to ensure stability during the storage period (Sutton, 1997).

Cereal straws, principally barley and wheat, are also a source of forage for ruminants, and can form a significant part of the diet for those animals with more modest performance targets (e.g. suckler cows, beef stores). Treatments with either sodium hydroxide or gaseous ammonia are used to upgrade straw quality, although such practices are increasingly questioned on economic grounds. Finally, consideration should be given to the role of legumes. The lucerne area in the UK remains

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low, for, whilst many farmers appreciate its value, difficulties associated with crop establishment and management, as well as harvesting and storage of the crop, all of which influence both forage yield and quality, remain major limitations to its widespread use. However, an increasing number of farmers are considering the possibility of growing lucerne, especially for inclusion in dairy-cow diets. Again, although the high nutritional value of white clover, especially in grass swards, is recognized, its poor spring growth and lack of persistency under frequent-cutting regimes tend to restrict its use to low-output per hectare and alternative farming systems. Red clover is of relatively minor importance.

From this brief outline of the types of forage available, it follows that the nutritional value of such feeds will vary in terms of their ability to support the maintenance and production requirements of ruminant livestock. This contrasts with many other primary feed sources, including cereal grains and pulses, where variation in nutritional value is relatively small. Principal factors affecting forage nutritive value include forage species, as indicated above, as well as prevailing climatic and management conditions. These include soil type, seasonal variations in the incidence of sunshine and rainfall, fertilizer treatment and stage of growth at harvesting (by animal or machine). For ensiled forages, postharvesting procedures with respect to field wilting, additive application and ensiling conditions are important. Equally, for hays, weather condition, length of the curing period and degree of mechanical handling have a marked influence on the quality of the end material, while drying temperature is important for artificially dried forages. The intention of this chapter is to examine the importance of determining the nutritional value of forages destined for feeding to ruminants, with a comprehensive review of how forage nutritive value is currently assessed. Finally, the chapter will provide some direction as to the way in which forage evaluation should develop, suggesting the potential contribution of newer technologies whilst drawing attention to possible pitfalls. To achieve this overall objective, an initial review of the processes of forage utilization by ruminants will be presented as background to the evaluation of forages as feeds for ruminants, as well as establishing a basis on which current techniques have been developed and future ones may be considered.

Nutrient Digestion and Utilization in Ruminants

Nutrient degradation

The principal reason why non-ruminants and preruminants are unable to utilize significant quantities of forages is that, like other mammals, they do not possess the enzymes capable of breaking down the complex β-linked polymers that form plant cell walls. In ruminants, however, the principal site of digestion in regard to forages is the rumen, where the feed is retained for substantial periods of time and subjected to extensive microbial fermentation under anaerobic conditions (Beever, 1993). Following hydration of the ingested feed, the microbial enzymes break down the complex dietary polysaccharides, namely, cellulose and hemicellulose, which are the principal components of the cell walls. The major end-products are the constitutive sugars, principally glucose derived from cellulose, but both hexoses and

pentoses, with variable amounts of uronic acids from hemicellulose, reflecting its more heterogeneous nature. The diet will usually contain other carbohydrates, including water-soluble carbohydrates, such as fructosans, which are found extensively in grasses, and starch, which occurs principally in cereal grains. Whilst these fractions could be potentially digested in the small intestine by enzymes of host-animal origin (Armstrong and Beever, 1969), they too will be subjected to the action of microbial enzymes whilst resident in the rumen.

However, not all ingested carbohydrate is degraded in the rumen, as both the rate and extent of polysaccharide breakdown are influenced by composition of the feed, as well as the nature of the microbial enzymes and accessibility of the dietary polysaccharides to these enzymes. This can be illustrated with feeds containing significant amounts of lignin, indicative of advancing plant maturity. Such forages have impaired rates of cellulose and hemicellulose degradation, for, whilst composition of these fractions will be similar to that in less mature forages of the same species, it is the ingress of lignin into these structures as the plant matures that acts as a major obstacle to their digestion by microbial enzymes (Chesson, 1988). Equally, the nature of the starch in the diet, particularly the relative proportions of amylose and amylopectin, will influence the extent of starch digestion in the rumen. This is best illustrated by the starches in maize grain (and maize silage), which are less rapidly degraded than those in other cereals, including wheat and barley, due to an increased proportion of amylopectin, as well as a more complex protein : lipid matrix found in the endosperm of maize grains. In contrast, there is little evidence that the rate of degradation of water-soluble carbohydrate (WSC), which is primarily associated with cell contents, is influenced by either forage species or stage of maturity, although levels of WSC in the plant decline as forages mature (Beever *et al.*, 1972).

Hexose disposal

Following the initial phase of carbohydrate breakdown, the released constitutive sugars are subjected to one of two metabolic processes, both occurring in the rumen. It is generally assumed that all released sugars undergo microbial fermentation, and, indeed, this is an important route of hexose and pentose utilization. The primary aim of this fermentative pathway is the provision of adenosine triphosphate (ATP), essential for the energy demands of the microbes for maintenance and growth (Issacson *et al.*, 1975). However, the rumen is largely anaerobic and consequently ATP yield per mole of carbohydrate fermented approximates to 10–12% of that achieved under aerobic conditions. Thus, to meet microbial ATP demands, a significant proportion of the available hexose and pentose will be fermented. As such, carbohydrate fermentation comprises a significant component of rumen metabolism (Beever, 1993). It is during this process that significant amounts of carbon dioxide are released, with part used in the removal of ruminally produced hydrogen through the synthesis of methane. This process involves methanogenic bacteria, which proliferate in those environments where excess hydrogen is produced. However, most microbes have some requirement for preformed hexose to support the synthesis of microbial biomass, in particular the synthesis of microbial

protein, but also the synthesis of microbial nucleic acids, lipids and polysaccharides (Black *et al.*, 1980/81). Whilst part of the microbial protein component is derived from preformed amino acids, *de novo* synthesis from ammonia and hexose is often the preferred route in overall microbial metabolism (Nolan, 1975) and has been shown to be preferred by amylolytic compared with cellulolytic bacteria. When carbohydrate availability is high (e.g. increased starch feeding), microbial polysaccharide synthesis, as an alternative route for hexose disposal, can be quite significant. There have been no direct attempts to establish the partition of hexose disposal between fermentation and direct incorporation. However, as illustrated by Beever (1993), this can have a significant impact on the net production of fermentation end-products, including the quantity and composition of ruminally derived volatile fatty acids (VFA).

VFA production and utilization

As indicated, the major event associated with the ruminal fermentation of dietary derived monosaccharides is the production of VFA, principally acetate, propionate and butyrate. In most dietary situations, acetic acid is predominant and, with butyric acid, reflects forage-rich diets, in which cellulolytic bacteria proliferate. In contrast, amylolytic bacteria dominate on starch-enriched diets, where increased propionic acid levels are normally observed. This distinction between acetate and butyrate, on one hand, and propionate, on the other, is important with respect to the glucose economy of the animal (MacRae and Lobley, 1982). The overall stoichiometry of carbohydrate utilization by fermentation in the rumen has been established (Baldwin *et al.*, 1977; Murphy *et al.*, 1982) and was recently summarized by Beever (1993), for three contrasting diet types based on high amounts of forage, cereal or molasses. These are presented in Table 2.1, where a number of interesting comparisons are apparent. The most obvious differences relate to the

Table 2.1. The estimated stoichiometric yield of VFA, ATP, carbon dioxide (CO_2) and hydrogen (H_2) (mol mol^{-1} hexose) with the resultant levels of methane (CH_4) production, from the ruminal fermentation of three diets based on different carbohydrate types. Values in parentheses refer to net productions after conversion of available hydrogen to methane.

	Diet types		
Products	High-fibre	High-cereal	Molasses-enriched
Acetate	1.34	0.90	0.94
Propionate	0.45	0.70	0.40
Butyrate	0.11	0.20	0.33
CO_2	1.53 (0.92)	1.30 (0.92)	1.60 (1.06)
H_2	2.44 (0.0)	1.50 (0.0)	2.14 (0.0)
CH_4	(0.61)	(0.38)	(0.54)
ATP	(4.62)	(4.38)	(4.54)

amounts of individual VFA produced, with high acetate levels on the high-fibre diet and relatively high levels of propionate on the high-cereal diet, although acetate was the predominant acid for all diet types. As a consequence of these changes, carbon dioxide yield was reduced on the high-cereal diet, with a net transfer of carbohydrate carbon to VFA carbon of over 78%. Corresponding values for the other two diets were lower (average 74%), representing an increased loss of carbon. The importance of these differences is appreciated when it is recognized that, for average-yielding dairy cows, the amount of carbohydrate fermented in the rumen may approach 50 mol day^{-1}. Associated with these changes were lower yields of hydrogen on the high cereal diet, the difference being most pronounced in comparison with the high-forage diet. The reason for this reduction is related to the utilization of hydrogen in the production of propionate from pyruvate (the initial three-carbon molecule derived from glycolysis). Propionate production can therefore be considered an important sink for the disposal of ruminally derived hydrogen. As a consequence, methane production, which is largely in stoichiometric balance with net hydrogen production, was highest on the fibre-rich diet, with reductions of between 12 and 38% on the molasses-enriched and high-cereal diets, respectively. However, despite such changes, ATP yield mol^{-1} carbohydrate fermented was not significantly affected by diet type, although the highest values were associated with acetate-dominant fermentations.

Following absorption, propionate is utilized extensively by hepatic tissues to support glucose synthesis (Reynolds *et al.*, 1998) and, in most situations, constitutes the primary source of glucose, given that net glucose uptake from the ruminant alimentary tract is generally minimal (Armstrong and Beever, 1969). Indeed, the gut utilizes significant amounts of arterial glucose and, apart from those few instances where small-intestinal starch digestion may be quantitatively important, the gut as a whole is a net utilizer, rather than a net provider, of glucose. In contrast, ruminally derived acetate is largely unchanged by hepatic metabolism and may be augmented by endogenous acetate production in the liver. The posthepatic supply of acetate to peripheral tissues constitutes a major part of the total energy available to the animal and may be either oxidized to produce ATP or used as a substrate in the production of long-chain fatty acids (see following section). While ruminally derived butyrate is quantitatively metabolized to β-OH-butyrate during absorption through the rumen epithelium, in posthepatic tissues it has a similar metabolic fate to that of acetate.

Fatty acid metabolism

Collectively, acetate and β-OH-butyrate make a significant contribution to the synthesis of fatty acids for deposition as triglycerides in adipose tissue or secretion in milk. This comprises chain elongation with two-carbon units up to a maximum of a 16-carbon unit and in part contributes to the preponderance of short- to medium-chain saturated fatty acids in ruminant products. At the same time, preformed fatty acids, either of direct dietary origin or from mobilized body fat (especially in the case of dairy cows), can be used for the provision of ATP by oxidative metabolism or the synthesis of triglycerides. With respect to dietary fatty acids,

however, one important distinction between ruminants and non-ruminants is the ruminal hydrogenation of dietary fatty acids. Ingested fats are relatively inert with respect to rumen metabolism, but, after ruminal hydrolysis, a significant proportion of the released fatty acids will be hydrogenated, due to the prevailing hydrogen-rich (i.e. reduced) environment. This constitutes a further route by which a significant amount of ruminally derived hydrogen may be disposed of, a feature not taken into account in the estimates of methane production presented in Table 2.1. Furthermore, as the degree of saturation of fatty acids entering the small intestine of ruminants usually exceeds that of ingested fatty acids (Outen *et al.*, 1975), the degree of saturation of the fatty acids found in ruminant products is generally greater than that found in non-ruminant products. Equally, fatty acids originating from the diet or from mobilized tissue usually comprise longer carbon-chain lengths compared with those synthesized *de novo* from acetate or β-OH-butyrate, with most, although not all, being accounted for as C-18 isomers.

Protein metabolism

One other major metabolic activity in the rumen relates to the utilization of dietary protein to support the synthesis of microbial protein. This has been the subject of considerable research effort, where a series of well-defined studies, conducted principally with sheep, established the quantitative contribution of microbial and undegraded dietary protein to small-intestinal protein supply (Smith and McAllan, 1970; Beever *et al.*, 1974). Following ingestion, dietary protein is subjected to degradation by microbial proteases, the principle end-products being amino acids, together with ammonia, which arises from deamination of the resulting amino acids or the catabolism of non-protein nitrogen (e.g. urea) derived from dietary or endogenous (i.e. saliva) sources. The extent to which dietary protein is degraded in the rumen has been the subject of much debate and, whilst reliable measurements of this process still need to be established, it is accepted that degradation rate is a function of protein structure. With fresh forages, ribulose-1,5-biphosphate carboxylase (Rubisco or fraction 1 protein) is the principal protein and several studies have shown that this protein is highly susceptible to the action of microbial proteases (Mangan, 1982), resulting in a relatively short half-life for the protein and rapid accumulation of ammonia in rumen fluid. Furthermore, at high levels of feed intake, outflow rate from the rumen increases, resulting in an increased proportion of dietary protein surviving intact to the intestines, despite having the potential to be digested in the rumen.

Microbial protein synthesis is dependent upon the availability of a continuous supply of precursors, principally ammonia and amino acids, but the process is energy-consuming and a supply of ATP, provided principally from the fermentation of dietary carbohydrates, is essential. This led to the concept of synchrony of nutrient release in order to ensure maximum rumen microbial protein synthetic rates, as well as optimal utilization of degraded nutrients. However, whilst the theory is well founded, definitive experimentation in support of the hypothesis is still required (Sinclair *et al.*, 1993; Witt *et al.*, 1997). Ultimately, microbial biomass passes to the small intestines, either attached to undegraded feed particles or free-floating within

the fluid phase of the digesta. During transit through the small intestine, host enzymes promote significant digestion of the proteins to peptides and amino acids. In turn, these will be absorbed across the intestinal wall, the ultimate contribution directly from peptides being considered to be minimal. As with glucose, gut tissue utilizes significant amounts of individual amino acids, probably from arterial supply, and thus net portal appearance of amino acids is unlikely to equate with small-intestinal loss of amino acids (MacRae and Beever, 1997; Wray-Cahen *et al.*, 1997).

Prediction of Animal Performance

Against this background of nutrient digestion and assimilation, in which dietary nutrients can be substantially modified, due to events principally within the rumen, prediction of animal performance remains relatively complex. Indeed, this task has become increasingly difficult, as the demands placed upon systems have become more prescriptive. When the importance of forage evaluation was first recognized at the turn of the century, the issues were large and apparent. It was relatively easy to establish the superior nutritive value of, for example, grass hays compared with cereal straws and, in the well-quoted studies of Armsby, Kellner and others, establishment of the nutritional advantages of barley grain compared with a range of different forages was quite straightforward. This situation prevailed until the middle of this century, when some of the more subtle differences between forages were appreciated. This led to considerable research effort focusing on three issues: namely, those influencing forage intake, digestibility and utilization. In this respect, a substantial research effort was directed towards consideration of forage digestibility. The resultant data were used effectively in the estimation of animal performance, through prediction of forage intake and the efficiency with which digested nutrients were utilized for maintenance and production. However, it became apparent that measurements of feed or forage digestibility were not sufficient to enable prediction of animal performance with an acceptable degree of precision. This led to in-depth examination of the processes of digestion, with specific focus on events occurring within the rumen. The outcome of this research has been highly rewarding in relation to protein metabolism, where it is now possible to provide more balanced diets to meet the 'protein' requirements of both rumen microbes and the animal. However, there has been no parallel comprehensive evaluation of the dietary carbohydrate fraction, although, with most diets, this will contribute to over 60% of total absorbed nutrients. Equally, only limited attention has been given to an evaluation of dietary lipids, even though these may constitute between 10 and 15% of the total absorbed energy on diets designed for high-producing animals. In partial mitigation, the Cornell net carbohydrate and protein system (CNCPS), as proposed by Sniffen *et al.* (1992), offered some improvement with respect to an appreciation of the importance of difference carbohydrate sources. However, the focus provided by the CNCPS is largely related to consideration of the digestive aspects of different carbohydrate and protein sources and how these influence overall supply of metabolizable energy and protein to the animal. To date, no feed evaluation systems that describe the individual end-products of digestion, other than total amino acids, as included in the plethora of metabolizable protein

systems have been developed. This is despite it being well established that the composition of the non-protein part of the total absorbed-energy fraction can have a significant effect on overall efficiency of nutrient utilization by the animal and, in particular, product composition. Such models have only been proposed for research purposes (Baldwin *et al.*, 1977, 1987a; Black *et al.*, 1980/81; France *et al.*, 1982), and, to date, none have been modified for on-farm use.

Whilst progress with respect to protein evaluation, as encompassed in the UK metabolizable protein (MP) scheme and other similar systems, has been considerable, with respect to the prediction of energy utilization, many of the systems proposed over 20 years ago are still in use. The UK metabolizable energy (ME) system, as first outlined by Blaxter (1962), has been extremely valuable in terms of prediction of gross animal events, and the energy unit of this system (MJ ME kg^{-1} diet DM) has been widely adopted within the livestock industry. Many of these data were derived from energy calorimetry studies involving sheep or, in limited instances, beef or dairy cattle, with most of the latter data being derived from US or Dutch studies. Rationalization of such data led to a system of energy evaluation which, at the time of its development, could be supported on conceptual grounds, with many of the proposed relationships being defendable on the basis of best statistical fit of the data. However, acceptance of linearity of response for all functions of energy utilization, at both above- and below-maintenance levels of feeding, has been increasingly questioned as difficult to justify on biological grounds. Equally, some of the distinctions drawn in relation to the efficiency of utilization of ME for different forages, appear to have been based rather more on data availability than on biological grounds, the differences between long and ground and pelleted forages and between spring and autumn forage being cases in point.

At the same time, similar systems were proposed by other countries in Europe, Australia and the USA, often with net energy as the preferred energy unit. However, as systems of animal production become more sophisticated, with greater emphasis on end-product quality to meet the demands of the consumer, such systems have been shown to be lacking in several respects, and it is likely that these will need to be replaced. Indeed, there is already debate as to how such systems should be refined, given the aim of improving the predictability of animal response whilst avoiding unnecessary complication. However, there is no consensus on how alternative systems of feed evaluation should be constructed and, given that most national funding agencies seem to be no longer interested, all future initiatives are likely to come from those actively working in the field. One obvious area of improvement would be systems that predict animal response to deliberate perturbations in nutrient input. Such an approach would be of critical importance in attempting to optimize product output per unit of feed input. Development of this approach would, of necessity, lead to the abandonment of models of feed evaluation based on estimation of the nutrient requirements of the animal and subsequent formulation of a diet to meet such needs. However, in this scenario, the required inputs would be largely unchanged from those currently in use, as it would be the model, rather than the inputs, that would be most affected. Such moves towards the development of nutrient response models are highly supportable, but there is a growing opinion that suitable progress towards reliable prediction of animal performance will only be achieved through recognition of the importance

of individual nutrients. Undoubtedly, ME has been a useful nutritional component, but lack of any qualitative description of ME is now a significant limitation. The absence of such information could allow the less informed nutritionist to conclude that wheat and wheat straw would provide similar levels of production (both amount and type) when supplied in equal amounts of ME to the animal. This issue was discussed in a recent Technical Committee on Responses to Nutrients (TCORN) publication (AFRC, 1998), when a number of options to provide a staged development of alternative models were advanced.

Consequently, all aspects of feed evaluation designed to predict animal performance are at an interesting stage of development. On the one hand, there is sufficient information to indicate that current methodologies are inadequate and refinements are required. On the other, however, uncertainty exists with respect to the degree to which systems of feedstuff evaluation will need to change. This ranges from those who advocate minor revisions to existing frameworks to others calling for more radical initiatives, to include recognition of the importance of individual nutrients, as occurs in most non-ruminant feeding systems.

Forage Evaluation: Current Practice

All systems of feedstuff evaluation aim to provide information regarding the capacity of individual feeds to meet the nutritional demands of the animal and, as such, represent some degree of compromise with reality. Undoubtedly, the most precise way to establish the nutritional value of any feedstuff would be to feed it to appropriate animal classes and to observe the level of animal production achieved, but such an approach is neither practical nor justifiable on cost grounds. However, in any system of feed evaluation, it is important to recognize that the ultimate arbitrator of nutritional value will always be the animal, and consideration of this should, at all times, outweigh expediency of laboratory operation in the refinement of current methodologies.

Digestibility and dietary carbohydrates

An excellent example of a systematic approach to the establishment of a feed evaluation system was the development of the first routine *in vitro* system by Tilley and Terry (1963). Recognizing the importance of diet digestibility as an index of nutritional value, these workers established a comprehensive *in vivo* database of forage digestibilities, from which suitable predictive procedures could be developed. To achieve this, a programme of work was undertaken to provide a wide range of forages (both grasses and legumes), which were systematically fed to mature sheep at maintenance levels of feeding to obtain measurements of whole-tract digestibility. Subsequently, these feeds were subjected to an *in vitro* technique, comprised of two stages to simulate ruminal and postruminal digestion, and a series of equations to predict whole-tract digestibility for different diets were proposed. Subsequently, workers in other countries extended this approach to develop suitable prediction equations for tropical forages, and collectively this work formed the basis on which

many feedstuffs were evaluated for feeding to ruminant livestock, in both temperate and tropical climates.

Establishment of the technique led to the development of the concept of forage D, or DOMD, value, defined as the content of digestible organic matter in forage DM, and studies by Givens *et al.* (1989, 1990, 1992) and Moss and Givens (1990) led to a series of equations to predict DOMD (%) as indicated below:

Fresh grass	DOMD = 12.0 + 0.857 IVD
High-temperature-dried grass	DOMD = 27.3 + 0.998 IVD
Grass hays	DOMD = 17.2 + 0.710 IVD
Grass silage	DOMD = 10.0 + 0.870 IVD

where IVD (*in vitro* digestibility) is the DOMD estimate obtained by the method of Tilley and Terry (1963).

Estimates of D value have also been widely used in systems of grassland management to optimize the use of grazed grass or the stage of harvesting of grass for ensiling. This concept was also adopted by plant breeders as a screening procedure to examine the nutritional value of new forage cultivars. Equally, DOMD values derived from IVD estimates have been used to predict forage ME contents. A value of 0.15 has been adopted to convert DOMD to ME (i.e. ME (MJ kg^{-1} DM) = 0.15 DOMD%) for fresh and dried grass and hay, and a higher coefficient of 0.16 for high-protein legumes and fermented feeds (see Beever *et al.*, 1999), whilst estimates of forage IVD have also been used to predict forage intake – yet another example of the utility of this index of nutritive value.

With the passage of time, possible alternatives to the *in vitro* system as proposed by Tilley and Terry (1963) have been suggested. As the Tilley and Terry procedure may contain pepsin-insoluble material and microbial residues, Goering and Van Soest (1970) introduced a final washing procedure, using a neutral detergent solution, and termed their end-point estimate the predicted true digestibility. In another development, Jones and Hayward (1975) replaced rumen fluid as the source of microbial enzymes for the ruminal phase of digestion with commercial enzymes. This approach was to be commended, as it offered the possibility of reducing methodological variation attributable to the source of rumen liquor, permitting improved standardization of the method, whilst eliminating the need for surgically modified animals as rumen-fluid donors. However, there are a number of drawbacks to this technique. First, there were doubts as to whether the use of only two enzymes with specific activities was sufficient to provide an accurate measure of digestion, in comparison with the highly complex array of enzymes present in the rumen ecosystem. Secondly, there was a lack of standardization between different batches of commercial enzymes, and consequently the technique has had limited usage. Another modification has been the use of fresh faeces as an alternative microbial inoculum to rumen liquor (El Shaer *et al.*, 1987). Evaluation established that this approach could be used to determine forage digestibility. However, while feedstuffs were ranked in a similar order, as the extent of digestion was generally less than that observed with rumen liquor, alternative calibration formulae were required. It is likely that long-term application of this method will be limited to laboratories where fresh rumen liquor cannot be obtained, a position that prevails in many developing countries.

With the recognition that forage fibre composition was a major determinant of forage quality, with the potential to predict intake and nutritive value, attention turned to developing laboratory methods for forage fibre analysis. Until the mid-1960s, fibre content of feeds, including forages, had been determined largely according to the Weende system of feedstuff evaluation, but there was increasing concern as to the value of crude fibre levels determined by this methodology. By sequential use of neutral- and acid-detergent solutions, the method of Van Soest and Wine (1967) provided, for the first time, a relatively inexpensive and routine method for determining different fibre fractions, namely neutral-detergent fibre (NDF), comprising cellulose, hemicellulose and lignin, and acid-detergent fibre (ADF), comprising cellulose and lignin. A further step involving acetyl bromide was developed to determine lignin content in the ADF fraction. This latter detail has not been widely adopted for routine feedstuff analysis, as it is recognized, on the basis of its relative complexity, that there are no ideal methods for routine analysis of lignin. However, other fibre fractions, especially ADF or modified ADF (MADF), have been used extensively in equations designed to predict forage digestibility, as proposed by Givens *et al.* (1989, 1990, 1992) and Moss and Givens (1990), as illustrated below (as g kg^{-1} DM):

Fresh grass	DOMD = 967 – 0.950 MADF
High-temperature-dried grass	DOMD = 1012 – 1.243 MADF
Grass hays	DOMD = 1023 – 1.222 MADF
Grass silage	DOMD = 996 – 1.04 MADF

The detergent fibre system does, however, have some limitations. Van Soest and Wine (1967) recognized that feeds which have been subjected to heat treatment may contain significant amounts of heat-damaged protein, which, based on its low solubility in detergent solutions, could be a significant contaminant in the isolated ADF fraction. This artefact was particularly evident with distillery by-products and, under such circumstances, it is important to take account of this if realistic estimates of ADF content are to be achieved. However, this potential problem has been turned to advantage, whereby the detergent method has been used to determine acid-detergent-insoluble nitrogen contents (ADIN) of feeds. This provides an estimate of the small-intestinal availability of ruminally undegraded dietary protein, an important component in several of the metabolizable protein systems. A further limitation is the estimation of NDF content in starch-rich feeds. During extension of the methodology from forages to use with other feeds, it was apparent that, under some circumstances, estimated NDF contents were too high and this raised the possibility of starch contamination. The extent of this will depend upon the amount and type of starch present in the original feedstuff, as those starches relatively resistant to chemical or microbial digestion will be more resistant to extraction by detergent solution. Consequently, a pretreatment with commercial amylases to digest the starch was developed, thus facilitating its subsequent removal by the detergent solution. In most forages, such procedures are not required, but, with maize and whole-crop silages, which contain significant amounts of starch, pretreatment of the sample is recommended if reliable estimates of NDF content are to be obtained.

In relation to a more specific evaluation of dietary carbohydrate fractions, one

area where uncertainty still remains is the estimation of starch content. With maize or whole-crop wheat silages, the determination of total starch content is essential if appropriate supplements that optimize total starch availability to the animal are to be developed. In the late 1960s, there was considerable effort towards the development of improved methods for determination of total starch content in different feeds. Based on a critical examination of available methods, MacRae and Armstrong (1968) concluded that many were not sufficiently specific and were thus subject to interference from moieties of non-starch origin. This resulted in an enzymatic method being developed by MacRae and Armstrong (1968), using a commercial amyloglucosidase to release the glucose units of starch, followed by determination of the released glucose, using a glucose-specific methodology (glucose oxidase). This methodology became the basis on which several laboratories conducted research to determine the digestion characteristics of different starch-containing feeds in ruminants (MacRae and Armstrong, 1969; Beever *et al.*, 1970), the only potential problem appearing to be some inconsistency in the commercially available amyloglucosidases. It is thus surprising that, over 25 years later, no standard methodology for starch analysis is available in the UK, and the position is remarkably similar in many other countries. Indeed, some laboratories appear to have reverted to techniques that were identified by MacRae and Armstrong as being both unsuitable and unreliable, whilst others have decided not to offer starch determination as a routine analysis. In a recent study, reported by Beever *et al.* (1996), nine laboratories in Europe (including the UK) and the USA were requested to determine the nutrient composition of two contrasting maize silages, including total starch content. Analysis of the resultant data indicated considerable variability, as illustrated in Table 2.2. Further investigation revealed the use of several different techniques, some relying on near-infrared reflectance spectroscopy

Table 2.2. Chemical composition of two maize silages harvested at different stages of maturity and analysed by up to nine different laboratories (units, g kg^{-1} oven-dry matter unless stated; means ± SEM; superscript denotes number of laboratories reporting).

		Low DM	High DM
Dry matter (g kg^{-1} fresh wt)[9]	Mean	276 ± 8.6	335 ± 9.3
	Range	264–290	323–348
Starch [8]	Mean	228 ± 35.6	261 ± 42.4
	Range	165–272	194–311
Crude protein [9]	Mean	101 ± 18.3	101 ± 7.5
	Range	57.0–119	84.0–108
ERDP [3]	Mean	61.3 ± 18.02	68.0 ± 4.16
	Range	27.0–88.0	60.0–74.0
DUP [2]	Mean	23.0 ± 2.00	21.0 ± 4.00
	Range	21.0–25.0	17.0–25.0
ME (MJ kg^{-1} DM) [6]	Mean	11.2 ± 0.55	11.5 ± 0.60
	Range	10.5–12.1	10.7–12.3

SEM, standard error of the mean.

(NIRS) rather than the more conventional chemical/enzymatic approach involving starch extraction and estimation. One US laboratory did not offer starch analysis, on the basis that they were dissatisfied with all of the available methods. Further, in relation to the evaluation of the carbohydrate fraction of forages, it is recognized that some forages may contain appreciable levels of WSC. Under most circumstances, this fraction is extensively and rapidly digested in the rumen, and consequently most analyses are restricted to determination of total WSC content, with no attempt to provide an analysis of the constitutive sugars. Suitable methods for the extraction of WSC exist, although, in estimation of the yield of reducing sugars, it is advisable to select an appropriate sugar standard (e.g. fructose/glucose), whilst ensuring that chemical moieties that could have a significant effect on the final colorimetric reaction are accounted for. When a more detailed analysis of the individual components of the WSC fraction is required, possibly in forage breeding programmes, suitable methods based on gas- or high-pressure liquid chromatography do exist, but these could never be contemplated for routine use.

Protein fractionation

For a significant part of this century, the accepted method for determining forage protein content was by estimation of total nitrogen content, using Kjeldahl methodology. However, this approach did not distinguish between nitrogen of protein and non-protein origin, and thus the resultant fraction was termed 'crude protein'. Attempts to rationalize this into digestible crude protein (DCP) or available protein (AP) were popular for some time, but reversion to crude protein was inevitable when users became dissatisfied with DCP or AP as indices of protein value. With increasing knowledge of dietary protein metabolism, especially in the rumen, Miller (1973) suggested development of a protein-rationing scheme, mirroring an earlier proposal by Burroughs *et al.* (1967) in the USA. Four years after Miller's proposals, the first version of the UK MP system was released, and a revision subsequently accorded some peer recognition (ARC, 1980). Since then a number of further revisions have been published (AFRC, 1984, 1993) and, despite several concerns, the concept of MP is now included in all UK feeding systems.

The main feature of the MP system is the recognition that the nature of the dietary protein will influence both the extent and efficiency of its utilization by rumen microbes. This will then determine the composition of the protein supply to the intestines and therefore the use of dietary protein by the host animal. Thus, the concepts of ruminally degradable and ruminally undegradable protein (RDP and UDP) were established, subsequently refined to effective rumen-degradable protein (ERDP) and digestible undegraded protein (DUP). To support such concepts, it was necessary to develop suitable methodologies to permit quantitative evaluation of the different fractions, and subsequently the *in sacco* technique, as proposed by Mehrez and Ørskov (1978), was recommended. Since then, this methodology has been used extensively to characterize a wide range of feedstuffs for ruminants. The method relies on the incubation of small amounts of test feed retained in artificial-fibre bags within the rumen of experimental animals consuming a feed similar to that being examined. Removal of feed samples at different time intervals and

analysis of the residues for total nitrogen content allow the pattern of nitrogen (N) degradation over time to be described. A number of different mathematical models have been developed to analyse these data (Ørskov and McDonald, 1979; McDonald, 1981; France *et al.*, 1993). In summary, three values for the degradation characteristics of the feed can be obtained, namely the rapidly degradable fraction (or *a* fraction), which may be significant in fermented feeds, such as grass silage, the slowly degradable fraction (*b* fraction), which will be digested over time, and the rate of degradation of this fraction (*c* value). Summation of the *a* and *b* fractions provides a measure of total dietary RDP content, whilst protein unaccounted for by these two fractions, $1-(a + b)$, is a measure of dietary UDP content. The quantity of fraction *b* protein degraded is determined by the time spent in the rumen with the feed exposed to microbial fermentation, itself a function of level of feeding and outflow rate. It follows that, at high rates of passage, a significant amount of the *b* fraction is likely to escape rumen degradation. The term ERDP therefore defines the quantity of N available for capture and utilization by the rumen microorganisms for growth and synthesis.

At the same time, it is recognized that the utilization of ERDP by the rumen microbes is influenced by energy availability within the rumen and, in this regard, ruminally digested organic matter (OM) was first proposed as a suitable measure. However, it was subsequently accepted that this was not a sufficiently adequate index of energy supply, and the alternative concept of fermentable ME (FME) was established. Defined as total dietary ME content, less the energy of dietary fermentation products, fats and oils (given that neither contribute significantly to energy supply for microbial metabolism), FME represented a conceptual improvement on previous schemes. However, it suffers from a major weakness, namely that, to date, no values of FME have been obtained from *in vivo* feeding systems. Thus, in a situation where the prediction of ME content may be questionable, the application of relationships between FME and predicted ME, as proposed by Chamberlain *et al.* (1993) and others, which were not based on validated estimates, only serves to add confusion and uncertainty to the ultimate prediction of microbial protein synthesis. Evidence of the potential magnitude of this problem can be obtained from Table 2.2, where further data regarding the two maize silages referred to earlier are presented. Whilst no estimates of FME content were obtained, variations in the estimates of crude protein, ERDP, DUP and ME contents were so large that, if applied to current feeding systems, they would lead to differences in the estimates of MP supply of sufficient magnitude as to make the whole scheme unworkable.

Thus, whilst scientific understanding of the metabolism of dietary proteins by ruminants has advanced considerably over the last two decades, there is still concern regarding the reliability of estimates of MP. More importantly, none of the major components of the scheme, namely the amounts of microbial and undegraded feed protein digested in the intestines, have been systematically validated against *in vivo* data. The absence of this validation clearly makes it difficult to accept or refute the many estimates of MP supply that are currently being reported. In this regard, a major revision to the value used to represent the efficiency of utilization of MP for milk synthesis was recently suggested by Mansbridge *et al.* (1999). Previous studies had suggested that the estimates of MP supply to support a desired level of milk production were too low, and this led to the suggestion that

the efficiency value of 0.68, as adopted by the Agricultural and Food Research Council (AFRC, 1993) was too high. In a carefully designed lactation study, Mansbridge *et al.* (1999) varied both protein supply and milk output, and analysis of the data provided an efficiency of protein utilization of 0.625, leading to their conclusion that this lower value should be accepted in all subsequent versions of the UK system. However, whilst Mansbridge *et al.* (1999) tested the system as it is currently being used, they did not provide any validation of their estimates of MP supply. Thus, it is not possible from this study to conclude that the efficiency value is wrong, when the error could equally reside in the computed MP inputs.

Furthermore, it is recognized that the *in sacco* technique (Ørskov *et al.*, 1980) has a number of potential weaknesses. It is difficult to standardize the technique, despite repeated attempts (Madsen and Hvelplund, 1994), this being particularly relevant in relation to the washing of feed residues to remove microbial contamination, whilst there remains concern over the choice of the most appropriate diet for the experimental animals. The technique relies on the use of surgically modified animals and, when the costs and other implications of surgical preparation and animal maintenance are taken into account, the method is relatively expensive and not conducive to routine use. There is also growing concern regarding the possible introduction of animal effects when using mature sheep to determine the digestion kinetics of protein metabolism of feeds for high-yielding dairy cows. Finally, whilst not particularly relevant to the evaluation of feed proteins, the *in sacco* technique has been extended to consider the rumen degradability of other nutrients, including starch and NDF, but often with quite variable and sometimes conflicting results. In this context, de Visser (1993) provided a very convincing data set relating to the degradation characteristics of the starch component of different maize silages, which suggested the presence of increased amounts of ruminally protected starch as the forage crop matured. In contrast, *in vivo* studies from this laboratory undertaken with lactating dairy cows to examine a range of different maturities of maize silage at harvest failed to establish the existence of large amounts of starch entering the small intestine of cattle. In this respect, the use of 'book values' for estimates of the rate of passage of digesta from the rumen in the estimation of the partition of digestion of ruminally degradable nutrients between ruminal and postruminal digestion may be worthy of reconsideration.

In vitro *gas production methodologies*

As indicated, rumen fermentation is associated with the evolution of gas, principally carbon dioxide and methane, with occasional amounts of hydrogen, whilst recently the occurrence of dimethyl sulphide has been reported. On the assumption that the quantity of gas released when feedstuffs are incubated *in vitro* with rumen fluid is closely related to digestibility, and therefore the energy value of the feed to ruminants, Menke *et al.* (1979) proposed a simplified system, based on gas production, to evaluate feedstuffs. In their system, fermentations were conducted in gas-syringes to allow the gas evolved to be retained and measured manually. The approach developed by Wilkins (1974) used a pressure transducer to measure accumulated head-space gases resulting from microbial fermentation. This technique

was subsequently exploited by Theodorou *et al.*(1993), who recognized the potential of the methodology to provide estimates of the rate and extent of feedstuff degradation. The technique has now been adopted by a number of laboratories throughout the world, as it undoubtedly offers considerable potential in relation to the throughput of samples, while reducing the reliance on surgically modified animals.

The earlier data were presented simply as cumulative gas-production curves, and this led to difficulties in data interpretation, with many of the more subtle changes between feeds tending to be either masked or lost when mathematical models were fitted. Subsequently, Mould *et al.* (1999) reconsidered this part of the technique in a series of studies designed to examine the effect of a feed additive on the rate and extent of rumen fermentation. Using appropriate corrections (negative controls), they derived profiles for the rate of gas production resembling gas chromatography curves. By using this approach, it became possible not only to 'fingerprint' a feed into its main carbohydrate components but also to provide an estimate of the extent of degradation of each of these components. This greatly increases the value of the technique when attempting to identify small differences between feedstuffs. A further simplification of the methodology was the abandonment of measuring gas volume directly (syringe) for measurement of changes in gas pressure (Mauricio *et al.*, 1999). This, together with a direct data entry system, which reduced operator-associated errors and greatly increased the capacity of the system, has provided a simplified technique for routine use, capable of screening large numbers of substrates.

However, concerns remain over the degree of interpretation being placed upon the data provided by such methodology. There have been few attempts to validate the results with *in vivo* data; indeed, it would be almost impossible to do so. Thus, such data are only comparative, ideal for screening purposes, from which more comprehensive research, possibly involving animals, may be required, before definite recommendations can be made. Gas produced during the ruminal fermentation of feeds represents only a small component of total nutrient flux in a rumen ecosystem. Consequently, changes in gas yield per mole of carbohydrate fermented (see Table 2.1), as well as the partition of degraded carbohydrate between fermentation and microbial incorporation, can have a significant bearing on total gas yield. As Menke *et al.* (1979) concluded, although a high correlation was found between cumulative gas production and OM digestibility, a feedstuff with a low rate of gas production may have a higher *in vivo* digestibility than that suggested from gas production data. Thus, *in vitro* gas production values alone provide little direct information, apart from estimating rate of fermentation. These therefore need to be examined together with simultaneously derived estimates of both rate and extent of degradation to permit estimates of fermentation efficiency to be obtained. It is a matter of concern, therefore, to note that the level to which the technique is now being developed is well beyond its realistic capabilities. Proposed as a first approximation to measurement of *in vivo* ruminal events, it is being overcomplicated by attempts to measure parameters such as protein degradation, ammonia evolution, microbial protein synthesis and the survival of feed protein (Cone and Van Gelder, 1999). Thus, a powerful but, as yet, not fully validated technique is being used to develop further data sets which have not been, and are unlikely ever to be, validated: a very different position from that adopted by Tilley and Terry (1963), as discussed

earlier. This is a very questionable basis on which to advance the science of feed evaluation, adding unnecessary complication when the first, and highly laudable, intent was to overcome the complexities of the cow. If current efforts continue, the cow will need to be reconsidered as a seemingly simple experimental tool on which to evaluate feedstuffs for milk production.

Near-infrared reflectance spectroscopy

Since its development, much has been reported about the potential benefits of this technology, particularly for laboratories involved in routine feedstuff analysis, where rapid, comprehensive and relatively inexpensive analyses are claimed (Offer *et al.*, 1998). NIRS is a physical method, which depends on the measurement of light absorption by the surface of a sample using wavelengths in the infrared region of the spectrum (1100–2500 nm). The absorption spectrum produced depends on the chemical bonds within the constituents of the feed sample and it is therefore possible to identify specific regions of the spectrum associated with different chemical entities, such as protein, fibre, starch, etc. To achieve satisfactory estimates of the nutrient content of feeds, however, it is first necessary to calibrate the apparatus against standard reference samples that have been analysed by more routine 'wet chemistry' methodologies. Several workers reported satisfactory estimates for components such as crude protein, and Barber *et al.* (1990) provided useful predictions of *in vivo* OM digestibility for grass silage, compared with more conventional approaches involving *in vitro* techniques or wet chemistry (for more details, see Beever *et al.*, 1999). These studies were subsequently extended by Givens *et al.* (1997) for a wider range of forage types. However, the problem appears to be related to the way in which the technique has been exploited beyond its capabilities, similarly to the gas technique discussed earlier. Prediction of rumen protein degradability by NIRS is never likely to be a simple process, given that most feeds contain several different types of protein, all of which are likely to have different degradation profiles. Furthermore, to use this technique in conjunction with *in sacco* measurements of protein degradability, which have never been validated against *in vivo* observations, is an enormous step of confidence and an approach that should be adopted with caution. A similar situation prevails in relation to the determination of FME contents by NIRS, a procedure used by several laboratories, for which no validation with *in vivo* estimates has been attempted. Consequently, whilst NIRS has much to offer, it should be used only where there is established confidence in the data, and its use to predict *in vivo* events from a questionable set of *in vitro* data is only likely to bring disrepute to a potentially useful technique.

Future Developments in Forage Evaluation

Undoubtedly, feedstuff evaluation is an established component of livestock feeding in the UK, along with most developed countries. It is only through access to reliable information regarding feed quality that rational decisions relating to the feeding of ruminant livestock can be made. This is particularly true with respect to

forages, for reasons discussed earlier. However, for the full potential of feedstuff analysis to be realized, several important issues need to be addressed. Some of these will be discussed in this section, along with possible recommendations, accepting that feedstuff evaluation is an evolving science, in which further revisions will be dependent upon the way in which systems of meat and milk production from ruminants develop. In this chapter, as well as in other reviews (e.g. Beever and Cottrill, 1993), concern over the adequacy of present feed rationing schemes, which generate much of the current interest in feed evaluation, has been expressed. Possibly the most crucial issue is the need to consider models that represent animal response, rather than simply meeting animal requirements. Before addressing longer-term aspects, however, it is pertinent to consider how various options may be developed to improve current systems.

Immediate requirements

All feedstuff analysis has a cost, which is always borne by the farmer, even if indirectly through the services of a feed or advisory company. Thus, livestock producers are entitled to question the value of their investment in feedstuff analysis in terms of enhancing the profitability of their business. This would be difficult to test objectively and the best that can be expected is a general perception that their investment is financially rewarding to their business. To achieve this, however, they need to be satisfied with both the analyses they receive and the interpretations placed upon them. Reorganization within the UK Ministry of Agriculture, Fisheries and Food (MAFF) in the late 1980s and privatization of the Agricultural Development and Advisory Service (ADAS) caused considerable fragmentation of the industry in relation to feedstuff analysis and nutritional consultancy. Many more laboratories are now offering such services, backed by an ever-increasing number of consultancy organizations and private consultants. But do all laboratories provide information the farmer wants and that can be used with confidence? Equally, do all laboratories report their data in a format that is easy to comprehend? Undoubtedly, many do, but in too many instances interpretation of the data can be difficult. For estimates of ME content, possibly the most important parameter with respect to forages, several laboratories report more than one value, depending upon the methodology used. An informed consultant may be able to reconcile such issues, but a difference of more than 1 MJ kg^{-1} DM, such as illustrated in Table 2.2, will be difficult for many farmers to ignore, irrespective of which value they believe. Equally, many laboratories report data which are of little or no interest to farmers. It is likely that farmers are already aware of those silages of inferior quality before they receive the silage analysis, which puts in question whether all of the data relating to the volatile components have to be reported, when lactate and acetate levels would probably suffice. The most obvious reason why such data are provided is because they were produced in the laboratory and thus the laboratory felt obliged to report it. All laboratories must be encouraged to be more discriminating in the data they report, as well as paying attention to the presentation of the data.

But there are deeper issues, relating to the plethora of different techniques being used to derive the analytical data. Some of these concerns have already been

expressed, the position regarding starch analysis being an excellent example. Currently, there is no agreement over the most appropriate methods to use for all analyses and, whilst most laboratories seek to acquire 'good laboratory practice' status, this does not necessarily guarantee that the data they generate is accurate. Some laboratories have resorted to techniques that were questioned many years ago, whilst others have increased their reliance on indirect methods, such as NIRS. The validity of data produced using NIRS for chemical entities such as crude protein, starch, etc. will never be better than the databases used to establish the calibration curves. There have been many instances where these have been shown to be inadequate, use of grass-silage-derived relationships to evaluate the energy component of whole-crop wheat or maize silages or even fresh grass being suitable examples. But it is the almost uncontrolled use of NIRS to estimate biologically important components, such as ERDP, DUP and FME, which is of greatest concern, with highly questionable databases of *in vitro*-derived information being used with scant regard to the lack of any validation with *in vivo* events. Reference has already been made in Table 2.2 to variation in the estimates of ME content provided by those laboratories involved in the study. Despite this, there was total agreement between laboratories that the later-harvested crop had a higher ME value. This gives little reassurance, however, when *in vivo* determination of the ME contents of the two feeds fed to dairy cows in respiration calorimeters found a higher ME content for the earlier-cut crop. An immediate explanation was the higher gross energy (GE) content of this feed, indicative of an increased extent of fermentation occurring during ensiling of the wetter and more immature crop (Table 2.3). Thereafter, no differences were observed between the two feeds with respect to the partition of GE to faecal, methane or urine energy. Thus, for feeds that have undergone

Table 2.3. Comparison of *in vivo* and laboratory-derived estimates of the metabolizable energy content of two maize silages harvested at different stages of maturity (units, MJ kg^{-1} oven-dry matter unless stated).

	Silage maturity	
	Low DM	High DM
Laboratory estimates*	11.2	11.5
In vivo†		
Maize		
GE content	18.9	17.6
ME content	10.9	10.1
Total mixed ration		
DE / GE (kJ MJ^{-1})	680	676
DE content	12.9	12.2
ME / GE (kJ MJ^{-1})	579	576
ME / DE (kJ MJ^{-1})	850	851

* Mean of six reporting laboratories.
† Not corrected for level of feeding effects.
DE, digestible energy.

different degrees of fermentation, some measurement of GE content to take account of different levels of volatile components may be necessary, if more reliable estimates of ME content are to be obtained.

In summary, the often heard remark that the analytical data were 'unbelievable' is not acceptable and urgent rationalization is needed to provide agreement on appropriate methods, resisting those which have not been validated against *in vivo* observations. However, this can only be achieved through coordination at national level. Additionally, with the level of concern over some of the analyses being provided, it should become a requirement of all laboratories to provide a brief résumé of the methods used, be it NIRS or wet chemistry. This may be of little immediate value to the farmer, but it would provide an important audit trail for the consultant regarding possibly aberrant data. Equally, all laboratories should report only on those parameters where they have sufficient confidence in the methodology, whilst positive encouragement should be given to their committed involvement in schemes designed to examine interlaboratory comparisons. Such procedures are already in place with respect to the analysis of milk constituents, which has major implications with respect to both milk price and quota management. Given that the financial implications of feedstuff evaluation are at least as great, the lack of appropriate standardization is unacceptable.

Longer-term needs

Whilst initiatives similar to those outlined above will provide better support for current systems of feed rationing for ruminants, there is a growing opinion that more radical changes in models of ration formulation are required. This is particularly evident from the current interest being shown in the UK for both the French PDI system and CNCPS. This should not be construed to suggest that such models have been adequately validated or that they necessarily perform better than UK equivalents; rather, they contain certain elements and representations considered to be superior to those in current UK systems of energy and protein rationing. It is not the intention of this chapter to review the strengths and weaknesses of such systems, as others have attempted this elsewhere. The more challenging task is to decide which type of system should be developed. Certain opinion suggests that serious revision of current systems would be adequate, but adoption of this approach would be difficult if one of the agreed challenges was a model of animal response rather than animal requirement. To meet this objective, a new model of energy and protein rationing is required, with an implicit representation of individual nutrients to provide the important characterization of ME and, to a lesser extent, MP that is not evident in present systems. Equally, any new system will require more comprehensive representations of postabsorptive energy and protein metabolism. While these are partially recognized in the rumen element of the current MP system, there is no evidence of nutrient interactions at the tissue level. Such approaches can be found in the models of rumen and whole-animal metabolism proposed by Baldwin and colleagues, the most notable efforts being those of Baldwin *et al.* (1987a, b, c). These models were quite successful in predicting overall nutrient utilization and lactational performance in dairy cows, but were criticized

in relation to the required dietary characteristics, which were too comprehensive when compared with those expected from routine feedstuff evaluation. However, in the development of these models, there were no attempts to consider if some of the inputs could be ignored, or at least aggregated, without serious loss of resolution in the models. Thus, it is difficult to provide a definitive list of required feed characteristics, but, in the light of present knowledge, it is possible to suggest some of the most likely candidates.

For those nutrients that the animal ultimately uses for maintenance and production costs, the simplest nutritional representation would be rumen VFA (both lipogenic and gluconeogenic), long-chain fatty acids, glucose and amino acids. Considering these in turn, partition of rumen VFA into lipogenic and gluconeogenic substrates is an obvious necessity, given their contrasting metabolic fates, with further distinction of lipogenic VFA into acetate and butyrate, if only to provide compatibility with *in vivo* data. There is no justification for representing other VFA, such as isobutyrate and valerate, as these are never found in significant amounts in any diets. Propionate is the sole gluconeogenic VFA and, given the crucial role that glucose plays in the overall nutrient economy of the animal, this will need to be represented in all models of nutrient digestion and postabsorptive metabolism. However, as VFA do not occur naturally in most feeds, only with fermented feeds, such as grass silage, will it be necessary to take account of these, acetic acid being the only one of nutritional importance. This will be referred to later in relation to silage analysis.

Long-chain fatty acids, on the other hand, occur in most feedstuffs and, whilst levels are generally low, especially in forages, they may account for up to 15% total ME supply in rations designed for high-producing animals. When augmented with fatty acids from mobilized body tissue in early-lactation cows or underfed animals, free fatty acids become important to the animal's overall energy metabolism. In addition, there are those situations where incomplete fatty acid metabolism can induce nutritional disorders, such as fatty liver syndrome and ketosis. There is also considerable interest, on the grounds of human health, in modifying the fatty acid composition of ruminant products, specifically to increase mono-unsaturated fatty acid levels and the ratio of ω-3 : ω-6 fatty acids. In this respect, both grazed and ensiled grass have been suggested as possible sources of increased ω-3 fatty acid levels. However, unless the market for such products increases dramatically, routine procedures to provide highly comprehensive fatty acid analyses for forages cannot be contemplated. Instead, there may be benefit if an agreed method for determination of total fatty acid content could be established, especially if associated with a rudimentary characterization of the saturated : unsaturated fatty acid ratio. Currently, most laboratories rely on ether extraction, but this method is limited, due to its non-specificity. Other methods, including those based on direct methylation of fatty acids (Outen *et al.*, 1975), may be worth considering, although a final gravimetric determination would be needed to replace existing methodology, which involves relatively time-consuming gas chromatography.

There are few, if any, situations where glucose occurs in significant amounts in the feed, the exception being fresh forages. However, as starch is of increasing interest with respect to the formulation of diets for ruminants, this should be a priority area for analytical technique development. At this point, it would be useful to

identify those features of the methodology developed by MacRae and Armstrong (1968) which several laboratories are dissatisfied with, whilst at the same time establishing which of the current methods should be abandoned as being unreliable. Undoubtedly, one important feature of the method of MacRae and Armstrong (1968) was the use of glucose oxidase to determine the released glucose units. Given the high specificity of glucose oxidase, it is unlikely that this is a major area of concern, and thus the problem must be associated with solubilization and hydrolysis of the starch during the initial stages of the method. Starch composition varies quite markedly between different starch sources, particularly the relative proportions of amylose and amylopectin, and it may be desirable to re-examine the initial solubilization procedures as proposed by MacRae and Armstrong (1968) with a wider range of substrates. Thereafter, the main cause of concern must be the specificity, as well as the uniformity, of commercially available amyloglucosidases. This has been recognized as a potential problem in the past, but it was never viewed as being of sufficient severity to cause the technique to be abandoned, especially in favour of much less specific methodologies. Remaining with starch, advances in the protein nutrition of ruminants which led to the concepts of ruminally degradable- and ruminally resistant proteins have recently been extended to starch, leading to nutritional entities such as ruminally fermentable starch (RFS) and ruminally resistant or digestible undegraded starch (DUS). This interest has developed on the unproved hypothesis that starch digested in the small intestines to glucose would be utilized more efficiently by the animal than starch subjected to ruminal fermentation with the resulting hepatic conversion of propionate to glucose. In studies in this laboratory, in which identical amounts of additional starch were provided by infusion into the rumen or the small intestine of dairy cows, posthepatic glucose output showed no effect of treatment, suggesting that the liver exerted considerable control on whole-body glucose status (Reynolds *et al.*, 1998). Equally, the same study failed to establish any difference in overall energy utilization between the different routes of administration. Such data raise doubt regarding the metabolic value of different starch entities such as RFS and DUS. However, interest in such fractions is likely to continue in relation to improving the efficiency of nutrient utilization within the alimentary tract.

Reference was made earlier to the importance of managing energy (carbohydrate) and protein (nitrogen) availability in the rumen in order to optimize microbial synthetic efficiency, and it is recognized that the provision of carbohydrates with different degradation characteristics will be important in this regard. The ability to balance contrasting starch sources, such as wheat and maize, when fed in conjunction with different forages will ensure a more even supply of energy for the microbes, thus avoiding periods of excessive VFA production and substantially reduced rumen pH values. It is known that high starch feeding can significantly depress rumen pH and values approaching 5.0 have recently been reported in this laboratory. On such occasions, overall fibre digestion in the rumen will be reduced, as shown by Beever *et al.* (1989), and ultimately this may lead to a significant reduction in whole-tract digestibility of fibre. Provision of a more continuous supply of degradable carbohydrate will minimize such events, whilst in such situations it may be possible to exploit the ability of the small intestine to digest starch. This would have the effect of reducing overall starch load within the rumen, and

research in this laboratory has shown that most dairy cows can successfully digest over 2 kg starch entering the duodenum before overall digestive efficiency is compromised and faecal starch levels increase. To provide suitable methodologies to describe the degradation characteristics of dietary starches, however, two important issues have to be addressed. First, as current *in sacco* methodologies are not suitable, a reliable method that could be used with concentrate feeds that are normally ground prior to pelleting must be developed. Equally, all data on the rate and extent of starch digestion in the rumen obtained with *in vitro* procedures must be validated against *in vivo* data. This would also apply if the methodology were extended to examine aspects of dietary fibre digestion in the rumen.

Remaining with this theme, serious consideration should be given to those procedures currently being used to characterize dietary proteins, and more reliable and repeatable alternatives to the *in sacco* technique should be developed. In this respect, artificial rumen techniques, such as Rusitec (Czerkawski and Breckenridge, 1977), have not been fully exploited and there may be benefit from developing these further, provided, once again, that the data are validated with *in vivo* observations as necessary. Currently, the fractionation of dietary crude protein is limited to that which is or is not of protein origin, with no attempts to identify specific proteins or, more specifically, individual amino acids. It would be difficult to justify major new initiatives in such areas at present, but the issues may need to be reconsidered if environmental pressures on livestock production systems call for tighter controls on nitrogen losses as urine and faeces. When most systems of milk production can claim a milk N output of no more than 300 g kg^{-1} dietary N intake, there appears to be considerable scope in the management of protein in ruminant diets to reduce both dietary input and waste output without compromising milk N output.

Returning finally to the issue of silage analysis, which undoubtedly is the main area of interest with respect to forage evaluation, a number of important suggestions have already been made in this chapter. One possible area worthy of attention is in relation to the determination of true DM content. This will require quantification of the volatile components and, whilst gas-chromatographic techniques are available for determination of most of the major components, such methodologies are not conducive to rapid and routine feedstuff evaluation. This situation is further complicated by the need to quantify lactic acid levels, which, in some silages, can comprise as much as 100 g kg^{-1} of total DM, or over 20% of the energy released in the rumen. Whilst lactate is not utilized to any great extent as an energy source, either directly or after conversion to acetate or propionate by the rumen microbes, it is extensively used in postabsorptive metabolism (Gill *et al.*, 1986). It is also an important component in the estimation of FME, given that this entity, as defined by AFRC (1992), is derived by discounting the ME contribution of fermentation acids and that of oils and fats from the total ME content. However, there is a wider issue relating to the analysis of silage volatiles, as several have been identified as being important in the prediction of silage intake. Finally, until feed rationing schemes move towards a fuller description of individual nutrients, serious consideration should be given to the provision of more reliable estimates of forage ME contents. Currently, these form a major component of all ration formulation schemes and yet, despite such initiatives as 'ME✓' (Offer *et al.*, 1996), which is widely used in the UK, there are many highly questionable values being produced by different

laboratories. The problem may be due, in part, to the failure to recognize different GE contents, as discussed earlier, but another more fundamental issue may be the predictive relationships that are being used. Many were based on grass silage and appear to have been extended to other forage sources with little regard for the wider implications. Furthermore, the use of sheep to develop such relationships needs to be examined, given the emphasis now placed on the accurate ration formulation of bovine animals and, in particular, high-yielding dairy cows. Recent studies in this laboratory, as reported by Beever *et al.* (1998a, b), showed measured ME intakes of over 310 MJ day^{-1}, equivalent to more than five times the maintenance level of feeding. An error of only 5% in this estimate is equivalent to an under- or overprediction of 3.0 l day^{-1} of milk of standard composition. Equally, errors of the magnitude reported for the two maize silages in Table 2.2 would, if applied to the total diet, equate to an underprediction of milk yield of 8.0 l day^{-1} when comparing the extreme estimates.

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3 Intake of Forages

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Introduction

Forage is a major component of ruminant rations. In temperate regions, systems range from extensive – in which ruminants harvest the forage themselves, to maximize the utilization of the forage – to intensive systems – in which forages are offered with high levels of concentrates, to maximize production per cow. Forages in these systems include rough grazing on hills and uplands, cultivated high-quality grass and legumes, which are grazed or conserved as hay and grass, legumes and maize conserved as silage and straws from cereal crops.

In the tropics, forages tend to be highly variable in quality and quantity, from cultivated forages, such as *Pennisetum purpureum*, through the leaves of fodder trees to poor-quality stovers of maize, sorghum and millet during the dry season. The lack of resources of poor farmers in many of these systems means that concentrate use is limited and, in recent years, there has been increased interest in the use of high-quality legume straws or tree fodders as supplements to poorer-quality forages.

Many authors (e.g. Illius, 1998) have suggested that intake is probably the single most important variable determining animal performance and voluntary intake is generally correlated with the amount of nutrients that can be extracted from a feed, i.e. its digestibility. For forages, digestibility is largely determined by features of the plant, but potential digestibility, and hence potential intake, may not be achieved, due to interactions between feeds or between one or more feeds and the animal itself. To understand, and hence predict, these interactions requires an understanding of the mechanisms that control intake and thus this subject has been studied in detail over the last few decades. Recent reviews of the topic include Gill and Romney (1994), Jung and Allen (1995), Forbes (1995a, b; 1996), Provenza (1995a, b), Allen (1996), Illius and Jessop (1996), Weston (1996) and Wilson and Kennedy (1996), amongst others. The present chapter does not attempt to cover all aspects in such detail, but rather summarizes the information and highlights the aspects that are most important for forage feeds. The chapter

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starts by considering the main principles of intake control, prior to considering forage factors that determine potential intake. This is followed by summaries of the effects of interactions with animal factors and between dietary components. The chapter ends with sections on prediction and manipulation of intake and priorities for further research.

Principles of Intake Control

In order to understand the principles of intake control, it is important to answer the questions: (i) why do animals eat? and (ii) why do they not eat all the time?

It is generally accepted that animals eat to supply the tissues with the nutrients required to fuel physiological processes of maintenance, growth (including fat deposition in mature animals), milk production and work. However, given the variety of feed components eaten by animals, it is unlikely that the composition of the nutrients supplied will exactly meet the ratio of nutrients required by the animal. Thus, meeting the requirements of one nutrient is likely to result in a deficiency or excess of another. Animals stop eating to limit metabolic or physical discomfort and thus the animal has to decide at what point the disadvantages of the deficiencies or excesses of some nutrients outweigh the advantages of trying to meet the animal's energy requirements, which are thought to be the main intake 'driver' (Emmans, 1997).

In the experimental situation, animals have been shown to be able to make nutritionally wise choices between a range of foods, confirming the theory that animals are monitoring supply and demand of vital nutrients. For example, poultry (Shariatmadari and Forbes, 1993) and pigs (Kyriazakis *et al.*, 1990) were able to select a diet meeting their requirements for protein from foods containing different levels of protein. Similar observations have been made in sheep (Kyriazakis and Oldham, 1993) selecting between diets varying in protein, although it is more likely that diet selection in ruminants is a response to changes in the rumen environment (Cooper *et al.*, 1995), rather than metabolic requirements of the whole animal.

The data considered above all relate to daily intake, while much of the work on factors that stop the animal from eating tend to have been measured at the end of a single meal. The vast literature on experiments to measure intake demonstrate that animals do appear to reach a relatively stable energy intake on a constant diet, but this can be achieved by very different patterns of intake within a day. Early work on factors that limited intake focused on single factors, such as temperature (Brobeck, 1948), blood glucose concentrations (Mayer, 1953) and fat levels in the body (Kennedy, 1953). However, there is plenty of evidence (e.g. Fig. 3.1; Thiago, 1988) that gut fill, for example, varies during the day and is not at a maximum at the end of each meal. Balch and Campling, back in 1962, had concluded that 'food intake is unlikely to be regulated by any single mechanism and that, through the central nervous system, oropharyngeal sensations, gastric contractions and distension, changes in heat production and changes in the levels of circulating metabolites may severally be indicated'. One solution was to envisage integration of different signals within the brain, but argument still remains as to how and where

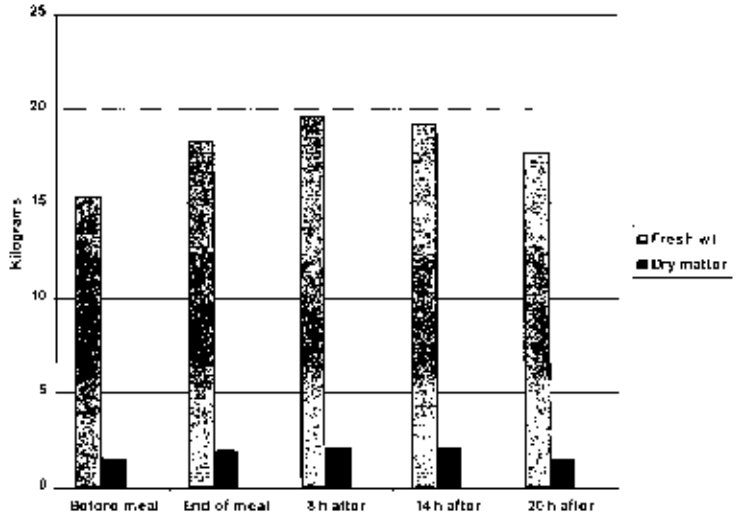

Fig. 3.1. Changes in the amount of fresh and dry digesta in the rumen of steers (live-weight approx. 120 kg) offered hay *ad libitum,* with time of offering a meal (Thiago, 1988).

such integration would take place. Anil *et al.* (1993) and Mbanya *et al.* (1993) reported, consistently, additivity between effects of volatile fatty acids (VFA) infused into the rumen and distension of the rumen, but not for stimuli of two different acids. It may be that it is the ratio of acids relative to the optimal ratio for the metabolism of the animal in question that is important, as discussed by Illius and Jessop (1996), but that ratio may vary within a day and it is not yet clear how an animal could keep count of cumulative metabolism over a 24 h period. This difficulty led Gill and Romney (1994) to hypothesize that factors determining the size of individual meals are related to optimizing nutrient supply, relative to daily metabolism, but this has yet to be confirmed.

It has long been assumed that, for forage diets, it is the 'bulk' of the forages that primarily limits intake, i.e. a combination of the volume and the time during which the undigested food remains in the gastrointestinal tract. Since the original proposal (Lehman, 1941, quoted by Van Soest, 1994) that the presence of undigested feed residues in the gastrointestinal tract limited intake, there has been extensive evidence to support the theory and this has been summarized recently by Allen (1996). Conrad *et al.* (1964) suggested that intake was restricted by gut fill up to a breakpoint in digestibility, beyond which the relationship between intake and digestibility became negative and controlled by the animal's requirements. The breakpoint is thus dependent on the energy requirements of the animal and on the relationship between digestibility and the nature of the constraint exerted at low digestibility for the particular feed under study. A better understanding of this latter relationship should lead to increased accuracy of prediction, particularly of diets with a number of components.

Forage Factors that Affect Intake

'Physical' factors

Plant structure

Physical factors are generally taken to refer to factors that directly influence the initial gut volume occupied by ingestion of a feed and the rate at which that volume is decreased by digestion and onward passage. The content of fibrous cell walls is a major factor in this respect, since these structures are less soluble and take up more space than the cell contents. Forages contain a large proportion of their organic matter (OM) content (35–80%) as cell walls, which provide the structural integrity of the plant. The structural carbohydrates, principally hemicellulose, cellulose and pectins, are broken down by microorganisms in the rumen, which enable the ruminant animal to take advantage of an energy source that is not able to be efficiently utilized by monogastrics. Distribution of the different molecules within the plant and the linkages between them will be important factors affecting the ease with which the microorganisms can break down the cells (Jung and Allen, 1995) and thus the space occupied in the gastrointestinal tract. In addition, physical characteristics of the cell wall or fibre particles themselves, such as tissue origin, shape, buoyancy and specific gravity, affect the rate at which particles are broken down and the ease of passage (Wilson and Kennedy, 1996).

Resistance to comminution (reduction in particle size) is positively related to fibre content; however, relationships between fibre measured using neutral detergent solution (NDF) and dry matter (DM) intake (DMI) are not always consistent. Reid *et al.* (1988) found a significant effect of forage class (C-3 or C-4 grass or legume) on the slope and intercept for regressions of DMI on NDF for both cattle and sheep, indicating that the fill effect of NDF may vary with different forages. This may be explained by the difference in distribution of the different structural polysaccharides. Minson (1990) observed that for groups of forages with similar DM digestibility, fibre content is greater in legumes compared with grasses, temperate compared with tropical grasses and leaf compared with stem. Wilson and Kennedy (1996) suggested that the lower digestibility of tropical grasses compared with temperate grasses and legumes reflects an interlocking and therefore more rigid cell structure. These authors also suggested that the greater digestibility of legumes compared with grasses may reflect leaf length. Grass particles are inherently long and buoyant, with a low functional specific gravity (FSG), and easily entangled, while chewed legume vascular particles are short and chunky with high FSG and therefore likely to escape the rumen quickly. Thus, potential intake is dependent not only on the fibre content, but also on the original structure of the plant and the way in which it breaks down during digestion.

The DM content of feeds may also influence the space occupied within the gut. Prewilting of grass prior to ensiling has consistently been shown to give silages with higher intakes compared with unwilted material from the same sward (Peoples and Gordon, 1989; Teller *et al.*, 1993), sometimes by as much as 44% (Romney *et al.*, 1997). One explanation is that the effectiveness of chewing during eating and hence the rate of particle breakdown was enhanced with the drier material. However, advantages in terms of animal production have been less evident in

recent years (Forbes, 1995b), as the control of fermentation in wet silages has improved and grass is cut at an earlier stage of maturity, giving material with higher digestibility, for which the effects of wilting are not as great. Advocacy of wilting continues mainly as a strategy to reduce effluent rather than to improve nutritive value.

Sward structure

In grazing animals, the structure of the sward can restrict intake not only in terms of the space taken up in the gut, but also by limiting the amount of herbage which the animal can actually harvest within a 24 h period. Characteristics of the grazed sward, such as plant density and height, can influence intake through their effect on ease of prehension and thus bite size, which has been shown to be a major factor influencing daily herbage intake (Hodgson *et al.*, 1991). Positive relationships between bite size of sheep and sward height have been observed (Allden and Whittaker, 1970; Penning *et al.*, 1991), while Stobbs (1973) and Chacon and Stobbs (1976) concluded that the sward bulk density and leaf-to-stem ratio were the major factors affecting bite size and intake of cattle. Cushnahan *et al.* (1998) conducted a study on lactating cows fasted for 5 h to enable measurement of potential intake and observed that pregrazing sward height was the major characteristic influencing bite depth and bite volume, but bulk density determined the weight of herbage per bite. Thus, for grazing animals, not only the plant structure, but also the sward structure need to be considered when predicting intake.

'Metabolic' factors

Not all of the differences in intake between forages can be explained by factors that could be anticipated to influence the space occupied in the gastrointestinal tract. For example, it is generally accepted that conservation results in reduced intake (Forbes, 1995b). However, following improvements in silage-making techniques, the low intake of silage compared with hay observed in the past is considered to be less of a problem.

The organic acids and nitrogenous substances, such as amines, produced during the silage-making process and additives used to improve the silage have often been implicated in the reduced intakes observed when fresh grass is ensiled. Ruminal infusions of individual silage constituents or mixtures of them (e.g. Buchanan-Smith and Phillip, 1986) have indicated that no single constituent is responsible for the low intake, but the additive effects of a number of substances may be important.

Interaction between Forage and Animal Factors

Animal size

As discussed earlier, intake is thought to be driven by an animal's requirement for energy and thus, across species, size is the factor most closely correlated with

intake. Larger animals consume greater quantities of food; however, the relationship is not isometric but scales allometrically with body mass, and intake is commonly expressed on the basis of metabolic body weight, or live weight $(LW)^{0.75}$. It is not surprising, either, that parameters related to the processing of fibre have also been found to be related to animal size. Welch (1982) found that rumination time per gram of NDF decreased exponentially with LW. Illius and Gordon (1991) showed that time taken to comminute large fibre particles and small-particle retention time scale with $LW^{0.27}$, while the digesta load of gastrointestinal-tract compartments is isometric with LW. These allometric relationships account for much of the intake variation attributable to mass (Illius, 1998) and can be incorporated in models to improve prediction of intake. Illius and Allen (1994) showed that, by scaling large-particle breakdown rate, small-particle passage rate and capacity according to animal size, variation in DMI explained by their model increased from 35 to 61%. However, growing animals appear to be able to consume a greater quantity of feed than mature animals of the same weight and intake appears to be related allometrically to $LW^{0.6}$, as opposed to $LW^{0.75}$, which is generally assumed for mature animals. This difference reflects the effect of metabolic rate and hence physiological status on intake.

Physiological status of the animal

Physiological status affects energy requirements and hence intake. As mentioned earlier, the point at which the relationship between intake and digestibility changes from positive to negative varies with the energy requirements of the animal and appeared to be higher for higher-yielding cows (Conrad *et al.*, 1964). In lactating animals, where nutrient demand is high, the rapid removal of metabolites from the blood may reduce the degree of stimulation of chemoreceptors from the same amount of absorbed nutrients (Forbes, 1995b), or rate of passage may be faster, reducing the bulk effect. Campling (1966) observed that intake is normally higher in lactating compared with dry or pregnant cows. Ingvartsen *et al.* (1992) observed a reduction in intake due to pregnancy, which was consistent with results from earlier literature. Ingvartsen *et al.* observed a decrease at least 12 weeks prepartum and hypothesized that hormonal regulation was implicated, as well as physical limitations resulting from the increased size of the fetus restricting space in the rumen. Evidence to support this hypothesis was provided by Coppock *et al.* (1972) who observed a greater decline in intake in late pregnancy for concentrate compared with forage rations and that intake was markedly reduced in the last 3 months of gestation in heifers fed a complete diet of finely ground roughages and concentrates (Aitken and Preston, 1964).

Grazing animals

Animal factors can also influence grazing intake. Intake of grazing animals is dependent both on intake rate (i.e. bite mass $\times$ bite rate) and time spent grazing (Allden and Whittaker, 1970). Although sward structure and herbage mass will

affect these parameters, as discussed above, animals are able, within certain limits to alter grazing behaviour, increasing grazing time where herbage is sparse and increasing intake rate. The effect of physiological status on grazing intake has also been shown; for example, when herbage availability is high, increases in intake of up to 460 g OM kg^{-1}, increases in milk yield have been obtained (Stakelum and Dillon, 1991). The effects have been observed on intake rate and/or on grazing time.

Intake rate

Bite mass and bite rate are not independent. Newman *et al.* (1994) point out that, for a given forage requiring a given time of mastication, an increase in bite mass will cause an increase in time of mastication, decreasing bite rate and resulting in intake rates that are similar. The fact that animals are able to alter intake rate is demonstrated by the observed increase in rate in response to fasting (Greenwood and Demment, 1988; Dougherty *et al.*, 1989). This observation also demonstrates that animals rarely graze at their maximum intake rate, although there have been some indications that animals increase intake rate when grazing time is restricted (Romney *et al.*, 1996), once they 'learn' that they will only be allowed to graze for a limited time. Newman *et al.* (1994) suggested that, where cattle increased intake rate through an increase in bite rate, this may reflect a decrease in the degree of mastication. In contrast, sheep increased intake rate by increasing bite mass and apparently have limited scope to alter degree of mastication, either because of plant characteristics, such as shear strength or fibre content, or because of digestive constraints (i.e. sensitivity to particle size).

Time spent grazing

Where sward structure limits bite mass and therefore intake rate, grazing time can be altered to compensate for decreased bite size. However, there appears to be an upper limit to the amount of time a ruminant will spend grazing. Weston (1996) notes observations of grazing times of 13 and 15 h for sheep and cattle, whereas Forbes (1996) suggests that ruminants are unwilling to eat for more than 12 h day^{-1}. Therefore, if bite size falls below a certain limit, animals will not be able to achieve maximum intake capacity. Illius (1998) suggests this occurs as a result of an upper limit to oral processing time, which encompasses prehension, mastication and rumination. However, this limit may in turn be dependent on physiological state, since increasing milk yield was shown to increase grazing time by Journet and Demarquilly (1979).

Experience and learnt aversions

A number of authors have shown that ruminants decrease intake of foods in response to a malaise resulting from ingestion of toxins, such as alkaloids (Thompson and Stuedemann, 1993), condensed tannins (Provenza *et al.*, 1990) and glucosinolates (Duncan and Milne, 1993), and of poor-quality silage (Buchanan-Smith, 1990). The aversion to a particular food increases with severity of the illness (Provenza, 1995a) and decreases with increasing delay between food

ingestion and illness caused by that food (e.g. Ralphs and Cheney, 1993). Aversion to feeds diminishes as time passes and the recuperative process counteracts the aversion (Provenza, 1995a), resulting in cyclical intake of nutritious feeds containing toxins, a sharp decrease being followed by a gradual increase. A similar response is also observed in animals consuming grain, which appears to be a result of malaise caused by organic acids produced in starch digestion.

Provenza (1995a) pointed out that, despite the presence of toxic feeds where ruminants are allowed free choice of feeds, there are relatively few cases of toxicosis and that animals are able to limit intake of nutritious plants containing toxins, dependent on the concentration of the toxin. He concluded that animals are able to recognize feeds based on previous experience. Thus, preference for a particular food depends not only on taste, but also on the consequences of that food for the internal environment (Provenza, 1995b).

Interaction between Diet Components

Supplementation can be considered as a means of increasing nutrient supply to animals that are unable to consume sufficient nutrients as forage because of physical fill limitations. Supplementation tends to have a positive effect on overall DMI, but may have positive or negative effects on intake of the basal forage.

Negative effects on DMI

Often there is a substitution effect, where intake of the fibrous feed decreases to an extent that varies with the digestibility of the basal feed, among other factors. In some cases, the effect might be explained according to simple rules of additivity, where fibre is replaced on a 1 : 1 basis; however, this cannot be used as a general explanation. Forbes (1995b) noted that substitution rates for dairy cows can vary between 0.4 and 0.8, with a mean of approximately 0.5. Although some work has shown a linear effect with increasing level of supplementation (Thomas, 1987), other work has shown an increase in substitution rate as level of supplementation increased (e.g. Sutton *et al.*, 1992) and a positive relationship with forage quality (Forbes, 1995b).

Supplements that are high in readily fermentable carbohydrate may have a greater effect on inhibition of fibre intake than more slowly fermentable supplements, through depression of digestibility of the roughage fraction. Rapid fermentation results in an inhibition of cellulolysis, which has been variously attributed to low pH (Terry *et al.*, 1969) or feedback inhibition of key fibre-digesting enzymes (Murphy, 1989).

Positive effects on DMI

In some circumstances, supplementation can be used to increase intake of a poor-quality feed by supplying a limiting nutrient. Wilson and Kennedy (1996) noted

that, for forage diets, the rate of microbial fermentation is depressed if ruminal ammonia concentration drops below 50 mg nitrogen (N) l^{-1}.

Meanwhile, Minson (1990) suggested that, for feeds with a crude protein (CP) content of less than 62 g CP kg^{-1} DM, fibre digestion is inhibited and reports a number of trials in which intake of forages have increased by 14–77% following provision of supplementary protein. Where ammonia N concentration limits microbial fermentation, supply of N to the microorganisms increases OM digestion in the rumen, which increases breakdown and rate of passage of a poor quality forage, thereby removing the physical constraint and allowing the animal to consume more feed. Leng (1990) noted that farmers in developing countries recognize the benefit of adding small amounts of green forage to poor-quality roughage diets and cites authors who have demonstrated that supplementation of a straw-based diet with forage of high digestibility boosts digestibility of the basal feed, even when levels of supplementation are low. He suggested that this may be through provision of a highly colonized fibre source to seed bacteria on to the less digestible fibre or through increasing rumen ammonia concentrations above a critical level.

A summary of plant and animal factors affecting forage intake is given in Table 3.1.

Prediction of Intake

Improved accuracy of prediction of intake has been the goal of many scientists in response to the needs of the livestock industry, but, given its multifactorial nature, what degree of accuracy can be achieved? In a review of intake prediction, Poppi (1996) concluded that the purpose for which the prediction equation is to be used should determine the level of accuracy required. The Agricultural and Food Research Council (AFRC, 1991) suggested a target of within 10% of actual intake for the purposes of practical ration formulation. In view of the dominant effect of animal size, referred to above, all prediction equations take account of weight, most relating intake to $LW^{0.75}$, and, for dairy cows, milk yield is often included, although, if energy requirements are driving intake, it should be potential rather than actual yield which is used. In terms of feed components, however, there is less unanimity of approach, and a number of approaches are summarized briefly below and in Tables 3.2 and 3.3.

Chemical composition

The most common chemical components used to predict the intake of forages include a measure of the cell-wall content, with acid- or neutral-detergent fibre fractions (ADF and NDF) (Van Soest, 1965) being the most frequently cited.

In vitro *digestibility*

The relationship between rate of digestion and intake through its effect on rate of passage has resulted in a number of authors using *in vitro* measures of rate of

Table 3.1. A summary of some of the plant and animal factors affecting intake of forages, and circumstances where high or low intakes can be expected.

	High intake	Low intake	Reference
Plant factors			
Fibre content	Low fibre	High fibre	Van Soest (1965)
Forage class	C-3 grasses (temperate), legumes	C-4 grasses (tropical)	Reid *et al.* (1988) Wilson and Kennedy (1996)
DM content	High DM silages	Low DM silages	Peoples and Gordon (1989) Teller *et al.* (1993) Romney *et al.* (1997)
Sward structure	High plant density Optimum sward height for animal species grazed	Low plant density Sward height too low for animal species grazed	Hodgson *et al.* (1991) Penning *et al.* (1991) Cushnahan *et al.* (1998)
Conservation	Good fermentation of silage	Poor fermentation of silage	Forbes (1995b)
Animal factors			
Physiological status	Growing animal Lactating animal	Late pregnancy Mature unproductive animals	Conrad *et al.* (1964) Campling (1966) Ingvartsen *et al.* (1992)
Size	Large animals	Small animals	Illius and Allen (1994) Illius (1998)
Intake rate	Forage characteristics optimize bite mass and minimize mastication	Forage characteristics limit bite mass and increase mastication	Newman *et al.* (1994)
Previous experience	Supplement Licks	Presence of alkaloids, condensed tannins, glucosinolates	Provenza *et al.* (1990) Duncan and Milne (1993) Thompson and Stuedemann (1993)

digestion and *in situ* measures of degradability to predict intake. Parameters used have included gas volumes or DM disappearance at specific times during the fermentation, as well as parameters derived from fitting curves to the gas produced. Blümmel *et al.* (1997) calculated a partitioning factor (PF), reflecting the variation of short chain fatty acid production per unit of substrate degraded and showed this to account for 11% of the variation in DMI. Table 3.2 shows that many authors

Table 3.2. Review of the use of *in vitro* and *in situ* parameters in intake prediction equations.

Authors	Feed type	*In vitro* gas production or *in situ* degradability	Parameters used*	DM intake	R^2
Blümmel and Becker (1997)	54 straws and legume-hay-supplemented straws	*In vitro*	*a*, *b*, *c* Whole substrate	g kg^{-1} LW	0.747
			*a*NDF, *b*NDF, *c*NDF NDF fraction	g kg^{-1} LW	0.822
Blümmel *et al.* (1997)	As above	*In vitro*	*a*, *b*, *c* and PF	g kg^{-1} LW	0.838
Kibon and Ørskov (1993)	5 browses	*In situ*	$A + B + c$	kg day^{-1}	0.99
Ørskov *et al* (1988)	5 straws	*In situ*	$0.572 + 0.0766(a + b)$ $-0.822 + 0.0748(a + b) + 40.7c$	kg day^{-1}	0.83 0.89
Khazaal *et al.* (1993)	9 legume hays + 1 grass hay	*In vitro* *In situ*	$-47.7 + 4.25a + 2.12b + 444.5c$ $10.3 + 0.53A + 0.70B + 199.4c$	g DM kg^{-1} $LW^{0.75}$ g DM kg^{-1} $LW^{0.75}$	0.630 0.776
Khazaal *et al.* (1995)	10 grass hays	*In vitro* *In situ*	$18.9 - 0.23(a + b) + 687c + 0.11CP$ $5.4 + 0.34(a + b) + 342c + 0.119CP$	g DM kg^{-1} $LW^{0.75}$ g DM kg^{-1} $LW^{0.75}$	0.947 0.917
Blümmel and Bullerdieck (1997)	9 legume hays + 10 grass hays	*In situ* *In vitro* *In situ*	$(a + b)$, c, PF_{48} $(a + b)$, c, PF_{24} $(a + b)$, c, PF_{48}	g kg^{-1} $LW^{0.75}$ g kg^{-1} $LW^{0.75}$ g kg^{-1} $LW^{0.75}$	0.597 0.744 0.597
Ferret *et al.* (1997)	11 maize silages	*In situ*	$-81.21 + 0.21a + 0.22B$	g kg^{-1} $LW^{0.75}$	0.932

*$B = (a + b) - A$ (soluble fraction) = potentially degradable insoluble fraction; *a*, *b* and *c* are constants in the equation $p = a + b(1 - e^{-ct})$, where p = DM degradation or gas production at time t; PF_x is the the partitioning factor reflecting the variation of short-chain fatty acid production per unit of degraded substrate after x h of incubation.

Table 3.3. Some feed parameters used for predicting intake.

	Parameter	Example references
Chemical composition	ADF, NDF	Van Soest (1965)
In vitro digestibility	Gas volumes DM disappearance Rate of digestion	See Table 3.2
Physical structure	Leaf proportion Bulk density Grinding energy Short term intake rate	Minson (1990) Minson (1990) Minson (1990) Romney *et al.* (1998)
NIRS	Spectral	Steen *et al.* (1995)

NIRS, near-infrared reflectance spectroscopy.

have shown high correlations between intake and *in vitro* or *in situ* parameters. However, it should be noted that, in most cases, groups of similar feeds were used. Romney *et al.* (1998) showed that, although high R^2 values between *in vitro* gas production and intake were observed for a range of hays and straws, poor correlations were observed when a wider range of feed types were examined. Discrepancies in predicted intake may relate to the effect of particle size and the physical structure of plant cells, which are not taken into account when ground samples are used. Cherney *et al.* (1988) showed that digestion rates increase as grind size decreases and Allen (1996) suggests that this may result in an overestimation of DMI when *in vitro* or *in situ* digestibility rates of ground samples are used as a predictor. This observation may suggest that some description or measure of physical structure and ease of particle-size breakdown should be used.

Measures of physical structure

Minson (1990) discussed a range of physical measurements that have been related to intake, including leaf proportion, bulk density and grinding energy. An alternative method proposed by Moseley and Manendez (1989) is the use of intake rate measured over 1 min periods. Romney and Gill (1998) have developed the idea further and have considered the use of short-term intake rate (STIR) values as a predictor of *in vivo* parameters, including intake, digestibility and rate of passage. It might be expected that STIR would be closely related to rate of passage and therefore intake, where physical structure and rate of passage were among the principal determinants of intake. These authors found that values were highly correlated with *ad libitum* intake of a range of feeds, when sugar-beet pulp, with a high level of water-soluble carbohydrates, was excluded.

Near-infrared reflectance spectroscopy (NIRS)

Work by Barnes *et al.* (1989) and Barber *et al.* (1990) has resulted in the establishment of standard procedures for the use of near-infrared (NIR) spectra to predict OM digestibility of silage. More recently, Steen *et al.* (1995) have reported R^2 values of 0.86 between NIR spectra and intake of individually fed beef cattle for 136 silages, and Givens and Gill (1998) suggested that NIRS shows potential for application to more complex situations, such as intake responses to forage combinations.

Models

Most of the prediction equations discussed above are based on empirical relationships between feed parameters and potential intake. In most cases, accuracy of prediction depends on the data sets used to develop them (Poppi, 1996) and, normally, they are only reliable when used to predict intake for similar feeds to those used in developing the equations.

Mechanistic models are useful for increasing the understanding of the underlying mechanisms and testing assumptions. If they are able to account for all factors predicting intake then they might be expected to be able to provide much improved predictions. However, they are mainly focused on explaining observed behaviour, rather than predicting what will happen under an unexplored set of circumstances. Few are able to provide useful predictions of intake at present. Even in models, often, the effects of a range of controlling influences are determined and the lowest prediction of intake taken as the correct one (Forbes, 1977; Poppi *et al.*, 1994) and few attempt to embrace the principles of integration. The inputs required are often complex and they remain primarily a research tool.

Manipulation of Intake

Strategies or technologies to alter the intake of animals will depend on the circumstances limiting intake and whether plant or animal factors or a combination of both are the first limiting. An assessment of whether the response in terms of production justifies the cost of improving intake must also be taken into account.

Low-quality roughages

Where low-quality feeds form the major proportion of the diet, intake is constrained by the rate at which the fibre is digested and passes through the gastrointestinal tract. For example, stovers or straws, which are fed particularly during the dry season in the tropics, tend to have a high fibre and low N content, which may limit the rate of fibre digestion. While supplementation with better-quality feeds can increase total intake, as referred to above, treatment of the basal feed itself or the way in which it is fed can also influence intake. Simple physical methods to increase intake include increasing the amount of forage on offer, to allow the

animal to select the more palatable or nutritious parts, thereby increasing the quality of the diet consumed as well as intake (e.g. Fernandez-Rivera *et al.*, 1994). Chopping of forage can also have beneficial effects in some cases, but Osafo *et al.* (1997), for example, reported positive effects on intake of chopping sorghum stover in sheep, but not in cattle. Chemical treatment with acid or alkali to break down fibres, with a view to increasing digestibility and therefore intake, has been tried and urea-ammonia treatment has received much interest in the developing world (e.g. Schiere and Ibrahim, 1989). However, use of the technology has been limited, for a variety of reasons (largely economic), except in China, where there has been a recent upsurge in its use (Finlayson, 1993).

Grazing systems

In grazing systems, stocking rate (the number of animals per hectare) can be manipulated to change the intake per animal and sward structure can be managed to maintain a height and density that maximizes intake, as referred to above.

Animal management

The intake of animals also depends on whether they are fed individually or in groups. Experiments to measure intake are often conducted with animals offered feed in individual pens or crates. In practice, farm animals are generally kept in groups and social factors, such as dominance order, can affect average intakes. In many situations, there is insufficient access to feed to enable all animals to have *ad libitum* access at the same time. Intake can be increased by simple husbandry methods to allow all animals adequate access and reduce wastage through soiling.

Manipulation of plant structure through breeding

New techniques of plant breeding mean that the 'ideal' plant can be bred in a much shorter time-scale than previously. The question remains, however, as to what constitutes the 'ideal'. Less fibre may theoretically increase intake, but this may not be ideal if the structure of the plant is changed in such a way as to reduce the efficiency of harvesting.

Examples of manipulating intake are given in Table 3.4.

Future Research

Research on intake is continuing at a number of levels, from metabolic studies, with the objective of identifying metabolites and hormones that initiate or inhibit intake to feeding experiments, to measure the intake of particular feeds under specific conditions. However, neither of these extremes are likely to produce major breakthroughs that will increase our ability either to predict or to manipulate

Table 3.4. Some examples of means of manipulating intake of poor-quality forages.

Manipulation	Mode of action	Reference
Supplement with small amounts of N	Increases rumen digestion and rate of passage where microbial fermentation is limited by N supply	Minson (1990) Leng (1990)
Increase offer rate	Allows animal to select more digestible portions	Fernandez-Rivera *et al.* (1994)
Chopping	Decreases particle size, increasing rate of passage. Can be ineffective if selection becomes constrained	Osafo *et al.* (1997)
Treatment with acid or alkali	Increases fibre digestibility	Schiere and Ibrahim (1989)

forage intake. Increased understanding of forage intake is more likely to arise from studies of factors influencing the rates of digestion and passage and of how nutrient requirements of ruminants and hence 'hunger drive' influence the rate at which an animal is prepared to eat.

At present the emphasis lies very strongly on experiments to study rates of digestion and there is very little research in the UK on factors affecting the passage of digesta down the tract. The explanation for this unbalanced emphasis lies in the increased simplicity of methods that measure rates of digestion. Nevertheless, interpretation of the results is not so simple without a better understanding of rates of passage. There is some evidence (Gill *et al.*, 1999) that fractional rates of passage are not the same for all components that move with the solid fraction and that they are not constant within any 24 h period, and this may have an impact on the pattern in which energy is supplied to the tissues. However, further research is needed to confirm initial findings. Further research on how forages and supplements interact and how mixtures of forages interact in the rumen and beyond would also help to improve ration formulation.

It is now more generally accepted that animals eat to meet their energy requirements, but there is less understanding of how the drive to meet energy requirements initiates and inhibits eating bouts, particularly in the grazing situation. This may be especially important for the development of management systems for feeding the high-genetic-merit dairy cow.

Many other research objectives could be proposed, but, in the current funding climate, proposals must be targeted at the funders' strategic objectives and there is insufficient space to list those here. Suffice it to say that there is a sound base for understanding the control of forage intake, as described above. Extrapolations can be made from existing knowledge to answer questions relating to unconventional feeds or management systems with different goals, although the accuracy of predicting

outcomes will be less than for feeds that have already been studied in detail. Future research should aim to generate information that will provide farmers with sufficient information to enable them to make management decisions in a world which, in economic and climatic terms, is changing more rapidly than ever before.

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4 Prediction of Energy Supply in Ruminants, with Emphasis on Forages

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Introduction

Energy and protein evaluation systems have been developed virtually independently. The systems use single descriptors of the energy or protein values of feeds for ruminants and assess animal requirements in units of the corresponding descriptor. The use of a single value for each feed, the additivity of the values when several feeds are used in a ration and the factorial approach to balance the estimated requirements of the animal for a target level of production and the nutrient supply from the diet are the main characteristics of the feed evaluation systems (Alderman, 1983; Van der Honing and Steg, 1990), with the aim to be as simple as possible in order to facilitate practical application. However, the simplicity of the systems may be in conflict with accuracy of prediction of animal performance over a wide range of variation of rations, and an appropriate modelling of the underlying physiological processes in the ruminant may be required. This chapter will focus on developments in the energy evaluation of feeds, particularly forages, with attention given to the role of *in sacco* and *in vitro* methods, in combination with mechanistic mathematical modelling, in predicting energy supply to the animal.

Predicting Energy Value and Digestibility in Current Feed Evaluation Systems

Current energy systems for ruminants are based either on metabolizable energy (ME) (CSIRO, 1990; AFRC, 1993) or on net energy (NE) (Van Es, 1978; Vermorel *et al.*, 1987; INRA, 1988; NRC, 1989, 1996). In the Agricultural and Food Research Council (AFRC) and CSIRO systems, a single value of the ME content (called M/D, in MJ ME kg^{-1} dry matter (DM)) is used for each feedstuff.

 Forage Evaluation in Ruminant Nutrition (eds D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed)

This value is determined for animals fed at maintenance levels of intake. The Dutch, National Research Council (NRC) and INRA feeding systems use indices of the energy value of feeds relative to a net production output. The determination of the NE value of a feed requires the measurement of the energy balance at two or more levels of intake, and the increment in NE gain resulting from an increment in the intake of the feed is commonly expressed as MJ kg^{-1} DM. However, no feed can be given a single NE value, because the value varies with the purpose for which energy is used by the animal (ME is used with different efficiencies for maintenance, growth and lactation).

The predictive capability of all energy systems relies, largely, upon the energy values assigned to each feed, as the animal requirements can be determined with an acceptable level of accuracy. Energy values (either in ME or in NE) and derived efficiencies of energy utilization have to be determined in balance trials, in which energy inputs (energy intake) and outputs (energy losses in faeces, methane, urine and heat produced) are measured (see Chapter 5). However, energy balance trials are expensive and cannot be conducted for all the forages and major bulk ingredients included in ruminant feeds; thus the energy value must be estimated from other variables.

The determination of feed digestibility is simpler than the determination of energy values in balance trials. Digestibility has been recognized as the main source of variation of the ME content of forages, given the large variability of their digestibility coefficients (values ranging from 0.35 to 0.80) (Minson, 1990; Hvelplund *et al.*, 1995) and the assumed relative constancy of the proportion of energy lost in urine and methane (19% of the digestible energy) (Tyrrell and Moe, 1975; CSIRO, 1990). On the assumption that, for most forages other than silage, the gross energy of the digestible organic matter (OM) is nearly constant, the Ministry of Agriculture, Fisheries and Food (MAFF, 1984) proposed the use of the digestible OM content of feed DM (DOMD or D value) as a useful index of M/D. But digestion trials to measure digestibility *in vivo* are also expensive and are not readily applicable to large numbers of feeds or when small quantities of each forage are available. Thus, the prediction of feed digestibility or energy values from compositional or *in vitro* information has become a necessity in all feeding systems. A predictor of forage nutritive value is a quality-related characteristic of the forage, which can be measured in the laboratory by simple methods. Using analytical results and energy values determined by feeding trials for a number of standard representative feeds, multiple regression equations can be derived statistically and used to predict the energy value of other samples.

A large number of different equations for predicting OM digestibility or energy value of forages from a variety of explanatory variables is provided in the literature (Van Es, 1978; Andrieu *et al.*, 1981; Minson, 1982, 1990; Barber *et al.*, 1984; Andrieu and Demarquilly, 1987; CSIRO, 1990; Givens *et al.*, 1990, 1992; AFRC, 1993; NRC, 1996). In these equations, forage digestibility (Table 4.1) or energy value are predicted from: (i) botanical features (including leafiness, botanical composition, growth or maturity stage), also considering the harvesting and preservation conditions; (ii) chemical composition; (iii) physical properties (density, spectrum for near-infrared reflectance); and (iv) *in vitro* measurements of digestibility (using either buffered rumen fluid or enzymatic solutions) (Minson, 1990; Hvelplund *et al.*, 1995).

Table 4.1. Ranges in correlation coefficients and in residual standard deviations (RSD)* associated with the prediction of *in vivo* digestibility from various predictors.

	Range in correlation coefficient	Range in RSD (%)
Chemical composition		
Crude Protein	+0.44 to +0.79	2.0 to 6.5
NDF	−0.45 to −0.80	2.4 to 5.1
ADF	−0.75 to −0.88	3.6 to 9.0
Lignin	−0.61 to −0.83	4.3
Alternative methods of estimating digestibility		
In vitro digestibility†	+0.94	1.6
Enzymatic	+0.94 to +0.99	1.8 to 3.2
In situ	+0.98	1.0 to 3.0
NIR	+0.95	0.6 to 2.7

* Data combined from Minson (1990), Carro *et al.* (1994) and Van Soest (1994).
† Tilley and Terry (1963).

Most of these equations are based purely on the statistical relationship between variables and the performance of regression methods facilitated by improved computing facilities, resulting sometimes in equations without biological meaning. One of the consequences of this empirical approach is that the large number of equations available in the literature differ significantly in the predicting variables, in the regression coefficients for the same predictors and in the estimated prediction error. Diverse equations have been obtained for separate classes of feeds and forages, and different laboratories may use different equations for the same combination of predicted and explanatory variables. These empirical prediction equations are a consequence of the specific data set from which they have been derived, so that they are only useful when the situation to be predicted is similar to the original data set. Thus, these equations have a variable degree of unreliability, due to environmental and species variation, interactions among plant species in mixed forages and the difficulty in accounting for basic cause–effect relationships between ruminal digestion and plant composition (Chesson, 1993; Van Soest, 1994). Any error in estimating digestibility is passed along to, and constitutes the largest error in, the final estimation of the energy value of the forage. Despite these criticisms, empirical equations are widely used in feed evaluation systems.

Towards New Approaches in Feed Evaluation

Because of the extensive research on feed evaluation carried out over the last decades, tables of the nutritive value of the forages mostly used in ruminant nutrition are available. These values have been either determined experimentally or predicted using empirical equations with an acceptable degree of precision. Conceptually, though, current evaluation systems have serious limitations, which have been generally recognized (Beever and Oldham, 1986; Gill *et al.*, 1989; Tamminga, 1992; Van Soest, 1994; Reynolds and Beever, 1995; Hanigan *et al.*, 1997).

Variation in energy supply

The first drawback of current systems is when energy content of a feed per unit of mass is used as its energy value, since this accounts for only a part of its potential to contribute to the nutrient supply of the animal and to influence nutrient utilization. Single energy values for a feed quote static conditions, whilst the energy supply from each unit of feed depends on a number of interrelated factors, including site of energy digestion, level of intake and interactions between individual feed substrates.

Site of energy digestion

When considering the major sites of energy digestion in ruminants, the digestive tract can be divided into three sections (Fig. 4.1): the reticulorumen, where digestion is by microbial fermentation; the abomasum and small intestine, where mammalian digestion by host enzymes predominates; and the large intestine, where the caecum is an important site of fermentation (although secondary to the reticulorumen). Microbial fermentation yields volatile fatty acids (VFA), together with

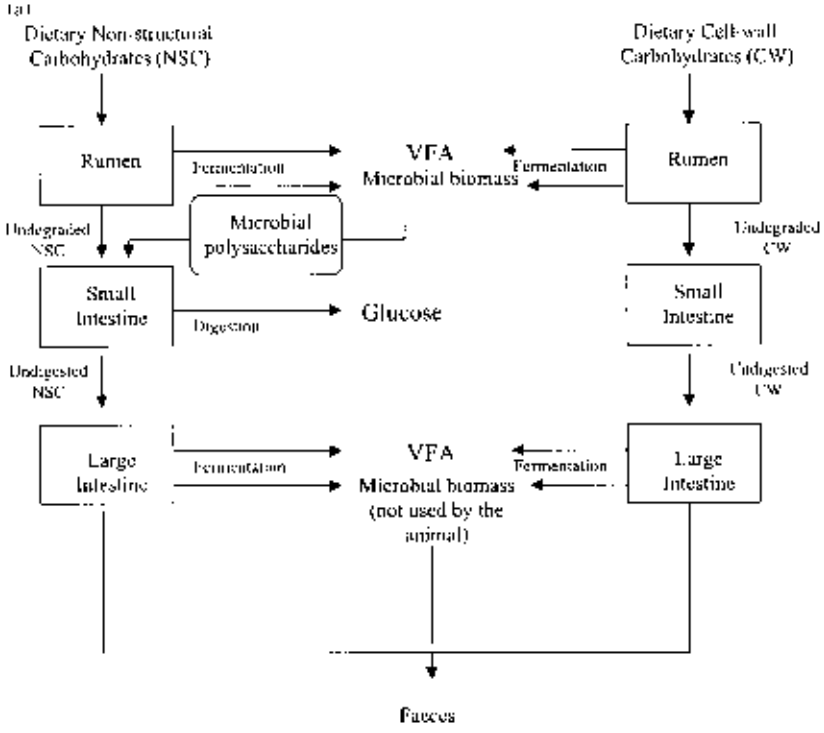
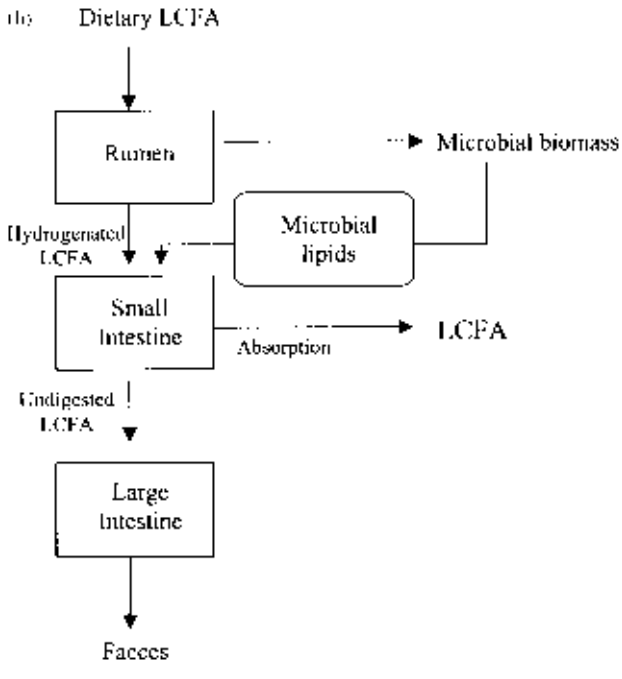
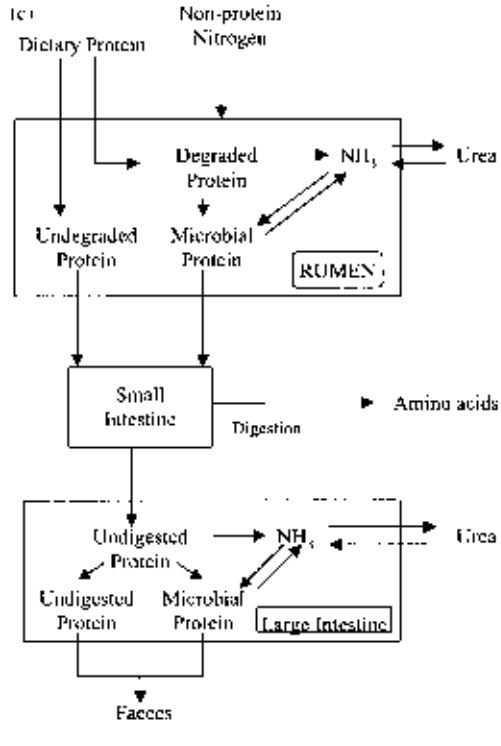


Fig. 4.1(a) (b and c opposite) Schematic representation of the digestion of (a) dietary carbohydrates, (b) long-chain fatty acids (LCFA) and (c) nitrogenous compounds by ruminants, showing the end-products of the digestion process at each site of the digestive tract. NH_3, ammonia.


(b)
Dietary LCFA
Rumen
Microbial biomass
Hydrogenated LCFA
Microbial lipids
Small Intestine
Absorption
LCFA
Undigested LCFA
Large Intestine
Faeces


(c)
Dietary Protein
Non-protein Nitrogen
Degraded Protein
NH_3
Urea
Undegraded Protein
Microbial Protein
RUMEN
Small Intestine
Digestion
Amino acids
Undigested Protein
NH_3
Urea
Undigested Protein
Microbial Protein
Large Intestine
Faeces

methane and fermentation heat, which have to be discarded when calculating ME from digestible energy (DE). A carbohydrate may be fermented in the rumen to VFA or digested in the small intestine to glucose, resulting in a different supply of energy from the same initial nutrient (Nocek and Tamminga, 1991) (Table 4.2). An increase in the molar proportion of propionate results in lower fermentation heat and methane losses, although the ratio between the latter remains almost constant (Blaxter, 1989). VFA molar proportions in the rumen from a given feed may differ widely with factors such as microbial growth rate, available substrates, level of feed intake and rumen pH (Dijkstra, 1994). Hence, the fixed factor used in many systems to calculate ME from DE (0.81) is variable (Fig. 4.2).

Level of intake and energy supply

The relationship between energy balance and ME intake is curvilinear, showing a diminishing-returns pattern, though a piecewise linear approximation is generally used (Fig. 4.3). Corrections to feed energy value for the effect of level of intake have been incorporated into some systems (Van Es, 1978; NRC, 1989, 1996; AFRC, 1993), whereas in others it is accepted that the systems have been specifically designed for certain feeding conditions, such as a fixed animal level of production, in which the range of intake levels is not large (INRA, 1988). The regression equations used in many energy systems have been developed from feeding experiments conducted largely with wethers at maintenance levels of feeding, and the validity of corrections for feed intake level for beef and dairy cattle based on sheep data has been questioned. Tyrrell and Moe (1975) reported for mature sheep limited variation of OM digestibility of four contrasting diets based on maize (88 to 92% of

Table 4.2. Differences in the supply of substrates from the gastrointestinal tract associated with changes in the site of digestion of dietary carbohydrates in dairy cows (from Sutton, 1985).

Cereal in the concentrate	Rolled barley	Ground maize
DE intake (MJ day^{-1})	164	158
Proportion of DE digested in the rumen	0.64	0.57
Starch intake (kg day^{-1})	5.75	6.37
Proportion of starch digested		
In whole tract	0.99	0.89
In rumen	0.90	0.67
Nutrient supply (MJ day^{-1})		
Acetate + butyrate	54	55
LCFA	10	14
Amino acids	31	27
Propionate	44	28
Glucose	7	14

LCFA, long-chain fatty acids.

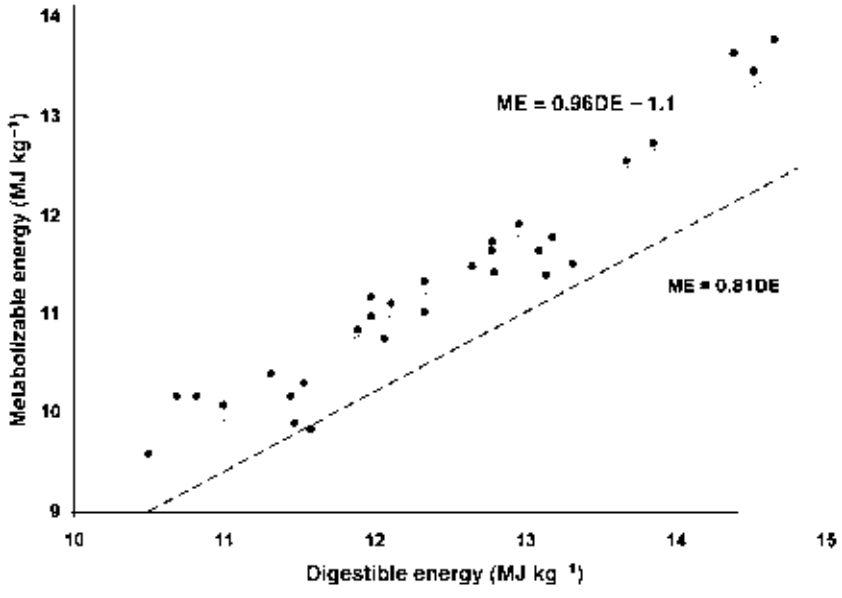


Fig. 4.2. The relation of metabolizable energy (ME) to digestible energy (DE) (adapted from Moe *et al.,* 1972). The commonly assumed factor 0.81 (dashed line) underestimates true ME, which is relatively greater for more digestible feeds.

intake), whilst the same diets fed to cattle resulted in OM digestibilities between 62 and 79% of intake. This clearly indicates the possible errors when extrapolating data on intake levels between species. Next, the use of a fixed correction for intake level is of particular concern in view of interaction between intake level and type of feed (Sutton, 1985). For example, ruminal starch digestion of barley diets decreased with an increase in level of feed intake in dairy cattle (91 and 89% at maintenance and three times maintenance, respectively), but this decrease in ruminal starch digestion was much more pronounced with maize diets (81 and 72% at maintenance and three times maintenance level, respectively) (Sutton, 1980).

Interactions between feed components

Important interactions between feed components exist which cannot be represented by a single energy value (Table 4.3). All the systems fail to consider the associative effects between feeds in a mixed ration, as they use the same energy value for a given feed, whether it is fed alone or in combination with others. However, an adequate combination of ingredients in the ration will result in a significant improvement in the supply of energy to the animal in relation to that obtained by each feed alone, probably due to an enhanced rumen fermentation or a higher efficiency of energy utilization. In contrast, the presence of high quantities of easily degradable soluble sugars and starch in forage or forage supplements will depress fibre degradation in the rumen, owing to a drop in rumen pH due to the accumulation of VFA (Tamminga and Van Vuuren, 1988). Some energy systems endeavour to correct the

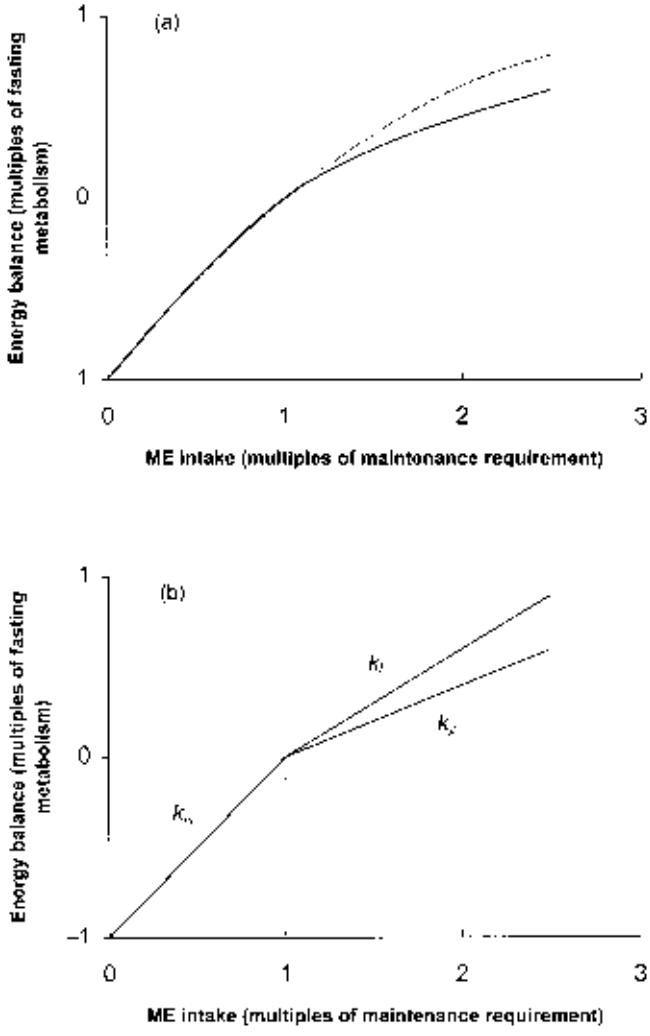


Fig. 4.3. Change in energy balance of the animal in relation to its metabolizable energy (ME) intake. Maintenance is the ME intake that results in zero energy balance. In (a) the dotted line is for a lactating animal whereas the solid line is for a growing animal. (b) A simplified representation of the non-linear relationship using piecewise linear models. The slopes of the lines represent the net efficiency of use of ME for maintenance (k_m), for lactation (k_l) and for growth and fattening (k_g). (Adapted from Blaxter, 1962.)

energy value in the presence of high dietary levels of non-fibre carbohydrates (Van Es, 1978). However, the effect of dietary non-fibre carbohydrates on fibre degradation appears to vary from one feed source to another. For example, replacing maize with barley was shown to have a negative effect on fibre degradability in dairy cattle (DePeters and Taylor, 1985). A possible interaction between carbohydrate

Table 4.3. Associative effects between feeds: influence of forage-to-concentrate ratio on the supply of energy-yielding substrates from the gastrointestinal tract (from Sutton, 1985).

Hay:concentrate	40:60	10:90
DE intake (MJ day^{-1})	157	164
Proportion of DE digested in the rumen	0.67	0.64
Nutrient supply (MJ day^{-1})		
Acetate + butyrate	66	54
LCFA	9	10
Amino acids	28	31
Propionate	24	44
Glucose	3	7

LCFA, long-chain fatty acids.

degradation in the rumen and the availability of rumen-degradable protein and non-protein nitrogen may occur because of the ammonia and branched-chain fatty-acid requirements of fibrolytic microbes. The major supply of ammonia and branched-chain fatty acids is through degradation and fermentation of protein. In dairy cattle, Sutton (1986) estimated an increase of 13 g kg^{-1} in fibre degradation per 10 g kg^{-1} DM increase of crude protein. Hence, the energy value of the feed depends on the whole diet and a single energy value will not necessarily suffice.

Animal requirements versus animal responses

An often recognized weakness of current energy systems relates to the prediction of animal responses (Beever and Oldham, 1986; AFRC, 1991; Reynolds and Beever, 1995; Hanigan *et al.*, 1997). The systems aim to balance the energy requirements of an animal by an appropriate supply of energy in the diet. As such, no attempt is made to consider how animal performance will respond to deliberate changes in the diet. Also, the systems are unable to predict body and milk composition. This is a major drawback in view of the demand for ruminant products of specified compositional standards by industry and consumers, as well as the need to reduce losses to the environment. Without due recognition of the response in production level and product composition to deliberate perturbations in nutrient supply, marginal cost response analysis is not possible.

Composition of the energy-yielding substrates

Energy has been considered as the primary driving force for animal production and dietary protein supply as permissive in allowing the influences of energy supply to be expressed (Thomas, 1990). Therefore, most feeding systems use distinct and independent energy and protein schemes. However, by using single and static

descriptors of energy (and protein), the present systems do not consider the composition of the energy- (or protein-) yielding substrates that make up the ME or NE supply (Beever and Oldham, 1986; AFRC, 1991; Reynolds and Beever, 1995). Animals do not metabolize energy (or protein) *per se*, but rather substrates and metabolites derived from the gastrointestinal tract. The availability of these substrates is also influenced by interactions between nutrients at specific sites of digestion and metabolism in different organs and tissues (MacRae *et al.*, 1988). The nature and amount of these available substrates determine how they are used and the proportion of energy they contain that is directed to the various production processes (Table 4.4). For example, Dijkstra *et al.* (1996) evaluated the supplementation of sugar-cane-based diets and simulated the availability of total energy and glucogenic, lipogenic and aminogenic nutrients for milk production (Fig. 4.4). These researchers showed that, in particular feeding situations, dietary energy did not limit milk production, but, rather, specific nutrients (lipogenic or aminogenic) were in short supply, a result that is impossible to predict using current feed evaluation systems.

The Mechanistic Approach

New feeding systems need to be based on the mechanisms that govern the response of animals to nutrients, dealing with quantitative aspects of the digestion and metabolism in the ruminant animal. From the drawbacks of current systems previously described, new mechanistic systems of feed evaluation need to represent the individual nutrients and substrates in organs and tissues, including the major interactions between nutrients and substrates and between organs and tissues. This is where mechanistic mathematical modelling can be applied to represent concepts and mechanisms quantitatively (France and Thornley, 1984; Baldwin, 1995;

Table 4.4. The calorimetric efficiencies of utilization for maintenance and growth of some end-products of ruminant digestion, given to sheep by constant rumen infusion (except values in brackets which are for substrates given by continuous infusion into the abomasum) (from Blaxter, 1962).

Substrate	Efficiency for maintenance	Efficiency for fattening*
Acetate	0.592	0.329
Propionate	0.865	0.563
Butyrate	0.764	0.619
Acid mixture 1†	0.872	0.581
Acid mixture 2‡	0.856	0.318
Glucose	0.940 (1.00)	0.545 (0.715)
Casein	(0.820)	(0.647)

* Increase in energy retained 100 kJ^{-1} of additional ME.
† Mixture 1 contained (mol^{-1} of total VFA): 250 mmol acetate, 450 mmol propionate and 300 mmol butyrate.
‡ Mixture 2 contained (mol^{-1} of total VFA): 750 mmol acetate, 150 mmol propionate and 100 mmol butyrate.

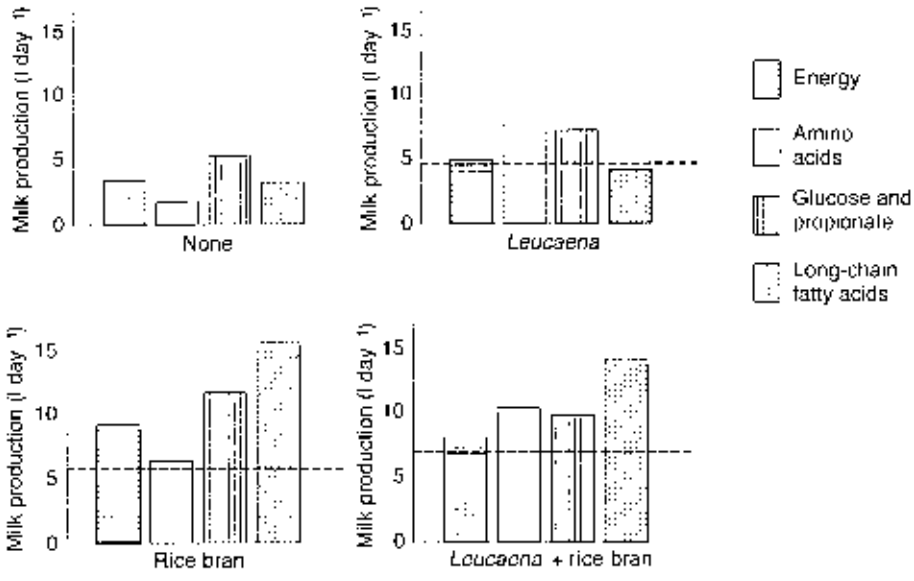


Fig. 4.4. Potential milk production according to simulated availability of energy, amino acids, glucose and propionate and long-chain fatty acids on unsupplemented basal diet (sugar cane and 40 g urea kg^{-1} sugar cane DM), basal diet supplemented with *Leucaena*, basal diet supplemented with rice bran, basal diet supplemented with *Leucaena* and rice bran. Simulations were performed using the model described by Dijkstra *et al.* (1996) and the dashed line represents average milk production observed with Brown-Swiss × Zebu cattle (Alvarez and Preston, 1976; Alvarez *et al.*, 1978).

Dijkstra and France, 1995). These models are constructed by looking at the structure of the system, dividing that structure into its key components and analysing the behaviour of the whole system in terms of its individual components and their interactions with one another.

The accuracy of prediction of animal response using mechanistic models may currently be lower than that achieved by the empirical methods used in practical application; hence, the use of the former models has been constrained mainly to research. However, these research models are very useful in evaluating the adequacy of current knowledge and data, identifying those areas where research efforts should be focused. In contrast with extant models for feed evaluation, new research findings can be easily incorporated and integrated into models constructed mechanistically, which will improve their ability to predict nutrient utilization and availability within the digestive tract (Dijkstra and France, 1995; Hanigan *et al.*, 1997). Therefore, many mechanistic models have been built on the achievements of previously reported models, introducing additional knowledge and detail, based upon newly emerging experimental information and specific failures of previous models to simulate reality (Baldwin, 1995). With this approach, it is expected that, in the future, mechanistic models will yield superior predictions of animal performance and will be more generally applicable than empirical models.

The Roles of *In Situ* and *In Vitro* Methods

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* results are restricted to the experimental conditions under which measurements are carried out. These trials cannot be considered routine in most laboratories and cannot be carried out for all the possible feeding situations found in practice. *In vitro* and *in situ* techniques allow manipulation of parameters defining the state of the animal. These techniques, if properly evaluated against *in vivo* observations, can be appropriate for studying the response of the animal, when one factor is varied and controlled, without the interaction of other related factors that could conceal the main effect. Examples of these techniques include the use of cultures to establish microbial growth yield and efficiency (for a review, see Dijkstra *et al.*, 1998a) and the use of *in vitro* rumen epithelium-cell cultures to study absorption and metabolism of VFA (reviewed by Bergman, 1990). Also, a number of *in vitro* and *in situ* methods have been developed to estimate the digestibility and extent of ruminal degradation of feeds and to study their variation in response to changes in rumen conditions. Thus, *in vitro* and *in situ* techniques may be used to study individual processes, providing information about their nature and sensitivity to various changes. This information is of great importance in the development of mechanistic models. Mechanistic modelling can be used to derive kinetic parameters from data obtained *in vitro* or *in situ*, which can then be incorporated into integrated models to simulate the whole system behaviour. An appropriate model can be a useful tool for linking the data obtained *in vitro* or *in situ* with the processes occurring in organs and tissues *in vivo*.

Predicting events in the rumen

Rumen function involves complex, dynamic interactions between numerous components and processes. Of crucial importance to the supply of energy-yielding nutrients from the ruminant alimentary tract is the presence of a microbial population in the rumen, which serves to degrade nutrients, with the associated production of VFA, and supplies a substantial part of the protein absorbed from the small intestine. Thus, regarding the rumen, major consideration should be given to quantification of the rate and extent of substrate degradation, of the kinetics of microbial growth and synthesis and of the amount and ratio of the different VFA produced and absorbed. Although these aspects will be discussed in separate sections, it is important to realize that there are important interactions between them, which must be represented in whole-rumen models.

Rate and extent of degradation

Kinetic degradation parameters are necessary for the prediction of feed digestibility and thereby the energy available in various circumstances. The amount of substrate degraded in the rumen is the result of the 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. 4.5) obtained using either the polyester-bag technique or the gas-production technique. In the first case, a time-course disappearance curve for each feed component is obtained *in situ* by measuring the amount of the remaining residue in the bag at several time points (see Chapter 9). Similar gravimetric methods have been employed in which the feed is incubated *in vitro* either with buffered rumen fluid (Chapter 7) or with enzymes (Chapter 8). Disappearance curves are used to evaluate the kinetics of degradation of feeds in the rumen, by assuming that disappearance from the bag equals degradation of feed in the rumen. The gas-production technique (Chapter 10) aims to measure the rate of production of fermentation gases that can be used to predict the rate of feed degradation, assuming that the amount of gas produced reflects the amount of substrate degraded.

To associate disappearance or gas-production curves with digestion in the rumen, models have been developed based on compartmental schemes and 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 (h^{-1}), after a discrete lag time T (h). The scheme is shown in Fig. 4.6, and the dynamic behaviour of the fractions is described by the differential equations:

$$dS/dt = 0 \qquad 0 \leq t < T \tag{1a}$$

$$= -\mu S \qquad t \geq T \tag{1b}$$

$$dU/dt = 0 \qquad t \geq 0 \tag{2}$$

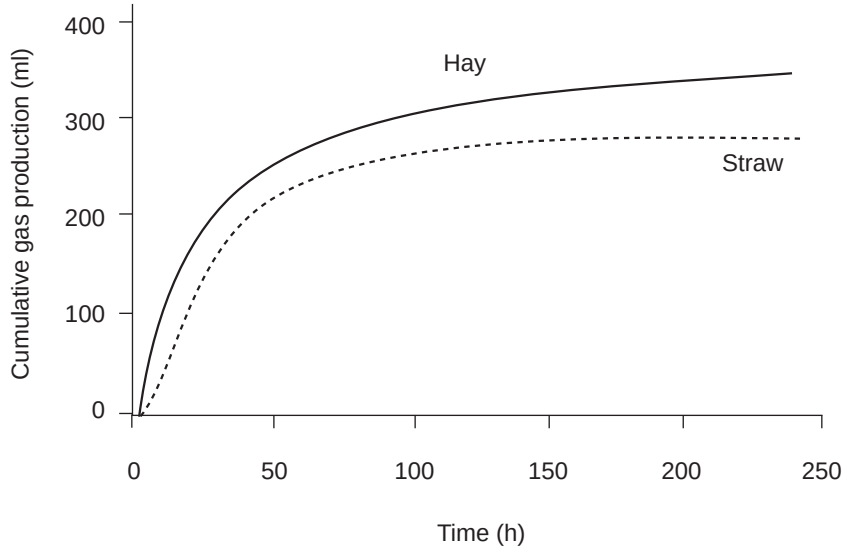


Fig. 4.5. Examples of sigmoidal and non-sigmoidal cumulative gas-production curves *in vitro*.

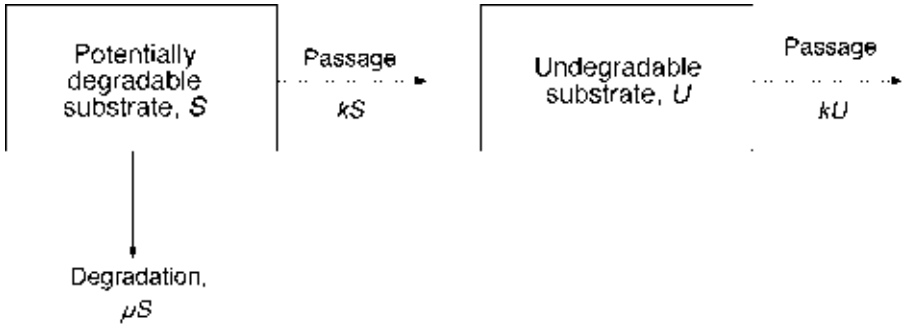


Fig. 4.6. The two-compartment model of ruminal degradation. Deletion of the dashed arrows gives scheme for disappearance during incubation *in vitro* or *in situ*.

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

Estimation of *U* is critical to the 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 sacco*, or estimated from non-linear fitting of degradation profiles. When degradation profiles are obtained by the polyester bag technique, the non-fibre components are assumed to contain a third fraction, called the soluble fraction (wash value, *W*). *W* disappears from the bag immediately after incubation begins and is assumed to be degraded instantly in the rumen. This simplification has been adopted because the technique is unable to measure the rate at which the *W* fraction would actually be degraded. Recently, some degradation rate values for this fraction, albeit very fast, have been reported (Weisbjerg *et al.*, 1998), and these could be incorporated in the models so that the portion of *W* escaping ruminal degradation can be taken into account. The loss of undegraded particulate matter from the polyester bag leads to overestimation of *W* and underestimation of the undegradable fraction. Estimation can be significantly improved by measuring the extent of the particle loss from the bag and applying mathematical corrections to the parameter estimates (López *et al.*, 1994a; France *et al.*, 1997). Using *in vitro* techniques allows degradation profiles with many 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). Such models contain a considerable number of parameters, requiring a large number of data points and complicating satisfactory parameter estimation, due to the limitations of the non-linear regression. On the other hand, experimental techniques may result in errors that have an important effect on the fractionation of feed components.

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 enzyme 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 the 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 energy supply to the animal from a given feed, because it can have significant effects on both the ruminal microbes (efficiency of microbial synthesis, changes in rumen environment and effects on relative numbers of microbial species and on cellulolytic activity) and the host (molar composition of VFA produced, VFA absorption rate, amino acid supply postruminally and feed intake). The fractional degradation rate can be considered an intrinsic characteristic of the feed, depending on factors such as the forage chemical composition, 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 the rate of particle-size reduction by rumination and microbial activity and ruminal conditions (pH, osmotic pressure, mean retention time of the digesta), which have a profound effect on microbial degradative activity. Associative effects of feeds in the diet can become very important. For example, the depressive effect of easily degradable non-fibre carbohydrates on the degradation rate of forage DM is generally recognized (Tamminga and Van Vuuren, 1988).

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. 4.7), and that only characteristics of the substrate limit degradation. This model has been extensively used, owing to its simplicity, but it is not capable of describing the large diversity of degradation profiles (see Fig. 4.5) which have been observed (Dhanoa *et al.*, 1995) and it 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 (France *et al.*, 2000) – for example, the generalized Mitscherlich model, the generalized Michaelis–Menten model and standard functions, such as the Gompertz and logistic (Fig. 4.7). 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. On substituting for the function proposed for μ and integrating, equation (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:

$$S = S_0 \times [1 - \Phi(t)] \quad (3)$$

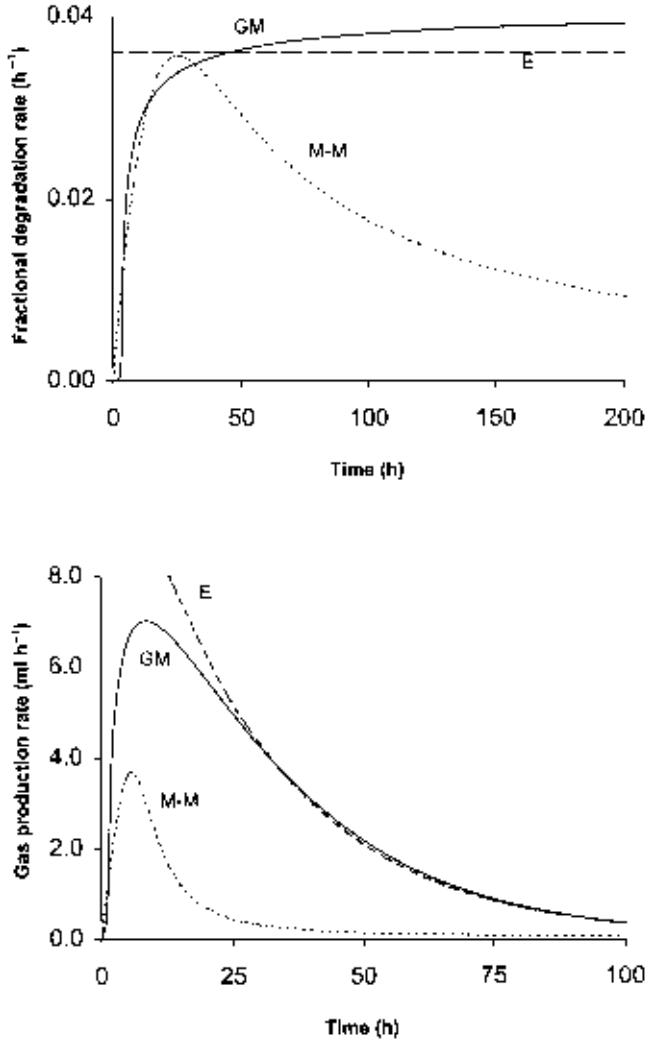


Fig. 4.7. Change in fractional degradation rate and in gas-production rate with time as represented by different mathematical models. E, exponential or simple Mitscherlich; GM, generalized Mitscherlich; M-M generalized Michaelis–Menten.

where S_0 is the zero-time quantity of the S fraction, $\Phi(t)$ is a positive monotonically increasing function with an asymptote at $\Phi(t) = 1$ (Table 4.5) and t is incubation time (h). *In situ* disappearance (D, g g^{-1} incubated) is given by:

$$D = W + S_0 - S = W + S_0 \times \Phi(t) \quad (4)$$

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

$$G = YS_0 \times \Phi(t) \quad (5)$$

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
Generalized Mitscherlich	$1-e^{-c(t-L)-d(\sqrt{t}-\sqrt{L})}$	$c+\frac{d}{2\sqrt{t}}$
Special case of generalized Mitscherlich	$1-e^{-c(t-L)}$	c
Generalized Michaelis–Menten	$\frac{t^c}{t^c+K^c}$	$\frac{ct^{(c-1)}}{t^c+K^c}$
Logistic	$\frac{1-e^{-ct}}{1+Ke^{-ct}}$	$\frac{c}{1+Ke^{-ct}}$
Gompertz	$1-\exp\left[\frac{b}{c}\left(1-e^{ct}\right)\right]$	be^{ct}

where G (ml) denotes total gas accumulation to time t and Y (ml gas g^{-1} degradable DM) is a constant yield factor. For each function, μ could be obtained from equations (1b) and (3) as:

$$\mu = -\frac{1}{S}\frac{dS}{dt} = \frac{1}{(1-\Phi)}\frac{d\Phi}{dt} \tag{6}$$

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 which is subjected to both passage and degradation, the rate of disappearance of S is given by (see Fig. 4.6):

$$\frac{dS}{dt} = -kS \qquad t < L \tag{7a}$$

$$= -(k+\mu)S \qquad t \geq L \tag{7b}$$

where k (h^{-1}) is the fractional rate of passage from the rumen and is assumed constant.

To obtain S, the solutions of these differential equations are:

$$S = S_0 e^{-kt} \qquad t < L \tag{8a}$$

$$S = S_0 e^{-kt} \times (1 - \Phi) \qquad t \geq L \tag{8b}$$

Using these equations, the extent of degradation in the rumen (E, g degraded g^{-1} ingested) is given by the equations:

$$E = \frac{W + \int_L^{\infty} \mu S dt}{W + S_0 + U} = \frac{W + kS_0 \int_L^{\infty} \Phi e^{-kt} dt}{W + S_0 + U} \tag{9}$$

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

$$E = \frac{\int_L^{\infty} \mu S dt}{S_0 + U} = \frac{kS_0 \int_L^{\infty} \Phi e^{-kt} dt}{S_0 + U} \tag{10}$$

for *in vitro* gas-production profiles.

These equations provide a general expression for calculating the extent of degradation and are applicable to any model expressed in the form of equations (4) and (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. The major assumption in the gas-production equations is that the rate at which gas is produced is directly proportional to the rate at which substrate is degraded, with constant yield factor Y. However, like any *in vitro* system of microbial activities, the partitioning of ruminally available substrate between fermentation (producing gas) and direct incorporation into microbial biomass may vary, depending on, amongst other things, the size of the microbial inoculum and the balance of energy- and nitrogen-containing substrates (Pirt, 1975). Indeed, Blümmel *et al.* (1997) showed an inverse relationship between gas production and microbial biomass yield. Clearly, during the course of fermentation, the amount of substrate becoming available per unit of microbial mass decreases, and hence (according to the growth and non-growth requirements as described by Pirt (1975)) relatively more substrate is expected to be used for gas production rather than incorporated into biomass, resulting in an increase in the yield of gas in the later phases of incubation. Furthermore, the pattern of fermentation can vary with respect to the yield of individual VFA and, from stoichiometric relationships, this undoubtedly affects Y in time. Whilst Y does not directly appear in the calculation of E (equation (10)), differences in Y during the course of fermentation might well affect the value of E. A further limitation of the gas-production technique is that it merely provides kinetic information on degradation of OM or of cell contents and cell-wall material, but cannot supply detailed information on the flow of aminogenic, glucogenic and lipogenic substrates out of the rumen.

Estimates of the extent of degradation of feeds in the rumen are invariably highly sensitive to the passage-rate values (reviewed by Dijkstra and France, 1996). The removal of digesta from the rumen is also one of the major processes control-

ling the intake of forages. Kinetics of passage have usually been studied using the faecal-marker excretion technique, enabling calculation of the mean retention time in the rumen and the whole tract. Rate of passage is, in part, an intrinsic characteristic of the forage (Aitchison *et al.*, 1986), but it is also influenced by factors such as particle size (affected by chewing and ruminating), particle functional specific gravity and water kinetics (Poncet, 1991). Main aspects of digesta passage have been identified (Kennedy and Murphy, 1988; Lechner-Doll *et al.*, 1991), but have yet to be quantified. Thus, the representation of outflow of digesta in the models of whole-rumen function is often much less detailed than the representations of substrate degradation and microbial synthesis processes. Some models have been developed to represent the dynamics of particle-size reduction to be applied to passage kinetics (Murphy and Kennedy, 1993), taking into account the particle size distribution in the feed and with a number of pools, each representing a different particle size, with rates of particle size reduction that determine the flow of particles from one pool to another. However, many of the mechanisms of fluid and particle outflow and their interactions with degradation through rumination and microbial activity have not been considered in mechanistic rumen models (for a review, see Dijkstra and France, 1996). Given the important effect of passage on extent of degradation, more quantitative research is required to allow proper integration of passage and degradation and improved prediction of degradation in the rumen.

Microbial synthesis

The partitioning of ruminally available monomers between fermentation (yielding VFA) and direct incorporation into microbial biomass may vary widely. This partitioning will influence the energy supply from the forage and the composition of the energy-yielding substrates. The different approaches to predicting microbial synthesis have been reviewed recently (Dijkstra *et al.*, 1998a). To understand aspects of microbial metabolism, physical analogues of the rumen ecosystem have been employed. The mathematical equations describing these cultures are helpful in discerning the essential parameters of microbial metabolism and in predicting the partitioning of substrate into microbial biomass and fermentation end-products. Most of the physical models for studying ruminal microbial metabolism have been based on the chemostat. The differential equations describing the dynamics of the chemostat are:

$$dX/dt = (\mu_G - D)X \quad (11)$$

$$dS/dt = CF - \mu_G X/Y - DS \quad (12)$$

where X (g) denotes the amount of biomass, S (g) the growth-limiting substrate, μ_G (h^{-1}) the fractional growth rate, Y (g biomass g^{-1} substrate) the growth yield with respect to S, D (h^{-1}) the dilution rate, F (ml h^{-1}) the medium inflow rate and C (mg substrate ml^{-1} medium) the concentration of S in the medium. The substrate consumption by the microbes is assumed to follow enzyme kinetics described by Michaelis and Menten (1913), and therefore the specific growth rate of biomass will be determined by substrate concentration, and can be represented by a rectangular hyperbola (Monod, 1942):

$$\mu_G = \mu_{G,max} /(1 + K/C) \quad (13)$$

where $\mu_{G,max}$ is the maximum value of μ_G (h^{-1}), and K (g substrate ml^{-1}) the saturation constant. K gives a measure of the affinity of the organism for the substrate. The $\mu_{G,max}$ and K values differ widely between microbial species and may be a major determinant of microbial survival in the rumen under conditions of competition (Russell and Baldwin, 1979). These equations show that faster growth rates are achieved when the substrate concentration is increased.

The overall rate of utilization of a substrate can be divided into the rate of usage for non-growth processes (maintenance) and for mass increase (growth). Based on this division, the yield factor in equation (12) is described by the double reciprocal equation of Pirt (1975):

$$1/Y = M/\mu_G + 1/Y_{max} \quad (14)$$

where M (g of S g^{-1} of X h^{-1}) is maintenance coefficient and Y_{max} (g of X g^{-1} of S) is maximum growth yield with respect to S. From equation (14), it is clear that Y decreases when maintenance requirements increase, and Y increases when μ_G or Y_{max} is increased.

Thus, the proportion of substrate fermented to VFA or incorporated into microbial biomass depends on factors that affect growth, maintenance and maximum growth yield (Fig. 4.8). Individual maintenance values can vary widely between species (Russell and Baldwin, 1979) and, since the composition of the rumen microbial population changes with changes in dietary characteristics, this may affect the proportion of the substrate being spent for maintenance. The microbial non-growth requirements are significantly increased when some nutrients other than carbohydrates are limiting growth. In such situations, the potential of microbes to produce energy from catabolic processes is in excess of their potential to use that energy for biosynthetic purposes (referred to as energetic uncoupling; Hespell and Bryant, 1979). Energy requirements per unit of microbial DM synthesized depend on the composition of microbial DM. For example, the biosynthesis of nucleic acids requires far more adenosine triphosphate (ATP) than does that of protein (Stouthamer, 1973). The outflow of microbial biomass to the duodenum is lower than that synthesized within the rumen, due to recycling of microbial biomass within the rumen (Dijkstra *et al.*, 1998b). This recycled biomass will be largely fermented to VFA again, and thus the degree of recycling within the rumen will affect Y as well. In their review, Dijkstra *et al.* (1998a) show that current protein evaluation systems do not take into account key aspects of microbial metabolism and will not predict microbial synthesis satisfactorily. In view of the relation between microbial biomass synthesis and VFA production from degraded substrates, this lack of adequate prediction is of relevance not just to protein nutrition of ruminants but also to energy evaluation, again highlighting the point that protein and energy evaluation systems should not be developed in isolation from each other if they are to cope with the energy and protein interactions within the animal.

VFA production

The VFA produced by fermentation of substrates in the rumen and subsequently absorbed represent the major source of energy, providing at least 50% of the total

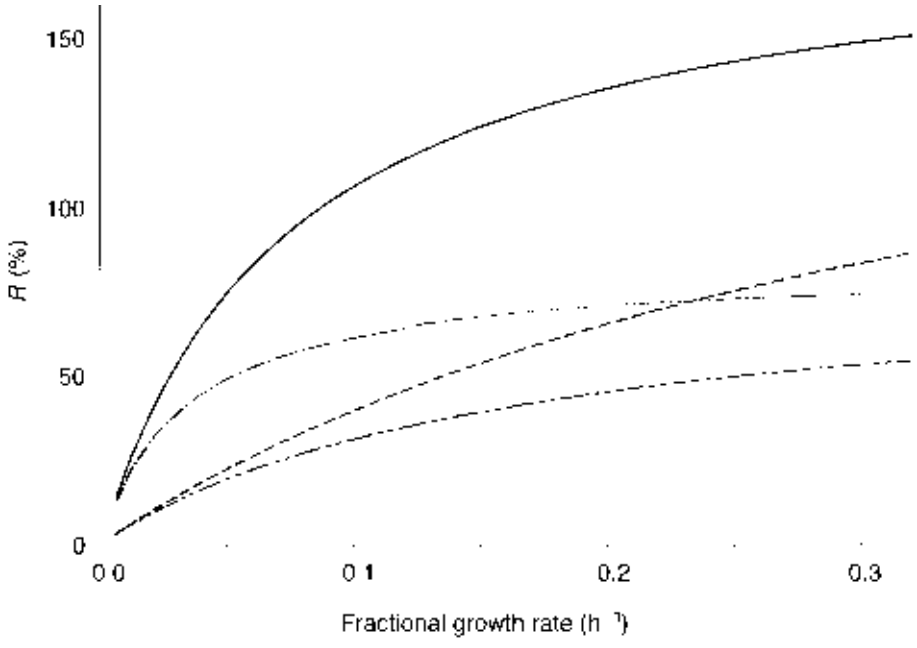


Fig. 4.8. Effect of fractional growth rate (μ_G), microbial maintenance requirement (M; low M = 0.04, high M = 0.20) and maximum growth yield (Y_{max}) in presence (Y_{max} = 0.65) or absence (Y_{max} = 0.45) of preformed monomers on the ratio (R) of carbohydrate incorporated into microbial biomass to that fermented to VFA. M = 0.04, and Y_{max} = 0.65 (——); M = 0.04, and Y_{max} = 0.45 (–·–); M = 0.20, and Y_{max} = 0.65 (------); M = 0.20, and Y_{max} = 0.45 (- - —— - -).

amount of digestible energy (Sutton, 1985). The relative concentrations of the individual acids (mainly acetic, propionic and butyric acids), commonly referred to as the fermentation pattern, are important determinants of energy utilization by ruminants. Methane production (which is an important energy loss) per mole of fermented hexose is variable, depending on the type of VFA produced (less methane if fermentation is directed to the formation of propionate). Within the host animal, propionate is used largely for glucogenesis, being the major source of glucose for the ruminant (Huntington, 1990). In contrast, both acetate and butyrate are used primarily as energy sources, and acetate is also the principal substrate for lipogenesis. This, in addition to the distinct effects of individual VFA on the hormonal balance in the animal, results in a significant effect of the fermentation pattern in the rumen on the partitioning of nutrients between organs and tissues (Bergman, 1990). Thus, diets that stimulate high propionic and low acetic acid concentrations in the rumen are linked to reductions in the milk fat secretion, associated with a repartitioning of nutrients within the body of dairy cattle (Sutton, 1985). Also, the balance between the supply of propionate and that of acetate and butyrate seems to influence the efficiency with which energy is used for productive purposes in dairy cows (MacRae and Lobley, 1982), though the effects

of differences in VFA ratios on energy efficiency for growth and growth composition are not consistent (Ørskov and MacLeod, 1990). Therefore, to predict the energy supply from a feed, it is important to estimate accurately the amount of VFA produced and absorbed and the molar proportions of the individual acids.

A number of techniques have been used to measure ruminal VFA production (Dijkstra, 1994). VFA production can be easily measured *in vitro* as the accumulation of VFA when the substrate is incubated in batch or continuous cultures. However, *in vivo* determinations are far more difficult, as VFA do not accumulate in the rumen and either are absorbed or flow out to the omasum. Techniques employing tracers are based on the application of compartmental analysis to interpret isotope dilution data, and some models have been derived based on simple or multiple pool schemes to estimate VFA production (France and Siddons, 1993). Concern exists over the variability and reliability of estimates obtained with ruminants using isotope dilution methods, particularly with dairy cattle (Sutton, 1985). Total VFA production depends upon factors affecting the ruminal digestibility of OM, such as the level of intake or the concentrate proportion of the diet, and also on factors affecting the amount of VFA obtained per unit of fermented OM, such as the fermentation pattern and the efficiency of microbial synthesis (discussed in the previous section). The type of substrate fermented is a major determinant of the type of VFA produced (Murphy *et al*., 1982), but a number of dietary factors, including the rate of fermentation and the level of intake, are responsible for specific interactions of the feed with the microbial population in the rumen and result in significant variations in the fermentation pattern. With forages, the major source of variation affecting the molar proportions of the VFA in the rumen is the OM digestibility, so that feeding more digestible forages is associated with lower proportions of acetate and higher proportions of both propionate and butyrate (Thomas and Rook, 1981). Treatments that change the rate of substrate fermentation may also give rise to marked differences in the fermentation pattern. Reductions in the particle size of forages by grinding are accompanied by less rumination and ruminal cellulolytic bacteria, resulting in a lower acetate-to-propionate ratio in rumen fluid. When starchy feeds are used, higher propionate concentrations at the expense of acetate can be expected, but the fermentation pattern is highly dependent on the source of the starch and its treatment during processing and also on the level of addition and the frequency of feeding of diets rich in sugars (Sutton, 1981).

Based on the well-known specific pathways of VFA production, Baldwin *et al.* (1970) suggested stoichiometric relationships between type of VFA produced and type of substrate fermented. This approach has been adopted in some mechanistic rumen models. Another approach that has been extensively incorporated into rumen models is that of Murphy *et al.* (1982), who provided a series of stoichiometric parameters relating to the yield of individual VFA for five specific dietary substrates. These relationships were not based on the biochemistry of rumen fermentation, but rather on statistical analyses of a large data set of ruminal VFA molar proportions observed for a range of dietary conditions, taking the solution that represented the best estimate on the basis of statistical criteria. Yet current approaches are insufficient in representing the fermentation pathways and in predicting VFA molar proportions accurately (reviewed by Dijkstra, 1994), confirmed

by the results of a comparative evaluation of rumen models (Bannink *et al.*, 1997). Probably, the mathematical representation of effects other than merely chemical composition of the diet needs to be considered, including dietary factors that induce changes in various parameters of the rumen environment (osmotic pressure, redox potential, pH, turnover rate) (Sutton, 1981). Dijkstra (1994) recommended that factors involved in the maintenance of the redox balance in the rumen (through reduction and reoxidation of nicotinamide adenine dinucleotide (NAD)) should be taken into account. These include ruminal pH, the composition of the substrate, the substrate availability and rate of depolymerization and the microbial species involved in the substrate fermentation. Prins *et al.* (1984) proposed that the pattern of VFA production from a given source and the amount of rumen-fermentable carbohydrate would be affected by the rate of flux of hexose through the microbial cell, which is related to the concentration of bacteria in the rumen (the lower the numbers of bacteria, the higher the flux per cell) and to the rate of fermentation of the substrate. The understanding of the complex relationships between dietary factors, rumen environment parameters and fermentation patterns could significantly improve the prediction of the molar proportions of VFA during fermentation under different dietary conditions.

In addition to VFA production rates, the prediction of VFA availability requires accurate estimates of VFA absorption as well. Mechanisms involved in the absorption of VFA across the rumen wall are not yet fully understood (Carter and Grovum, 1990; Rechkemmer *et al.*, 1995). Quantitative information on the absorption rates is also lacking. The rate of VFA appearance in the portal blood has been used to estimate the net VFA absorption into the bloodstream. However, the amounts of VFA appearing in the portal blood are not equal to the amounts produced and absorbed from the rumen, because the former also includes VFA produced and absorbed in the lower tract and because some of the VFA absorbed (in particular, propionate and butyrate) are metabolized by the rumen mucosa during the process of absorption. VFA absorption has also been measured in animals receiving infusions of VFA into the emptied, washed and temporarily isolated rumen. Using this technique, it has been observed that the absorption of VFA through the rumen wall accounts for up to 88% of the infused VFA, that the absorption rates are different for the individual VFA and that absorption can be affected by rumen factors, such as pH, osmotic pressure and VFA concentration (Dijkstra *et al.*, 1993; López *et al.*, 1994b; López and Hovell, 1996). Further experimental data would be required to characterize the kinetics of VFA production and absorption and of metabolism in the rumen wall, in order to estimate parameters that can be incorporated into mathematical models to account for the dynamic, integrated character of VFA production and absorption processes, allowing for a more accurate prediction of the VFA and energy supply to the animal.

Predicting events postruminally

Small intestine

Nutrients entering the small intestine can be digested by pancreatic, hepatic and enteric enzymes, the end-products being absorbed into the portal bloodstream. The

main energy supply to the animal at this level is from starch, protein and long-chain fatty acids (LCFA) (see Fig. 4.1). Some sources of starch are particularly resistant to rumen degradation, and considerable outflow of starch to the duodenum may occur (reviewed by Nocek and Tamminga, 1991). Then, information would be needed on the relative contributions of escaped soluble and insoluble feed starch and of microbial storage starch to the total amount of starch flowing out of the rumen. The amount of starch bypassing the rumen in a variety of circumstances can be predicted using the rumen models of Baldwin *et al.* (1987) and Dijkstra *et al.* (1992). Digestibility coefficients of starch in the small intestine (as proportion of the amount reaching the duodenum) are highly variable, ranging from 0.28 to 0.98 (for a review, see Toullec and Lallès, 1995). Some of the variation is attributed to the variation in amount of starch flowing out of the rumen. In general, as duodenal starch flow increases, the small-intestinal digestibility declines progressively, whereas large-intestinal digestion of starch increases (Beever, 1993). However, the most important factors affecting the site and extent of starch digestion are chemical and structural characteristics associated with the source of starch and the form of processing (Reynolds *et al.*, 1997). The digestion of starch in the small intestine has been considered of special importance for the supply of glucose to the animal. However, the net flux of glucose to the portal vein can only account for a small proportion of the starch digested in the small intestine (Reynolds and Beever, 1995), probably due to the utilization of glucose by the gut epithelial tissue. All these factors affecting the intestinal digestion of undegraded starch must be considered for any prediction of the supply of nutrients from this source. Only a few models have explicitly represented outflow of substrates from the rumen and digestion of substrates postruminally. If represented, the application of fixed intestinal digestion coefficients prevails (Baldwin *et al.*, 1987; Dijkstra *et al.*, 1996).

The absorption of LCFA is almost exclusively from the small intestine. Forages generally contain low amounts of LCFA, though, and hence these do not contribute large amounts of energy to the total energy absorbed from the small intestine. The use of feed supplements rich in LCFA is limited, because of the general adverse effect of LCFA on fibre degradation in the rumen (reviewed by Jenkins, 1993). Microbial LCFA are synthesized in the rumen *de novo* or incorporated from feed LCFA. The synthesis *de novo* may explain why the flow of LCFA into the duodenum exceeds the dietary intake of LCFA on diets containing low or moderate amounts of lipid (Doreau and Chilliard, 1997). Although saturated LCFA are absorbed more slowly than unsaturated LCFA, in general absorption in the small intestine is high, with true digestibility approaching 95–100% (Van Soest, 1994).

Amino acids flowing into the duodenum may form an important contribution to the energy absorbed. Microbial protein is generally highly digestible in the small intestine, with average values of 85% (Storm *et al.*, 1983). However, dietary protein digestibility in the small intestine may be far more variable, especially that of forage protein, and it varies between 50 and 99% of the escaped feed protein entering the small intestine (reviewed by Van Straalen and Tamminga, 1990). As with starch and LCFA, current feed evaluation systems and mechanistic models generally apply fixed intestinal digestion coefficients of protein. The models do vary significantly in predicting the flow of microbial and bypass protein from the rumen,

though, thus potentially allowing for considerable variation in the predicted contribution of protein to the energy supply of the ruminant.

Hind-gut

The large intestine may play an important role in the digestion of forages by ruminants. Although up to 95% of the total fermentative activity takes place in the rumen, an active microbial population is also present in the hind-gut (caecum and proximal colon), which may contribute, to a variable extent, to the degradation of carbohydrates and nitrogen compounds (see Fig. 4.1). Digestibility of structural carbohydrates in the large intestine of sheep (as proportion of the amounts entering into the large intestine) ranged from 19 to 49% (Tisserand and Demarquilly, 1995), and substrates absorbed in the caecum–colon may account for 4 to 26% of the total energy absorbed in the whole digestive tract. The proportion of substrates degraded and of digestible energy obtained in the hind-gut in relation to that in the whole digestive tract is greater if the digestibility of the forage is lower, the level of intake is increased, the level of inclusion of concentrates in the diet is increased or with ground and pelletized diets (Demeyer, 1991; Van Soest, 1994). In general, digestion in the hind-gut partly compensates a potential decline in whole-tract digestibility caused by any factor decreasing ruminal digestion of carbohydrates. This also stresses the importance of integrating digestive processes in the rumen and small and large intestines in mechanistic models to obtain approximate estimates of the energy and nutrient supply from the feeds consumed.

Degradation and fermentation processes of substrates in the hind-gut are rather similar to those in the rumen (Demeyer, 1991). After hydrolysis of the substrates, microbial fermentation yields VFA and microbial biomass. Total VFA concentrations in the caecum–colon are in the range of 60 to 90% of those typically observed in the rumen. Some differences between rumen and hind-gut VFA molar proportions have been reported. The molar proportion of acetate in the hind-gut is usually greater and less variable than that observed in the rumen, because in the hind-gut a larger fraction of the available carbohydrates are structural carbohydrates and reductive acetogenesis is not outcompeted by methanogenesis (Demeyer, 1991). Yet many of the research findings provide only qualitative descriptions of the digestive processes in the hind-gut, due to the difficulty of studying digestion and absorption in this compartment. From the available information, it appears that most concepts used for modelling rumen fermentation could be applied to the fermentative processes occurring in the hind-gut as well. Differences between the rumen and the large intestine in the harboured microbial populations are largely related to the shorter retention time of digesta in the hind-gut and to differences in the available substrates for the microorganisms. The retention time of digesta in the hind-gut may vary between 7 and 18 h (Grovum and Hecker, 1973), shorter than retention times in the reticulorumen under similar nutritional conditions. Similar bacterial species to those found in the rumen have been identified, although total counts are considerably smaller (Ulyatt *et al.*, 1975) and relative numbers also differ. No protozoa have been isolated from the ruminant hind-gut, and little is known about any fungal populations at this site. Substrates digested at the caecum–colon are those arriving from the ileum, comprising nutrients that have not been digested in the reticulorumen, abomasum and small

intestine and those contained in endogenous secretions and cells. These substrates are more refractory to digestion than those entering other segments of the gut, although their susceptibility to microbial attack may be enhanced following exposure to abomasal and intestinal digestion and by reductions in particle size relative to the feed. Any model to predict energy supply to the animal from the hind-gut has to be integrated in a model of the whole digestive tract, as substrates available for fermentation at this site depend on digestion and absorption in the rumen and small intestine. However, no explicit mathematical representations of hind-gut fermentation in ruminants are available, other than those in whole-animal models, where a fixed digestion coefficient or a fixed proportion of rumen fermentation is applied to predicted flows of nutrients into the hind-gut (Baldwin *et al.*, 1987; Dijkstra *et al.*, 1996).

Concluding Remarks

The prediction of energy supply from forages is primarily based on empirical ME or NE systems. Such predictions do not consider site of nutrient digestion within the ruminant and the major interactions between substrates, and take no regard of the nature of the metabolites that comprise the energy fraction. Moreover, the current systems aim to estimate feed requirements to meet the energy needs of the ruminant, whereas the response of the ruminant to dietary perturbations are of greater interest, along with the animal product composition and excretion of waste material. Data obtained using *in vitro* and *in sacco* methods provide additional information for estimating energy supply that is less expensive to obtain than by using *in vivo* methods. Such information may be of limited value, however, if regarded in isolation. In this chapter, it has been argued that mathematical integration of new and existing data and concepts into mechanistic models is essential in order to utilize the information optimally and to improve the prediction of energy supply to ruminants.

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5 Forage Evaluation Using Measurements of Energy Metabolism

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Introduction

Considering the evolutionary adaptations of the ruminant digestive tract, the fundamental importance of forages in ruminant nutrition is self-evident. In wild ruminants and many extensive ruminant production systems, forages often provide the sole source of dietary energy and protein. In these situations, forages are often harvested solely by the animal, but forages may also be mechanically harvested and conserved for use during seasonal changes in forage availability. In more intensive systems seeking maximum production, forages may be fed in conjunction with cereals and other concentrated energy or protein sources, but even in high-yielding dairy cows a minimal level of dietary fibre is required for the maintenance of rumen function and health. In these cases, the effectiveness of by-product fibre sources in maintaining rumen function is often evaluated relative to grass or legume forages (e.g. Clark and Armentano, 1997). In a similar manner, the first feeding standards for cattle developed by Thaer compared the nutritive value of feeds and expressed requirements relative to the fattening value of 'good meadow hay' (Flatt *et al.*, 1972).

Even before the development of Thaer's hay-equivalent system in 1809, ruminant nutritionists and husbandmen sought to develop comprehensive systems for evaluating and expressing the nutritive value of forages and other feeds relative to their ability to meet specific animal requirements. In the intervening years, a number of systems have evolved, but the need for refinement and improvement of current feed evaluation systems is as important today as 200 years ago. Current systems for evaluating the nutritive value of forages for specific production requirements are an improvement over their predecessors, largely on account of collective and integrated experience and technological advances, but they do have limitations. The ideal system of feed evaluation was described by Flatt *et al.* (1969a) as the 'Holy

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Grail' of animal nutrition, where, as for religion, a variety of approaches are used around the world, each believed by its users to be the one 'true' system.

Many of the current 'official' feeding systems compare the energetic value of forages based on their relative ability to meet the animal's requirements for net energy (NE) at a standardized level of intake or a specific physiological state. These systems are based on calorimetric measurements of energy balance within animals fed individual feeds or combinations of feeds, although in practice the energetic values of many feeds are estimated using other approaches. This approach has been the subject of much criticism, in part owing to the inability of current approaches to predict production responses in terms of intake, product composition or nutrient partitioning. Energy is not a nutrient *per se*; thus NE approaches represent an amalgamation of the metabolism of a variety of compounds into a single entity, combustible energy, which is measurable at specific points of loss or gain within the animal. This simplification of complex biological reactions is both a strength, in terms of applicability and versatility, and a weakness, in terms of predictability. Many now believe the newer 'religion' of mathematical modelling of nutrient metabolism will ultimately illuminate the path to predictable response, but in application such models will need to be 'fed', using practical criteria for evaluating individual feeds. Although highly desirable and the justification for untold sums of research funding, the one 'true' system capable of predicting production responses to changes in feed composition is as elusive today as the Grail for the crusaders.

This chapter will consider the use of energy-balance measurements for forage evaluation within the context of current feeding standards and research, as well as the limitations and merits of the approach, with an inevitable bias towards the author's background and previous employment.

Historical Perspectives

The work of Thaer and Einhof led to the development of the first 'official' hay-equivalent feeding system and was the basis of the 'Weende' system of feed analysis. This analytical approach was the basis of a 'total digestible nutrients' (TDN) system of feed evaluation and livestock rationing, whose origins were at the Weende Experimental Station in Germany in 1860 (Flatt *et al.*, 1972). The TDN approach that developed was used for rationing dairy cattle in the USA until it was replaced as the 'official' National Research Council (NRC) system for rationing energy in dairy cattle by the NE for lactation (NE_l) system (NRC, 1971). However, the TDN system is still widely used throughout the world. Indeed, because of the difficulties of obtaining measurements of energy balance in dairy cattle, many of the tabular values of ME and NE_l used in the current NRC publication (NRC, 1989) are based on TDN values, often obtained in sheep or steers.

Even as the digestible-nutrient approach was being developed, it was realized that digestible nutrients were not equal in terms of the production they could support and that losses of nutrients other than those in faeces must also be accounted for. In many instances, these differences were due to the efficiency of utilization of metabolizable energy (ME) for NE gain. NE systems for rationing ruminants are based on measurements of energy metabolism and balance, obtained by measuring

ME, by subtracting energy losses as urine (UE) and combustible gases (GE) from digestible energy (DE) and by measuring either heat energy (HE) loss or energy retained as product (RE). The use of calorimeters for measuring HE and NE balance by a number of individuals in Europe and the USA led to the development of two NE energy systems in the early 1900s: Kellner's starch-equivalent system, used widely in Europe, and Armsby's estimated NE system in the USA, which ultimately had limited use as an adjunct to the TDN system. However, both of these systems were based on a limited database of experimental measurements. The limitations of the TDN and starch-equivalent systems and the paucity of their database eventually led to the development of the current NE systems in use today, which were based on an explosion of animal calorimetry studies beginning in the late 1950s. A driving force behind the establishment of a calorimetry facility for dairy cattle at the US Department of Agriculture's Research Center in Beltsville, Maryland, was the scientific debate over the reasons why TDN from concentrates supports higher milk production than TDN from forages (Huffman *et al.*, 1952). Conjointly with the development of large databases on NE metabolism in farm animals, chemical methods of feed evaluation were also developed and refined in an attempt to explain and predict animal responses to changes in diet composition and quality. At the Beltsville laboratory, these efforts led to the simultaneous development of the NE_l system for rationing dairy cows and the neutral-detergent fibre (NDF) system for analytically describing fibre fractions in feeds (Goering *et al.*, 1970). The NDF approach was a leap forward in the evolution of chemical procedures for forage evaluation (Reid, 1994), but the need for enlightened approaches to assessing forage and feed quality and predicting their effects on animal production are as relevant today as for Thaer.

Methodology

Techniques for measuring energy metabolism have been described and critiqued in numerous reviews and books (Flatt, 1969; Blaxter 1971; McLean and Tobin, 1987). As mentioned previously, there are two general approaches to determining NE. One is to measure or estimate HE and calculate RE by subtracting HE from ME and the other is to measure RE, but a measure of ME will also be required to estimate HE. Since the days of Lavoisser's ice calorimeter (McLean and Tobin, 1987), measurements of HE have been obtained using calorimeters that house the experimental subject. Two approaches have typically been used: direct measurement or indirect estimation of HE. As the name implies, direct calorimeters obtain direct measurements of the HE dissipated by the animal, using a variety of technological approaches, such as measuring the difference in the temperature of water entering or leaving a jacket surrounding the chamber, or thermocouples. Indirect approaches include estimation from respiratory exchange or carbon and nitrogen balance. Carbon and nitrogen balance simultaneously estimates RE, but still requires measurement of carbon dioxide and methane losses.

Owing to the historical complexities of direct calorimeters, especially for use with large ruminants, the majority of the calorimeters in use in the later half of the 20th century have been indirect calorimeters. These can be either closed- or

open-circuit, but the problem of removing large quantities of moisture and carbon dioxide in closed-circuit systems has meant that most of the calorimeters used for large ruminants are open-circuit. In open-circuit systems, estimates of respiratory exchange are obtained by measuring the difference in gas concentration between incoming and outgoing air and the flow rate of air through the chamber. These measurements are then corrected for effects of temperature, barometric pressure, humidity and the residual volume of the chamber (Flatt, 1969). HE can then be estimated, based on relationships between nutrient oxidation and heat production, with adjustments for the type of nutrient oxidized, based on carbon dioxide production and urinary nitrogen loss. For ruminants, further adjustments are made for incomplete oxidation of carbohydrates, based on methane losses. The constants for these relationships were recommended by a European Association of Animal Production (EAAP) subcommittee on constants and factors (E. Brouwer, K. Blaxter, K. Nehring and W. Wohlbier) and condensed into a single equation, reported by Brouwer (1965).

Whichever approach is used to determine HE, complete energy-balance measurements also require the measurement of DE and UE, using digestion trials, and GE, using respiratory exchange. Gaseous energy losses are mostly represented by methane, but in some cases hydrogen and ethane may also be lost (Flatt, 1969). Digestion trials and measurements of HE may be conducted simultaneously, if the calorimetry system employed is suitable for separation and collection of faeces and urine, or obtained on separate occasions. If this is the case, then effects of level of intake and feed quality must be considered carefully, as changes in intake can have immediate effects on HE, as well as on DE and ME. This is especially a problem in evaluating forages, where day-to-day variations can be substantial, depending on the source, method of harvesting and conservation and experimental management.

Another approach to estimating NE is to use comparative slaughter techniques. To determine RE over a period of time, the body composition of two groups of animals is determined at the beginning and at the end of a prescribed experimental period. DE is determined separately, with GE often estimated. This has obvious limitations for lactating animals, but indirect (specific gravity) estimates of changes in carcass composition are the basis of energy feeding standards for growing beef cattle in the USA (NRC, 1996). However, numerous comparisons between comparative slaughter and respiration calorimetry have found that estimates of HE are higher and thus NE is lower when measurements are obtained by comparative slaughter (Johnson, 1986; Waldo *et al.*, 1990). There are a number of reasons for this discrepancy. One concern with the use of calorimeters is that they require the subject to be confined in a sealed chamber for extended periods, which can elevate HE by causing stress if proper adaptation is not employed. More importantly in the comparison of respiration and comparative slaughter calorimetry, activity is limited when animals are confined to chambers and thus maintenance costs and HE are reduced. Another concern with digestion trials and balance approaches is that errors of measurement are cumulative; thus losses of energy and nitrogen during faecal, urine and scurf collection tend to cause overestimation of their retention in the animal (Martin, 1966). The confinement of animals in chambers also means that traditional respiration-calorimetry approaches cannot be used to measure NE of animals under field or grazing conditions.

Other techniques can be used to estimate respiratory exchange in subjects that do not require total confinement, but at the expense of the precision which can be achieved with a rigorously operated chamber system. Examples include head chambers or face-masks, which would not estimate total GE and also require restriction of activity. Approaches that have been applied to the study of grazing ruminants include tracheal cannulas or isotope-dilution procedures. Tracheal cannulation techniques for measuring energy exchange of ruminants have a history extending over a 100 years (Flatt, 1969). Attempts to use the techniques for measurement of HE in grazing cattle (Flatt *et al.*, 1958) and sheep (Young and Webster, 1963) have been successful, but concerns about the weight of equipment the animal is required to transport and the effects of the cannula on animal health have limited the use of the technique. As for face-masks, the approach does not measure total GE and carbon dioxide loss.

Isotope-dilution procedures can be used to estimate body carbon dioxide production, using labelled carbon dioxide (Corbett *et al.*, 1971) or doubly labelled water (McLean and Tobin, 1987), or body composition (Andrew *et al.*, 1995; Crooker *et al.*, 1998). The doubly labelled water approach is widely used in studies of energy metabolism in 'free-living' humans and appears an attractive option for studies in grazing ruminants, but the agreement between this approach and established calorimetry procedures has not justified the widespread adoption of the procedure. One concern with the use of labelled water dilution in ruminants is the effect of the large and variable gut and milk pools on the dilution profiles obtained (Crooker *et al.*, 1998).

Practical considerations

The limited application of alternative procedures has meant that the evaluation of the energetic value of forages and their effects on animal energy metabolism have largely been obtained using chamber calorimeters and conserved forages. Measurements for fresh herbage have been restricted primarily to zero grazing, which has often required the freezing of large quantities of fresh material to ensure consistent quality over the course of the experiment. However, like other methods of conservation, freezing has effects on forage quality (Minson, 1990). In addition, the cost and difficulties of maintaining calorimeters for large ruminants has meant that measurements from sheep are often used to provide tabular values of the ME or NE value of forages for cattle. However, digestibility and metabolizability of a variety of feeds is higher in sheep than in cattle, due to comparative differences in rumen function (McDonald *et al.*, 1995). Similarly, effects of level of intake are an important consideration, especially when using tabular values from maintenance-fed sheep for rationing lactating dairy cows (Tyrrell and Moe, 1975).

Measurements of HE are not required to estimate ME, and some NE systems assign energy values to feeds in terms of ME and then use constants for the efficiency of ME use for a productive function in rationing the animal to arrive at NE (Alderman and Cottrill, 1995). Thus, in evaluating forages, an ME value is used as the term of reference. Calorimeters are expensive and labour-intensive to operate, but, if calorimeters are not available, then a digestion trial may be conducted and

methane losses estimated. The equations derived by Blaxter and Clapperton (1965), based on DE and intake level, are often used for this purpose. However, for dairy cows, a summarization of data by Moe and Tyrrell (1979) found that GE losses were best predicted from amounts of digested soluble residue (the more soluble and readily digested carbohydrates), hemicellulose and cellulose. In a more recent analysis of data from cattle obtained at Beltsville, the equation of Moe and Tyrrell (1979) predicted methane output more closely than any others available (Wilkerson *et al.*, 1995). However, this agreement may reflect the fact that both data sets were obtained in the same laboratory, using similar types of feeds. Another concern is the effect of feeding fats on methane production, which may not be adequately addressed in the prediction equations currently available. There are numerous other approaches for the prediction of ME based on laboratory analyses, which are addressed in other reviews (e.g. Barber *et al.*, 1989; Alderman and Cottrill, 1995). A major concern for the evaluation of conserved forages is the ability to determine volatile components of ensiled material at feeding. Whether fed fresh or conserved, in practice forage quality varies considerably from harvest to harvest, or even day to day; hence the need for rapid, accurate and economical lab-based methods of assessing forage energy value.

In contrast to most concentrates, the energetic value of many forages can be determined by feeding the test forage as the sole diet component. However, this is often not the case in studies with higher-yielding dairy cows. In the case of comparisons or determinations for concentrates (e.g. Andrew *et al.*, 1991) or for forages fed to lactating dairy cows (e.g. Casper *et al.*, 1993), substitution trials are often conducted (Armsby and Fries, 1918; Tyrrell and Moe, 1975). A variety of approaches can be used, but often, in measuring ME or NE values for a specific diet component, all other components of the diet are kept as constant as possible whilst only changing the test component. This can be extremely difficult to achieve if attempts are made to equalize parameters, such as crude or rumen-degradable protein, ME, minerals or fibrous components, such as NDF or acid-detergent fibre, but it represents a creative challenge for the researcher. The associative effects of combining feeds on their digestibility and metabolizability are due to a multitude of factors, such as energy and nitrogen interactions within the rumen, pH, intake and other effects on microbial dynamics. However, these effects must be considered in interpreting substitution trials and applying tabular energy values for individual feeds in diet formulations (Tyrrell and Moe, 1975; Moe, 1981).

Digestibility

Of the losses of dietary energy measured in determining energy balance, the most variable is faecal energy loss; thus DE accounts for the largest proportion of the variation in NE between diets and feeding criteria. In summarizing the results from 543 energy balance trials, Moe *et al.* (1972) found that DE accounted for 86% of the variation in NE measured. Digestibility of forages is determined largely by structural factors, such as the degree of lignification, but is also influenced by factors such as physical processing, level of intake, diet protein concentration and other associative effects within mixed diets (McDonald *et al.*, 1995). All of the fac-

tors that determine digestibility, then, have a major impact on the ME the ruminant can derive from a specific feed.

A particular problem in formulating rations for dairy cows is the effect of intake on diet digestibility. A number of studies have shown that increasing intake reduces dry matter (DM) digestibility, but the response varies with the type of diet fed. For example, in heifers fed pelleted diets containing either 75% lucerne hay and 25% concentrates or the inverse proportions, increasing intake from just above maintenance to nearly twice maintenance levels numerically depressed the digestibility of the 75% concentrate diet to a greater extent than the 75% lucerne diet (Reynolds *et al.*, 1991). By increasing rate of particle passage from the rumen, pelleting tends both to lower overall digestibility and to exacerbate effects of intake level on digestibility. However, similar responses have been observed in lactating dairy cows fed lucerne hay-based diets differing in concentrate level (Tyrrell and Moe, 1975). These observations have suggested that increasing the level of cereals in the diet exacerbates depressions in digestibility with increasing intake, but the response is not consistent across all studies (Tyrrell and Moe, 1975). Of the diet components measured, cell-wall components are often most affected. Many of the associative effects of grain feeding on cellulose digestion are known to be associated with, if not mediated by, depressions in rumen pH (Mould *et al.*, 1983). If feeding grain and subsequent effects on rumen acid load reduce cellulolytic activity of the rumen, then the increased rate of passage with increased intake may have a greater impact on cell-wall digestion than in the absence of starch in the diet. Increasing intake reduces methane production to a greater extent as the proportion of concentrate in the diet is increased (Blaxter and Clapperton, 1965), which reflects shifts in the microbial population of the rumen.

One consideration is that studies specifically designed to measure effects of intake level on digestibility often compare the relative digestion of diets in lactating dairy cows at maximal intake and non-lactating animals at maintenance (Tyrrell and Moe, 1975). These comparisons are appropriate, considering that many tabular values for DE, TDN or ME were obtained in steers or sheep, but they tend to magnify the problem relative to changes that may occur across a range of intakes within a group of lactating cows. For example, Bines *et al.* (1988) found that the digestibility of grass hay-based diets was lower in lactating cows than in immature, dry cows, but that, within lactating cows, moderate variations in level of intake had no effect on diet digestibility. This was especially true as level of concentrate in the diet increased. However, elevation of intake did depress digestibility in the non-lactating cows. This suggests either that the response to intake level is curvilinear or that physiological factors other than level of intake *per se* may play a role in the lower digestibility of diets fed to lactating cows compared with dry cows.

It has long been known that there is a positive relationship between dietary crude protein (CP) concentration, DM digestibility and ultimately intake level in ruminants (Schneider and Flatt, 1975). Averaged across a number of studies, the response is roughly 0.01 units of digestibility (1%) for each unit change in diet CP% up to 16%, or higher (Oldham and Smith, 1980). The response appeared to be similar in US and UK studies, although it was suggested that the response might be lower for diets with higher digestibility and CP content. This was more often the case in the UK studies surveyed, where diets tended to have a higher digestibility

than US diets because of the use of high-digestibility grass silages (Oldham and Smith, 1980). Increases in DM digestibility with increasing diet CP content can be attributed to the innately high digestibility of protein, or positive effects on microbial fermentation and digestion in the rumen (Tyrrell, 1980). Intake responses may be a result of both increases in digestibility and metabolic effects of improved amino acid supply (Oldham and Smith, 1980). Abomasal infusions of protein are as (or more) effective as dietary protein supplementation in improving grass silage intake (Chamberlain *et al.*, 1989); however, this response might be attributed to increased transfer of urea nitrogen to the rumen. In lactating dairy cows fed maize silage-based diets, feeding urea restored DM digestibility as effectively as an isonitrogenous amount of soybean meal, but only increased DM intake 1.3 kg day^{-1}, compared with an increase of 5.9 kg day^{-1} when soybean meal was fed (Tyrrell, 1980). It is known that urea is less effective than protein as a nitrogen supplement in dairy rations (Clark and Davis, 1980). This suggests either: (i) a protein, peptide or amino acid effect on microbial growth in the rumen; (ii) a metabolic effect of amino acids absorbed from rumen-undegraded feed protein in the small intestine; or (iii) negative effects of excess ammonia in the rumen or tissues such as the liver (Clark and Davis, 1980; Reynolds, 1992). For US dairy rations, the effect of diet CP level on milk yield is greatest when CP content is below 14% but continues to have a diminished effect as CP content increases to as high as 20% (Clark and Davis, 1980). In the UK, the response of DM intake to increasing diet CP content was on average 0.34 kg $unit^{-1}$ increase in CP% across all diets surveyed (Chamberlain *et al.*, 1989).

Retention of DE as ME

Unquestionably, digestibility accounts for the largest variation in ME or NE value of forages. However, forage type and quality can also influence the efficiency of DE use for ME, as well as the use of ME for RE. Methane losses are really digestive losses but are not accounted for when apparent digestion is measured solely by faecal output. Factors affecting methane output are related primarily to the availability of digestible carbohydrate fractions, rumen turnover and microbial population dynamics (Moe and Tyrrell, 1979). In addition to methane, UE losses are the other determinant, albeit a small one, when calculating the amount of DE available as ME in measurements of energy balance. UE losses are determined primarily by urea, which accounts for more than 70% of urine nitrogen (N) (Blaxter and Martin, 1962). However, other metabolites, such as hippurate, also contribute to the energy content of urine, such that the relationship between N and energy content varies with the diet fed (Blaxter *et al.*, 1966). Regardless, factors that increase liver urea production, such as excess rumen-degradable nitrogen, increase UE losses and have a small effect on the ratio of ME/DE. The ratio of ME/DE is normally quite high, especially for diets fed to lactating dairy cows (87%; Flatt *et al.*, 1969b), where GE losses as a proportion of intake energy are lower than for non-lactating animals fed poorer-quality diets at lower intakes (Blaxter and Clapperton, 1965). However, these differences are small, with GE and UE losses combined normally accounting for less than 15–20% of DE.

Efficiency of ME Use for RE

Forages compared with cereals

The lower efficiency of ME retention for forages compared with cereals was clearly demonstrated by Kellner in the development of the starch-equivalent NE feeding standards over 100 years ago (Armsby, 1903; Table 5.1). Oxen fed a supplement of straw or meadow hay had a higher HE increment than when fed an equivalent amount of ME from starch. This was related to the low digestibility of the forages, as alkali treatment of straw dramatically reduced the HE increment when it was fed (O. Kellner as summarized by Armsby, 1903). However, studies by Armsby and Fries (1918) in steer 'J' found a similar NE value for maize (corn) starch and lucerne (alfalfa) hay, which was lower than the value for starch reported by Kellner. This discrepency may have been the result of differences in the basal intakes used in these studies, as the efficiency of utilization of ME for maintenance is greater than the effciency of ME utilization for growth (Blaxter and Graham, 1955). In addition, there were differences in the correction factors used in calculating the results.

These and other conflicting results, often from studies using very limited numbers of observations (e.g. one animal), added confusion to the debate over the reasons for differences between the use of TDN from lucerne compared with maize for milk production. The debate was also clouded by the recent discoveries of a number of vitamins as essential nutrients (Huffman *et al.*, 1952). As mentioned, this debate in part led to the establishment of the Beltsville calorimeters and the conduct of a series of trials comparing the NE value of diets varying in lucerne hay and concentrate (maize, soybean and bone meals) proportion fed to lactating dairy cows. Initial work compared isonitrogenous diets containing 100, 75 or 50% estimated NE from lucerne hay (100, 84 and 63% lucerne hay on a DM basis; Coppock *et al.*, 1964a). They observed clear differences in the ratio of HE/ME across the three diets. When the ME required for maintenance was assumed (131 kcal $kg^{0.75-1}$) and tissue energy losses or gains were adjusted to zero, using efficiency constants, the efficiency of utilization of ME for RE was 54, 61 and 65% for the 100, 84 and 63% hay diets, respectively. Later studies compared similar diets containing 60, 40 or 20% lucerne hay (Flatt *et al.*, 1969) in trials which included the legendary cow Lorna (who achieved a milk yield of 49 kg day^{-1} whilst in the respiration chambers). At higher levels of concentrate inclusion (60% or more), there

Table 5.1. Tissue energy retention (RE) from supplemental ME in oxen fed fodder (from Kellner and Kohler, 1900, as cited by Armsby, 1903).

Supplement	RE/ME
Wheat straw	17.4
Meadow hay	41.4
Starch	61.5
'Extracted' straw	63.0

was no effect of level of forage in the diet on the efficiency of ME use for RE, but clear effects on the partitioning of ME between milk and TE. Thus, in lactating cows, the inclusion of relatively small amounts of concentrate improved the efficiency of ME use for NE_l, but there was little effect of further dilution of the forage component of the diet above minimal levels of concentrate normally fed to dairy cows in practice. For this reason, the effects of the diet forage to concentrate ratio on the efficiency of ME use for NE_l are acknowledged, but are considered too minor to merit inclusion in the NE_l rationing approach (NRC, 1989). As the efficiency of ME use for tissue and milk energy synthesis is similar in lactating dairy cows, the NE_l standards do not attempt to distinguish between the two processes (Moe *et al.*, 1972).

Forage type

In addition, type of forage can also affect the amount of ME lost as HE, and conversely NE (RE) gain. An often-used model in this regard is the comparison of grasses and legumes, where ME from grass is typically used less efficiently than ME from legumes. In growing cattle, the use of ME for RE was lower for cocksfoot grass (orchard-grass) silage than for lucerne silage (Varga *et al.*, 1990; Waldo *et al.*, 1990). Similarly, dairy cows fed diets based on cocksfoot grass silage consumed less DM, had higher HE/ME ratios, produced less milk and had lower TE gains than when fed diets based on lucerne silage (Casper *et al.*, 1993). More recently, a substantial body of data from lactating dairy cows have suggested that feeding grass silage as the sole forage source causes a large elevation of maintenance requirement compared with published values (Yan *et al.*, 1997).

Hypotheses

Solving the riddle of the basis for these differences in the efficiency of ME utilization between forages and concentrates and types of forages has been the 'Rubik's cube' of ruminant nutrition, practically since the discovery that ruminants derive their ME primarily from absorbed volatile fatty acids (VFA). Calorimetric studies suggested that the efficiency of utilization of ME from acetate was lower than for propionate or butyrate (Armstrong and Blaxter, 1957a, b), suggesting that, as feeding forages tends to increase ruminal acetate concentrations, the ratio of absorbed acetate : propionate was responsible for the increase in HE with high-forage compared with high-concentrate diets. It was later suggested that reductions in the efficiency of ME use would be expected when molar acetate proportions in the rumen exceeded 70% of total VFA (Blaxter, 1962). Measurements of ruminal VFA concentration in the studies at Beltsville supported this concept, with a linear negative relationship observed between energetic efficiency and ruminal acetate concentration (Coppock *et al.*, 1964b), but numerous VFA infusion and feeding studies have not (e.g. Ørskov and Allen, 1966; Ørskov *et al.*, 1969). One explanation for the disparity in the results obtained, first proposed by Armstrong (1965), is that the utilization of acetate for fat synthesis requires an adequate supply of reducing power in

the form of nicotinamide adenine dinucleotide phosphate (NADPH). The needed reducing equivalents will be more available if ample supplies of absorbed amino acids or glucose are present (Annison and Bryden, 1999). This concept was supported by the finding that the HE increment for acetate infused into the rumen was much lower in dry cows fed concentrate than in those fed lucerne hay alone (Tyrrell *et al.*, 1979).

In addition to variations in the relative proportions of acetate and glucose metabolized, other factors may contribute to differences in the efficiency of ME utilization. In the studies at Beltsville, growing steers fed grass silage had greater gut fill than steers fed lucerne silage (Waldo *et al.*, 1990). In addition, relative to increments in DM intake steers had a much greater increment in oxygen consumption by the portal-drained viscera (PDV) with increasing intake of grass silage compared with the increment for lucerne silage (Huntington *et al.*, 1988). This difference in incremental oxygen use by the PDV accounted for all the difference in incremental body oxygen consumption between the two forages.

In a comparison of high-forage and high-concentrate diets fed at two equalized ME intakes, heifers fed a 75% lucerne diet had greater PDV blood flow and oxygen consumption at equal ME to the 75% concentrate diet (Reynolds *et al.*, 1991). This increase in PDV oxygen consumption accounted for 66% of the difference in body oxygen use between the two diets at an intake near maintenance, and 84% of the difference at an intake near twice maintenance requirements for energy. These studies suggest that a large portion of the difference in ME loss as HE between forages and concentrates and between grasses and legumes is a consequence of increased oxidative metabolism by the PDV. This may be a result of changes in gut fill, the extent of rumination and the work of digestion. In addition, gut mass may be increased, especially the rumen, due to trophic effects of lipogenic VFA, physical abrasion of the epithelium or the work of digestion. Alterations in the profile of metabolites used may alter PDV oxygen consumption as well. In this regard, the PDV is a principal user of acetate, accounting for 50% of total acetate use in sheep fed lucerne at maintenance (Bergman and Wolf, 1971) and as much as 25% of body fat. In the study of Reynolds *et al.* (1991), feeding the high-forage diet increased the ratio of acetate to glucose released by splanchnic tissues (PDV plus liver) twofold compared with the high-concentrate diet (Reynolds *et al.*, 1993).

Excess dietary protein

Many immature, heavily fertilized forages and legumes can be high in rumen-degradable protein. Another consideration for the efficiency of utilization of ME from forages is the negative effect of excess nitrogen on HE and amino acid availability. In a summarization of early calorimetry studies at Beltsville, Tyrrell *et al.* (1970) found that excess protein consumption increased HE and decreased RE in lactating dairy cows. This reduction was equivalent to 30 MJ ME for each g of nitrogen consumed in excess of requirements, and this adjustment was included in the formula used for the calculation of NE_l (Moe *et al.*, 1972). Alternatively, feeding lactating cows protein at a level below requirement reduced RE through effects on DM digestion, as opposed to metabolic effects (Moe and Tyrrell, 1972).

The effect of excess nitrogen intake on HE has been attributed to the energy cost of urea synthesis and excretion, which Martin and Blaxter (1965) estimated to be 3.8 kcal g^{-1} ammonia nitrogen in sheep. This was greater than the theoretical cost of 3.2 kcal, which they attributed to the recycling of urea to the gut and the relatively small cost of renal excretion. However, feeding growing heifers 75% lucerne diets increased the amount of digestible nitrogen compared with a 75% concentrate diet providing equal ME, which markedly increased net PDV absorption of ammonia and liver urea release, but had no effect on liver oxygen consumption (Reynolds *et al.*, 1991). In other work, feeding urea in lucerne hay-based diets caused large (60%) increases in net PDV absorption of ammonia and liver urea release, with little change in the absorption of other nutrients, but had absolutely no effect on liver oxygen consumption (Maltby *et al.*, 1993). These observations may be due, in part, to the fact that, although urea synthesis is described as a cyclic process, it is not isolated from other metabolic processes in the liver and the flow of metabolites through other metabolic pathways will be reduced when urea synthesis is increased. In addition, the cost of urea synthesis in terms of adenosine triphosphate (ATP) is lower on a net basis when the generation of reducing equivalents from fumarate is considered (Reynolds, 1992). The real cost of urea synthesis in terms of ATP and oxygen use is much lower when the cycle is not considered in isolation.

In contrast to the studies just cited, feeding an increased level of soybean meal in a high-concentrate diet increased net PDV absorption of ammonia and liver urea release, as well as liver and body oxygen consumption in growing steers (Reynolds *et al.*, 1992). However, there was also an increase in PDV oxygen use, which was equal to the increase observed for the liver. Together, the PDV and liver accounted for all the increase in body oxygen use, and thus HE, when excess CP was fed. This suggests that there may be specific effects of feeding excess protein, rather than nitrogen *per se*, and that the origin of the increase in HE may not be restricted solely to urea synthesis in the liver. In this study, the increase in liver urea production with increased dietary CP level was associated with an increase in liver removal of methionine, tryptophan and lysine (Reynolds *et al.*, 1995). Effects of increased liver ammonia removal on the concomitant removal of amino acids has been suggested, based on observed changes in α-amino nitrogen (Reynolds, 1992) or amino acid (Parker *et al.*, 1995) metabolism. However, studies in sheep, using isotopic labelling of urea (Lobley *et al.*, 1995, 1996), have not shown conclusive evidence that an increase in ammonia absorption requires an increase in the deamination of amino acids (other than glutamate) to provide the aspartate needed for urea synthesis. If there is an effect of excess ammonia absorption on liver amino acid removal, the response is likely be influenced by the energy and protein status of the animal.

As mentioned previously, abomasal infusions of protein or essential amino acids have improved milk yield and intake in cows fed forages high in rumen-degradable protein (Oldham and Smith, 1980; Chamberlain *et al.*, 1989). However, these responses may reflect effects of excess rumen-degradable protein on the efficiency of microbial protein synthesis in the rumen, rather than effects of excess ammonia absorption on liver removal of absorbed amino acids.

Concluding Remarks

Measurements of energy balance form the basis of most current rationing systems for energy in ruminants and thus are the standard used in judging the effects of forage chemical composition on energy value for production. In this regard, many laboratory procedures for evaluating forage quality are used to predict the digestibility or ME value of forages *in vivo*. Measurements of energy balance are costly and time-consuming to obtain, whilst the resources for the maintenance of a sustained programme of calorimetry are at present limited: hence the need for current, as well as new, innovative, approaches to the evaluation of forage quality and energy value for production.

Current rationing systems for energy and protein fail to adequately predict the response of the animal, both in terms of product composition and nutrient partitioning. However, the systems were never intended to be a replacement for practical wisdom and experience, but a guide and basis for 'rational' decision-making. As discussed, the predictive limitations of current NE systems are, in part, due to the simplification of metabolism to a caloric basis, as well as the simplification of the effects of the animal's metabolism on the recovery of absorbed nutrients in a product to a series of efficiency 'constants'. If the 'nirvana' of a rationing system capable of predicting production response is to be attained, the need for more complex approaches is therefore suggested. This is the logic behind the development of models based on a prediction of the nutrients absorbed from the gut and their subsequent utilization by specific tissues in the animal (AFRC, 1998). If predictive models incorporating the complexities of nutrient metabolism are capable of predicting response, the challenge will be for them to maintain a practical basis at the level of feed-quality evaluation.

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6 The Measurement of Forage Digestibility *In Vivo*

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History

The first digestibility trials were conducted at the Weende Experimental Station at the University of Goettingen in Germany, and began before 1860 (Schneider and Flatt, 1975). Since then, a vast number of digestibility trials have been conducted throughout the world and reported in a range of scientific journals. A detailed account of the procedures involved in measuring digestibility *in vivo* was given by Schneider and Flatt (1975), while the special considerations involved in measuring forage digestibility were described by Minson (1981, 1990). The measurement of straw digestibility was described by Cottyn *et al.* (1989) and Givens *et al.* (1989). An overview of the subject was provided by McDonald *et al.* (1995).

Calculation of Digestibility

The proportion of a feed that is not excreted in the faeces is assumed to have been absorbed by the animal, and this is defined as the apparent digestibility of the feed. It is not the true digestibility, as in ruminant animals the methane that arises from the fermentation of carbohydrates is lost as eructation and is not absorbed (McDonald *et al.*, 1995). Also, in addition to undigested feed residues, the faeces contain enzymes and other substances secreted into the gut, which are not reabsorbed. Parts of the gut lining, which slough off as the feed passes through the gut, are also excreted in the faeces (McDonald *et al.*, 1995). The amount of this metabolic material that is unavoidably lost is directly proportional to dry matter (DM) intake, regardless of forage type (Minson, 1990). The amount of metabolic material secreted in the faeces has been observed to range from 0.098 to 0.129 g g^{-1} DM matter intake (Minson, 1990). Determining the true digestibility rather than apparent digestibility may be scientifically more accurate. However, no advantage has been demonstrated from the use of true digestibility in forage evaluation

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(Minson, 1990), as the loss of the metabolic secretions is a consequence of feeding the forage and therefore should be accounted for in the assessment of the forage's value to the animal.

A variety of means of estimating the digestibility of feeds have been developed, which are summarized here.

Direct method

To estimate the dry-matter digestibility (DMD) of a diet involves the complete collection over a period of time of all the faeces excreted by an animal once it has been adapted to a diet. The amount of feed dry matter consumed by the animal during this time is also recorded. This is the actual amount of dry matter consumed, and so is the difference between the amount of dry matter offered and the amount of dry matter rejected by the animal. The DMD is then calculated from the equation:

$$\text{DMD} = \frac{\text{DM intake} - \text{Faecal DM excreted}}{\text{DM intake}}$$

The digestibility of other feed fractions may be determined by substituting them for dry matter in the above equation. For forages, the proportion of digestible organic matter (OM) in the dry matter (DOMD or D value) is often calculated, as this gives a measure of the available energy in the forage dry matter. DOMD is calculated using the equation:

$$\text{DOMD} = \frac{\text{OM intake} - \text{Faecal OM excreted}}{\text{DM intake}}$$

Difference method

If a forage is being fed alone, then its digestibility may be determined using the direct method described above. However, it is necessary in many situations to supplement the forage with another feed, as described later ('Use of feed supplements'). This complicates the calculation of digestibility somewhat, and there are two main approaches that have been adopted to cope with this situation. In both approaches, it is assumed that there is no interaction between the feeds in terms of their digestibility.

The first approach is to feed the forage with another 'base feed' of known digestibility (McDonald *et al.*, 1995). The digestibility trial is conducted as normal, and the forage (the test feed) should constitute the major proportion of the diet. The DMD of the forage is then calculated using the equation:

$$\text{DMD of test feed} = \frac{\text{DM intake of test feed} - (\text{Faecal DM} - \text{Faecal DM from excreted base feed})}{\text{DM intake from test feed}}$$

The digestibility of the forage is therefore calculated by difference.

Regression method

The second approach is to feed the forage with a supplement (which may be another forage) at a number of different ratios between forage and supplement (Schneider and Flatt, 1975). The digestibility of each of these different diets is estimated. The diet's digestibility is then regressed with the proportion of the forage in the diet, and then the digestibility of the forage is estimated by extrapolating to zero inclusion of the supplement (1000 g kg^{-1} inclusion of the forage). This approach is illustrated in Fig. 6.1. In this example, the DMDs of four diets with forage inclusion rates of 200, 400, 600 and 800 g kg^{-1} were estimated. They were observed to be 0.72, 0.64, 0.56 and 0.48 respectively. When the inclusion rate of the forage was plotted against the observed digestibility, and the line was extrapolated to 1000 g kg^{-1} inclusion of the forage, it could be estimated that the digestibility of the forage was 0.40.

Givens *et al.* (1989) compared these three methods (direct, difference and regression) as a means of estimating straw digestibility. The largest variability was associated with the difference technique, while the direct method had the smallest variability when straw was fed *ad libitum*.

Indirect method

In some circumstances, particularly with grazing animals, it may not be possible to accurately measure either feed intake or faecal output, or both. The digestibility of the forage may still be estimated, if it contains a component that is known to be completely indigestible. This marker should also be unaltered during its passage through the gut, and it should be completely recovered in the faeces (Omed, 1986). If the concentrations of this component in both the feed and the faeces are

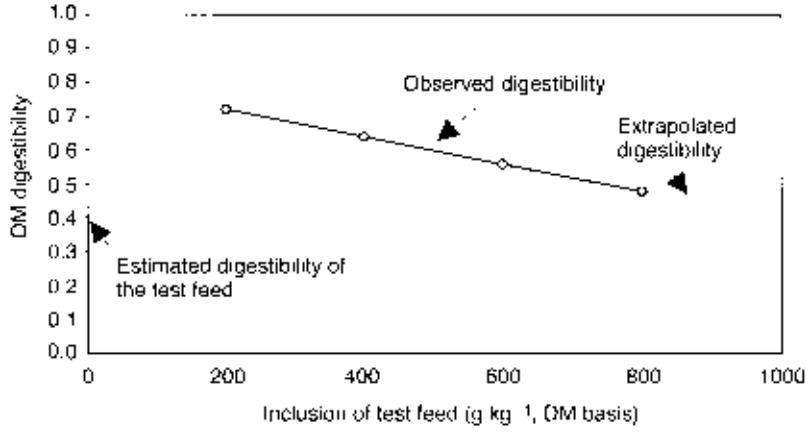

Fig. 6.1. Calculating the dry-matter digestibility of a test feed after feeding it with a supplement at different ratios of test feed:supplement.

then measured, the digestibility of the forage may be predicted. In this method, complete collections of faeces are not needed, so long as representative samples of faeces are collected. Representative samples of the forage that has been consumed are also required for the analysis of the marker.

The equation used to calculate digestibility in this instance assumes that all of the marker that is consumed is excreted. Therefore, at steady state:

$$\text{Amount of marker consumed per day} = \text{Amount of marker excreted per day}$$

$$[\text{Marker}]_{\text{Feed}} \times \text{Feed intake} = [\text{Marker}]_{\text{Faeces}} \times \text{Faecal output}$$

where marker concentrations are expressed in terms of g kg^{-1} DM and feed intakes and faecal outputs are expressed in terms of kg DM day^{-1}. Rearranging this gives:

$$\text{Faecal output} = \frac{[\text{Marker}]_{\text{Feed}} \times \text{Feed intake}}{[\text{Marker}]_{\text{Faeces}}}$$

and since:

$$\text{Digestibility} = \frac{\text{Feed intake} - \text{Faecal output}}{\text{Feed intake}}$$

then:

$$\text{Digestibility} = \frac{1 - [\text{Marker}]_{\text{Feed}} \times \text{Feed intake}}{[\text{Marker}]_{\text{Faeces}} \times \text{Feed intake}}$$

$$= 1 - \frac{[\text{Marker}]_{\text{Feed}}}{[\text{Marker}]_{\text{Faeces}}}$$

The marker may be a naturally occurring constituent of the feed, or it may be a chemical mixed into it. However, it is difficult to mix chemicals with forages, although the animals could be dosed with the marker each day. The disadvantage with this is that it increases the amount of handling that is required and adds to the stress of the animals. Constituents occurring in forages that have been used include lignin, acid-detergent fibre, indigestible acid-detergent fibre (IADF), acid-insoluble ash (AIA) (which is mainly silica) and some naturally occurring *n*-alkanes of long chain length (C-25–C-35). The possibility of using long-chain fatty acids has also been noted (Omed, 1986). The indicator most commonly added to feeds is chromium in the form of chromic oxide, Cr_2O_3, although a number of other markers, including dysprosium and granulated polyamide, have been used as well. The suitability of these markers is discussed later. One problem with external markers is that they can contaminate grazed forage and give biased results in future experiments. This was observed by Sprinkle *et al.* (1995) when chromic oxide was used as a marker.

The indirect, indigestible marker technique is easy to carry out and requires less work than estimating digestibility by the complete collection of faeces. If the concentration of the indicator in the feed is high, then this technique produces reasonably good results (Omed, 1986). However, the technique has limited usefulness in some circumstances, and it should only be used if there is a high possibility

of obtaining a representative sample of the forage consumed (Omed, 1986). There are three main sources of error associated with this method. The first is the estimation of the marker's concentration in the forage, if an internal marker is used. This error may arise either from the sampling of the herbage, or from analytical errors. Analytical errors may also be associated with the estimation of the marker concentration in the faeces. It was noted by Newman *et al.* (1998) that analytical errors in estimating the marker concentration in the forage and the faeces may tend to cancel each other out. However, most markers are not completely indigestible, and this third source of error may give rise to very unreliable estimates of digestibility.

Obtaining a representative sample of the forage is difficult, as grazing animals, particularly sheep, can be highly selective in the forage they consume. One approach that can be adopted is to fistulate the animal's oesophagus. A sample of oesophageal extrusa may then be taken and analysed for the indicator to estimate the concentration of the marker in the forage actually consumed.

Prediction of forage digestibility

This method relies on the observation that certain faecal components are related to the digestibility of the diet. If the digestibility of the diet has been measured using the direct method and the faeces from these estimations have been analysed for a particular component, then a regression may be established between the concentration of that component in the faeces and the observed digestibility *in vivo*. In subsequent experiments, spot samples of faeces may be taken (for example, in a grazing experiment), the faeces analysed for the particular component and, from these data, the digestibility of the grazed herbage may be predicted (Omed, 1986). A range of different faecal components have been used, including nitrogen, chromogen, crude fibre and methoxyl. The suitability of these markers will be discussed later.

The technique works better when a local regression is established, using either the same or very similar herbage to that under investigation (Omed, 1986). The general equations that have been established are less precise than expected (Omed, 1986), because herbage factors are a major source of variation.

Conducting a Digestibility Experiment

To obtain an accurate estimate of the digestibility of a forage, it is important that well-established techniques are adopted and also that the objective of the experiment is clearly defined, as this will affect the amount of forage that is fed and what supplements, if any, are included in the diet.

Experimental design

Although it is usually considered that the variation in digestibility between animals is low (Cottyn *et al.*, 1989), the accuracy of the estimation will be improved if some

form of crossover design is used. If there is likely to be an effect of time period, then a Latin-square design should be employed. This is particularly true with forages if they are being collected daily and are likely to change in composition during the experiment. If the animals are changing in physiological state during the experiment – for example, if they are lactating or actively growing – then a Latin-square design is also appropriate. However, the physiological changes likely to be encountered in mature wether sheep are small and, if these are the experimental animals, it is questionable what benefit there is in using a Latin square. Although Raymond *et al.* (1954) suggested there may be a decrease in digestive ability during winter, this has not been confirmed. Evans and Potter (1984) observed no effect of time period on the digestibility of cocksfoot grass (orchard-grass) fed to wethers. The advantage of not using a Latin square in this type of experiment is that an extra $(n - 1)$ degrees of freedom are gained, where n is the number of time periods used in the experiment.

The adaptation period

There are two stages to the digestibility trial, the first being the adaptation period and the second the collection period. The adaptation period is designed to ensure that a stable population of rumen microflora has become established, that the animals are eating approximately the same amount of feed daily and at the same time and that the residues being excreted in the faeces arise from the digestion of feed used in the experiment (Omed, 1986).

The variation in daily faecal output is reduced if the animals are fed at the same time each day. The more frequently they are fed, the smaller the variation in faecal output and so, ideally, animals should be fed hourly (Blaxter *et al.*, 1956). However, Raymond *et al.* (1953a) observed that there was little evidence that the frequency of feeding affected the digestibility of the feed, although it might affect the efficiency of utilization of digested feed. Ideally, if animals are only fed twice daily, this should be at 12 h intervals to reduce variation in faecal output (Schneider and Flatt, 1975). This is rarely done, and it is more usual for animals to be fed at the beginning and end of the working day. If this compromise is adopted, it is important that the feeding times are strictly adhered to (Schneider and Flatt, 1975).

The length of time for the animal to be adapted to its diet varies from 4 to 12 days. Normally, it has been shown that 6–8 days are required (Omed, 1986). Chenost and Demarquilly (1982) reported that an adaptation period of 10–14 days was required for the animals to obtain maximum level of intake, but this period could be reduced to 7 days if the animals were familiar with the forage. However, with straw, an adaptation period of 14 days was advocated by Cottyn *et al.* (1989). During this adaptation period, the animals should also be introduced to their digestibility cages if they are being confined (Omed, 1986).

Collection of faeces

Following the adaptation period, there is a collection period, in which the faeces are collected from each experimental animal. During this collection period, representative samples of the feed are also taken and the amount of feed refused is recorded. Representative samples of the refusals are also taken and analysed, so that the amount of each nutrient that was actually consumed may be calculated.

Complete collection of faeces

A complete collection of all the faeces excreted is required if the direct, difference or regression methods are being used. The faeces should not be contaminated with urine, and the collection should be made at the same time each day. The faeces taken each day should be bulked over the whole collection period and a sample taken for analysis. If sheep are used, then there is no need to subsample during the collection period. However, with cows, subsamples can be taken each day (*c*. 10% by weight) and added to the bulked sample (Minson, 1981).

Spot sampling of faeces

Spot samples of faeces are required if the indirect or prediction method is being used. Samples should be taken at least once and preferably twice a day. However, no difference in the estimate of digestibility was observed when samples were taken twice a day as opposed to four times a day when *n*-alkanes were used as a marker (Malossini *et al.*, 1994). If there is diurnal variation in the excretion of the marker (as is the case with chromic oxide), however, more frequent sampling is required.

Spot samples of faeces may be taken from each animal by taking a grab sample from the rectum. This does require that the animals are handled and can cause the animals stress. If they are grazing, then samples of the faeces can be taken from the field. The animals can be dosed with a coloured dye so that the faeces from individual animals can be identified. If this approach is adopted, it is important that the dye selected does not affect the digestibility of the forage or interfere with the marker being used to estimate digestibility.

The length of time of the collection period varies with the nature of the diet, shorter collection periods being required for more uniform diets. Typically, a period of 4–12 days is chosen, but the accuracy of the measurement increases with the length of the sampling period (Blaxter *et al.*, 1956). A collection period of 10 days is necessary for straw (Cottyn *et al.*, 1989).

Apparatus used in digestibility trials

Pens

Animals that are being housed for a digestibility trial need to be penned separately so that they can be fed individually. The arrangement of the pens and the design of the feed boxes need to be such that animals cannot reach the feed in their neighbour's pen. The walls of the feed box also need to be sufficiently high to minimize the amount of feed lost by animals, particularly cows, throwing their feed around. If the feed box is also designed so that the animal's head is restrained while it is feeding,

this will reduce the amount of feed that is pulled back into the pen, where it may also be lost. This may mean that the animal needs to be tethered during the digestibility trial. The inside of the feed box should be smooth, so that no feed can lodge in crevices.

Clean, fresh drinking-water should always be available, either via an automatic drinking bowl or by a water bucket that is filled two or three times a day.

Meshes

A metabolism cage that allows the separation of faeces and urine was described by Schneider and Flatt (1975). The animal stands on a mesh floor, which is situated over a screen that is small enough to retain the faeces. The faeces then collect on this screen, while the urine falls through to a sloped or funnelled pan, which directs it into a collection bottle below. The screen on which the faeces collect is removable, so that the faeces may be gathered from it each day. The disadvantage of this type of crate is that the faeces fall over a wide area on the screen below the false floor and become exposed to contamination by urine. Faeces that fall on the floor of the crate may also be trampled and ground by the animal, so that the small particles then pass through both screens (Schneider and Flatt, 1975). This problem may be partly overcome by reducing the size of the cage so that the animal cannot turn around. With male animals, the urine will then tend to fall in the middle of the cage and the faeces around it.

Bags

Instead of using meshes to separate the faeces and urine, bags attached to harnesses that are fitted to the animal may be used. Faeces collect in the bags, which are emptied once or twice a day, or more often if necessary. Bags may be made of canvas, although this is unsuitable if the feed is likely to produce wet faeces (Raymond *et al.*, 1953a). In this case, a rubber or rubberized canvas bag is better, although this will perish with time. Bags may also be lined with plastic bags, which facilitates the sample collection. The bag may be attached to the harness by clips, which are unfastened when the faeces are collected. Alternatively, the bag may be attached to the harness by buckles and a zip-fastener fitted to the bag (if it is made of canvas). Faeces are then collected by simply unzipping the bag and emptying the faeces into a bucket. The harness itself may be made of webbing or leather and consists of two straps, which go round the animal's girth (just behind the shoulders) and just in front of the flank. A strap runs along the animal's back and attaches the bag to these two straps. Smaller straps hold the bag in place by anchoring it to the rear strap across each side of the hindquarters. Care must be taken in fitting harnesses to ensure that they do not rub or chafe. The animals should be adapted to these harnesses for some days before the collection of faeces begins.

A bag of this type is suitable for collecting faeces from wether sheep. A much larger bag is needed for collecting faeces from steers. The advantage of these bags is that they can be used for grazing animals as well, although the presence of the bag may affect their grazing behaviour. With female animals, a plastic tongue needs to be attached above their vulva to direct faeces away from the urine. The faeces may then either be collected in a bag, or in a separate trough if the animals are penned.

Selection of animals

Obviously, the animals used in any digestibility trial should be healthy and, if they have been grazed, they should be wormed. They should be checked before being selected for a digestibility experiment and, in the case of sheep, their feet and teeth in particular should be examined (Givens and Moss, 1994). They should also be used to being handled and, if complete collections of faeces are being made indoors, they should be penned and handled before the beginning of the experiment (Schneider and Flatt, 1975). If they are to wear bags and harnesses, then they need to be adapted to wearing these.

Species

A wide range of species have been used to estimate forage digestibility, and these include golden hamsters, rabbits, goats, sheep and cattle (Minson, 1990). Equids, such as horses, donkeys and mules, have also been used, as have camels. The advantage of the smaller species is that they consume less feed and are therefore less expensive to keep. However, their digestive tract may be very different from the target species. If an estimate of forage digestibility by cattle is required, sheep can be used, and frequently are. Cottyn *et al.* (1989) conducted a number of experiments using maize silages, grass silages and grass hays to investigate whether cattle and sheep differed in their digestive capacity. They concluded that cattle digest poor-quality forages more efficiently, whereas sheep digest high-quality forages more efficiently. They suggested that, for most temperate forages, the difference in digestive capacity between the two species was so small as to be of no practical significance. However, they did suggest that, if sheep were used to estimate forage digestibility, a positive correction should be applied for organic-matter digestibilities (OMDs) below 0.65 and a negative correction for OMDs over 0.80 when predicting the digestibility of the forage by cattle. The size of the applied correction (Y) was:

$$Y \text{ (as a proportion)} = 0.348 - (0.0048 \times \text{Sheep OMD})$$

where Sheep OMD is the observed OMD of the forage by sheep.

A lack of data prevented these authors from providing similar correction factors for other feed fractions, although they did note that protein was usually better digested by sheep than by cows. They suggested that, for grass silage, grass hay and many mixed rations, a mean difference of 0.07 between sheep and cows should be used, as proposed by Van Es (1978). There appears to be no difference in the protein digestibility of maize silage by cows and sheep (Cottyn *et al.*, 1989).

Sex

Male animals are generally preferred, as it is much easier to separate the faeces and urine. However, if the digestibility of forage at high planes of nutrition is required, then high-yielding dairy cows should be selected (Schneider and Flatt, 1975).

Age

The age of the animal appears to have little, if any, effect on DMD. Raymond *et al.* (1954) observed small differences due to age, but acknowledged that they were

unlikely to be significant. They did observe some evidence that the efficiency of digestion in sheep increased at the rate of 0.01 unit (when digestibility is expressed as a proportion) per year up to the age of 2 years, with a further, smaller increase between 2 and 3 years. However, Givens and Moss (1994) observed that neither the age nor the body weight of sheep made any significant difference to the digestibility of dried grass, when sheep ranging in age from 3 to 8 years were studied.

Breed

The effect of breed on the DMD of dried grass was investigated by Givens and Moss (1994). They observed a significant (although small) difference between breeds of sheep, with the DMD of dried grass being 0.03 units higher with Cheviot sheep compared with Suffolk × Mule sheep. No significant differences were observed by Ranilla *et al.* (1998) in the DMD and fibre digestibility of good-quality forage at maintentance when fed to either Churra or Merino sheep.

Number

The number of animals that should be used depends on the expected difference between test feeds and on the degree of confidence that is required. Raymond *et al.* (1953a) cited Schneider and Lucas (1950) as stating that the greatest variation in the estimation of digestibility coefficients was between experiment and between experimenter. These authors calculated that the between-animal variation was quite small. However, Raymond *et al.* (1953a) presented a table that provided the number of replicates needed to detect differences between feeds when using a *t* test to analyse differences. These data are presented in Table 6.1.

These numbers could be reduced if a crossover design was used, as the between-animal variation could then be removed. Cottyn *et al.* (1989) noted that, if the feedstuff under investigation was fed alone (and the direct method was used), then three animals were sufficient. However, if it was fed in combination with another feed (and the difference method was used), then four, six or eight animals were required if the test feed constituted 50, 25 or less than 25%, respectively, of the ration.

Table 6.1. The number of replications that are required to obtain a significant result ($P < 0.05$) in a digestibility experiment using Student's *t* test (from Raymond *et al.,* 1953a).

True difference, σ (proportional digestibility)	Number of replications needed to detect σ with a probability of	
	0.8	0.9
0.010	28	38
0.015	14	17
0.020	8	11
0.025	6	7
0.030	4	6
0.040	3	4
0.050	3	3
0.100	2	2

Feed used in the experiment

If complete collections of faeces are being made, it is important that the amount and composition of the feed that is consumed in the experiment are accurately measured. If the indirect method is used, an estimate of the composition of the forage actually consumed is needed, as is an accurate estimate of the concentration of the marker in the consumed forage.

Use of feed supplements

The objective of the digestibility trial is usually to estimate the digestibility of a feed when offered to a healthy animal with a stable rumen. It is therefore important to ensure that the animal's health is maintained throughout the trial and that the diet is balanced. The diet should therefore be supplemented with an adequate mixture of vitamins and minerals. If the OMD of the feed under investigation is below 0.55 or greater than 0.75, then it cannot be fed alone, as to do so would jeopardize the health of the animal (Cottyn *et al.*, 1989). Even if the feed's digestibility is between 0.55 and 0.75, its physical structure may not maintain a healthy rumen function and it should be supplemented. It may also be the case, if high-yielding dairy cows are being used, that a forage may not be able to meet all of the cow's requirements, and supplementation is again required. Experiments investigating straws are particularly difficult, as the forage is so deficient in major nutrients, such as nitrogen (N) and sulphur (S). Cottyn *et al.* (1989) recommended that experimental diets should contain at least 200 g kg^{-1} long forage to prevent an abnormal fermentation, and that they should contain no more than 50 g kg^{-1} DM unsaturated fat and 100 g kg^{-1} DM saturated fat. They further recommended that the diets should contain (on a DM basis) at least 180 g kg^{-1} crude fibre, between 120 and 200 g kg^{-1} crude protein, less than 400 g kg^{-1} starch and less than 200 g kg^{-1} sugars. This means that, for many forages, some form of supplementation is required. The ways in which the digestibility of the supplement may be taken into account when calculating forage digestibility were described above ('Difference method' and 'Regression method').

Level of feed intake

The digestive capacity of animals tends to decrease as the level of feed intake increases. The digestibility of a range of concentrate ingredients was observed to decrease as feed intake was increased from maintenance to twice maintenance (Woods *et al.*, 1999). Givens (1990) noted that OMD decreased by 0.014 units per multiple of maintenance. However, Cottyn *et al.* (1989) concluded that the effect of increased feed intake had little or no effect on the digestibility of long or coarsely chopped forages.

Traditionally, digestibility trials required the feeding of exact quantities of feed over long periods of time. However, Minson (1981) raised the question as to whether, when comparing two forages, the same amount should be fed or whether each should be fed *ad libitum*. Blaxter *et al.* (1956) argued that feeding *ad libitum* necessarily meant that refusals would be left, and the assumption of steady state was invalid if there were refusals. Blaxter *et al.* (1956) therefore concluded that intakes should be limited during digestibility experiments to ensure that no refusals were

left. However, Minson (1981) pointed out that feeding close to *ad libitum* levels was a closer approximation to what would occur in the field. He therefore advocated feeding close to *ad libitum*, but did not remove all refusals each day. This 'recycling' of refusals was designed to reduce the amount of selection of forage by the animals. An alternative approach was adopted by Zemmelink (1980), who recommended offering animals four levels of feed rejection. This produced response curves between digestibility and the amount of excess forage offered. From these data, an estimate of the plant's digestibility, depending on the extent of selection the animal was able to practise, could be estimated. Laredo and Minson (1973) also suggested that the leaf and stem could be mechanically separated and then offered separately. However, this is a laborious process that is not easily mechanized. The leaf dust is lost, and the leaf fraction tends to contain leaf sheath and seed heads, which reduce the observed digestibility of the leaf fraction (Minson, 1981).

The object of the experiment must therefore be borne in mind when selecting the appropriate level of feed intake. If the objective is to estimate the digestibility of the whole forage, then feeding should be restricted to maintenance levels, which also has the advantage of being easier to standardize (Cottyn *et al.*, 1989). If the objective is to estimate the digestibility of the forage in a situation where the animal is able to practise a degree of selection, then the amount offered should be closer to *ad libitum*. However, even if this selection is allowed, it is not clear what relationship there is between the forage selection practised by grazing and penned animals (Minson, 1981).

Preparation of feeds

The forage that is offered to animals in a digestibility trial is chopped and mixed to ensure that representative samples of the forage are offered. However, this preparation may in itself affect the digestibility of the forage, although the effect is likely to be very small.

The forage offered to an animal in a digestibility trial may be either fed fresh or harvested before the experiment and preserved by drying or freezing it. It cannot be ensiled to preserve it, unless the object of the experiment is to estimate the digestibility of the ensiled rather than the fresh forage. Alternatively, the animal may be allowed to graze the forage, and the faeces must then be collected from the animal in the field. Each approach has both advantages and disadvantages, and none of them are ideal solutions.

With forages, the upper parts of the plant are more digestible than the lower parts, and animals will therefore tend to select the upper parts. This should be borne in mind when cutting forage for animals in a digestibility trial. It is also a factor that should be considered when sampling pasture that is being grazed by animals in a digestibility trial.

FRESH FORAGE. If fresh forage is to be fed to penned animals, then it needs to be cut each day. This does mean that any change in the composition of the forage during the period of the study may affect the estimation of the forage's digestibility, as the forage will not be of consistent composition throughout the experiment. Since the estimation of digestibility *in vivo* assumes that a steady state has been attained and that feed of the same composition is being fed throughout the experiment, this is a

potentially serious flaw in this experimental design. Cutting large quantities of forage can also be difficult when the ground is waterlogged, and it is not possible to compare forages grown in different years or at different times of the year (Minson, 1990).

However, if forage is to be cut daily, then it was recommended by Minson (1981) that forage was cut in the afternoon, and that sufficient forage was cut for both that evening's feed and also the following morning. Cutting in the afternoon avoids contamination of the forage with morning dew. The forage that is to be stored overnight should be kept on dry ice, as the carbon dioxide inhibits further tissue metabolism. Refrigerating the forage in the absence of dry ice is less satisfactory (Minson, 1981). An alternative means of keeping the forage overnight is to freeze it quickly, and then remove it from the freezer the following day and feed it (Raymond *et al.*, 1953b). This does mean that the forage will still be frozen when it is fed, which is likely to increase the amount of feed refused. This is likely to affect the estimated digestibility of the feed. It was pointed out by Minson (1981) that, if neither dry ice nor refrigeration was available, then the forage needed to be cut twice daily.

FREEZING. Harvesting all of the forage that is needed for the experiment beforehand is preferable to cutting fresh forage daily. This is because the composition of the forage will then not change during the experiment. After the forage has been harvested, it should be mixed thoroughly, bagged and frozen. The bags should contain enough forage for 1 day's feed per animal, and should be removed from the freezer the day before they are to be used. The effect of freezing forage has been observed to have negligible effect on the estimation of the DMD of both tropical and temperate forages (Raymond *et al.*, 1953b; Minson, 1990), although the composition of the feed will change to some extent and this has been observed to affect nitrogen digestibility.

DRYING. This method can also be used to preserve forages for use in a digestibility trial. As with freezing, it has the advantage of ensuring that the composition of the forage used in the experiment remains constant. It is important that the forage is dried rapidly, and that the temperature does not exceed 100°C (Minson, 1990). If the temperature rises above 100°C, then the forage will be damaged, because of Maillard reactions. If the temperature is below 100°C, the forage does not dry quickly enough, and this usually results in a decrease in DMD compared with fresh forage (Minson, 1990). It should also be noted that drying changes the physical structure of the forage, and the forage's physical structure can be an important component of its nutritive value.

It was recommended by Minson (1981) that forage should be wilted and then dried for 6 h at 100°C. The forage should then be blown with cold air for 8 h. High-temperature driers should not be used, as these cause a reduction in crude protein digestibility.

FREEZE-DRYING. This is a very good means of preserving forage, as it minimizes the changes in the chemical composition of the forage and removes the need to maintain cold storage facilities. However, freeze-drying is an extremely expensive means

of preserving forage and, as with conventional drying, the physical nature of the forage is changed during this process.

GRAZING. Instead of harvesting the forage and then offering it to the animal, the animal can be allowed to graze the material instead. This has the advantage of keeping the animal under more 'normal' conditions, since fresh forage will normally be grazed. However, the disadvantages of feeding cut fresh forages to the animal are also relevant to the grazing situation. The composition of the grazed forage will change during the experiment, and it is not possible to compare forages from different years or different seasons. It is also not possible to measure intake directly, and so either the indirect method of estimating digestibility must be used or some estimate must be made of the amount of forage consumed.

ENSILING. During the ensiling process, there are considerable changes to the chemical composition of the forage. Ensiling cannot therefore be used as a means of preserving forage for use in a digestibility trial if the objective of the experiment is to estimate the digestibility of fresh forage. However, if the digestibility of ensiled forage is to be estimated, then the silage should be mixed well before the experiment begins and stored frozen, as the composition of the silage can change throughout a clamp and its composition will also change with time.

Markers used in the indirect method of estimating digestibility

As was mentioned before, a range of markers has been used to estimate diet digestibility indirectly. The qualities of these markers are described here.

Silicon

Silicon is present as solid silica and monosilicic acid, and the ratio between these two species varies with plant species and plant maturity (Omed, 1986). Silica was first used as an internal marker in 1874, and its determination may now be carried out either colorimetrically or by atomic absorption spectroscopy (Omed, 1986). Recovery rates of silica close to 100% have been reported, and a good agreement between the digestibility predicted using silica as a marker and that observed by a complete collection of faeces has been observed (Duksin and Drozdenko, 1982). However, there is some evidence to suggest that a proportion of the silica is absorbed from the digestive tract and is excreted in the urine or found in the lymph nodes and urinary calculi. It has also been reported by Van Dyne and Lofgreen (1964) that silica can accumulate in the gut, which resulted in a variable recovery rate and a less accurate estimation of digestibility compared with lignin (Omed, 1986). Silica is not widely used as an indicator now, because of its possible absorption from the gut, and the risk of contamination of the herbage with soil which invalidates the estimated intake of silica. There is also some doubt about the accuracy of silica determination (Omed, 1986).

Acid-insoluble ash

Minson (1990) described work using ash insoluble in hydrochloric acid as a marker. Good recoveries of AIA and good agreement between *in vivo* OMD and predicted

digestibility were reported. A good prediction of digestibility was also observed in horses by Miraglia *et al*. (1999). However, unsatisfactory results were reported in other studies, with reported recoveries of AIA being 127% (Minson, 1990). The use of AIA was observed to be inferior to acid-detergent fibre methods of predicting DMD (Minson, 1990), although Pereira and de Queiroz (1997) reported a recovery of AIA of 101%. Dos Santos and Petit (1996) noted that, compared with AIA, a controlled-release bolus of chromic oxide gave a much better estimate of faecal output.

Lignin

Lignin is quite widely used as an indicator in digestibility trials and is regarded as a good indicator by many authors (Omed, 1986). However, there are a number of reports which provide evidence that lignin is not completely indigestible. The poor recovery of lignin in horses led Miraglia *et al.* (1999) to suggest that it was an inappropriate marker for measuring digestibility. Mauricio *et al.* (1996) observed that the estimated digestibility of dry matter, crude protein, neutral-detergent fibre (NDF) and gross energy was about 0.05 units lower using lignin as a marker, rather than making a total collection of faeces. Pereira and de Queiroz (1997) observed a recovery of lignin of only 58.3%. Van Soest (1994) outlined a number of factors that could affect the calculated recovery of lignin. These factors were: first, that non-lignin material in the feed could contaminate the lignin in the assay procedure; secondly, that some losses of immature lignin may occur; thirdly, that soluble phenolic matter may form; fourthly, that drying temperatures may affect faeces and herbage samples differently; and, finally, that some finely divided lignin in the faeces may not be recovered in gravimetric methods. It is also difficult to obtain a representative sample of the actual intake (Meijs, 1981). Many workers therefore have little confidence in the use of lignin, although Van Soest (1994) maintained that the technique worked well if the diet dry matter consisted of more than 60 g kg^{-1} DM lignin. It was also noted that, as the lignin content of the diet increased, the standard error of its determination decreased.

Chromogen

Chromogen is a plant pigment that is soluble in 85% aqueous acetone and consists mostly of chlorophylls and their degradation products (Omed, 1986). There have been reports of high (100.5%) recoveries of chromogen in the faeces and a very good agreement between the estimate of digestibility using chromogen and that observed from a complete collection of faeces (Omed, 1986). However, less satisfactory results have also been reported, particularly with autumn and winter forage (Omed, 1986). There are other difficulties associated with this technique as well. Chromogen is unstable in light, and so light must be excluded during the extraction and measuring processes. The use of the technique is also limited to forages with a high chlorophyll concentration, and this restricts its suitability to fresh, green herbage. The technique also requires standardization with indoor digestibility trials, and the wavelength required to measure the concentration of the pigment in the faeces varies with different plant species and stages of growth (Omed, 1986). These drawbacks have resulted in chromogen no longer being used to estimate digestibility (Minson, 1990).

Indigestible acid-detergent fibre

Since a proportion of the cell wall is digestible, the use of lignin as a marker has been questioned. If the potentially digestible fractions of the lignin and cellulose could be removed, the indigestible residue could be used as an indicator. The IADF fraction was studied by Penning and Johnson (1983) as an indicator for estimating digestibility. IADF was estimated by treating the forage and faeces with acid-detergent solution and then treating the residue with cellulase. The remaining fraction was considered to be indigestible cell wall, and Omed (1986) reported that Penning and Johnson (1983) observed that IADF produced satisfactory results over a range of samples. This was also observed by Dove and Coombe (1992). However, Minson (1990) noted that, in another study, only half the marker was recovered in the faeces and in further work the recovery was variable. The technique requires further development and validation to ascertain what causes these variable recoveries, but the method is simple to perform (Omed, 1986).

An alternative means of analysing IADF is to incubate the sample in the rumen for extended periods of time. Huhtanen *et al.* (1994) tried incubating samples for 228 h *in situ* or for 96 h in rumen fluid *in vitro*. The estimation of digestibility that these markers gave was very poor (Huhtanen *et al.*, 1994). However, Fondevila *et al.* (1995) suggested the use of rumen-undegradable NDF or dry matter as an internal marker. They observed that the estimated digestibility with these markers gave a correlation coefficient, with digestibility measured by total collection, of 0.80.

Long-chain n*-alkanes*

The long-chain *n*-alkanes of uneven chain length (C-19–C-35) found in the cuticular wax of forage plants can serve as markers and are increasingly used in the estimation of herbage intake. The recovery of these markers in the faeces increases with increased chain length, although even C-35 has a recovery of only 93% (Mayes *et al.*, 1986). This may limit their usefulness as markers for estimating digestibility, and it was concluded by Newman *et al.* (1998) that estimates of digestibility using *n*-alkanes were unlikely to be reliable. However, Dove and Coombe (1992) observed that the use of them accurately estimated digestibility, in a way that lignin did not. Another advantage with *n*-alkanes is that no cyclical pattern in their excretion has been observed (Mayes *et al.*, 1986). This would reduce the error of estimating faecal alkane concentration by taking only spot samples of faeces. The determination of *n*-alkanes is more accurate than that of lignin, and they are stable, whereas chromogen is not.

Chromic oxide

Chromic oxide is often used as an external marker. If it is added to the ration, it must be mixed in thoroughly, but if it is used in the powdered form it will separate from coarse feeds (Schneider and Flatt, 1975). It will then travel at a different rate through the gut compared with the rest of the diet. It can be mixed with paper pulp and ground to a similar particle size as the coarse feeds, and it will then mix better with the diet. This mixing of the chromic oxide with the feed is not possible in a grazing situation, of course. In this case, the animals need to be dosed with the chromic oxide. This can be achieved with ruminally cannulated animals by insert-

ing chromic oxide preparations into the rumen. J.F.D. Greenhalgh developed a means of impregnating paper with chromic oxide which releases the chromium more slowly into the gut. Alternatively, gelatin capsules containing chromium can be used. Luginbuhl *et al.* (1994) observed that, with a controlled-release bolus containing chromic oxide, a constant faecal excretion of chromium was observed after 8 days. A sharp peak in chromium excretion was then observed about 27 days after administration, and complete excretion was observed after 31 days in seven out of 24 lambs. The accuracy of estimation of digestibility did depend on the diet. It was very poor when a commercial pelleted diet was fed, but very good when lucerne hay was fed (Luginbuhl *et al.*, 1994). The use of a slow-release bolus of chromic oxide resulted in a grab sample taken once providing a reliable ($r = 0.96$, $P < 0.05$) estimate of faecal output (Dos Santos and Petit, 1996). However, a more frequent sampling regime would be advisable.

The recovery of chromic oxide is often not complete (Schneider and Flatt, 1975). If the true recovery rate can be estimated in a separate experiment, then the calculated digestibility can be corrected using this true recovery rate. Another difficulty with chromic oxide is that it exhibits diurnal variation in its excretion pattern (Schneider and Flatt, 1975). This means that random sampling of the faeces may not provide a truly representative sample of the faeces. The diurnal variation is reduced if the chromic oxide is administered more frequently (at least twice a day) or if its release into the gut is continual (by the use of chromic-oxide-impregnated paper or a bolus). It is also important that it is administered for at least 10 days before faecal samples are taken (Schneider and Flatt, 1975). Faeces should also be sampled frequently (at least four times a day) to obtain more representative samples.

When analysing samples for chromium, a calibration curve is required. This should use faecal samples from animals not ingesting chromium. These faecal samples can then be spiked with known amounts of chromium to generate the calibration curve. However, it is essential that these faecal samples are obtained from animals grazing the same forages as those used by the animals dosed with the chromium (Holt, 1993).

Granulated polyamide

Mahler *et al.* (1997) investigated the use of granulated polyamide as a marker. They administered the marker via gelatin capsules every 12 h, and observed that 4 days were needed to obtain a steady state. The marker recovery was 98% and was not affected by diet type or the level of feed intake, unless the diet contained less than 9.26 g N kg^{-1} OM. A good relationship was observed between digestibility calculated using this marker and that observed by making a total collection of faeces. To use this marker, a sampling schedule is needed that takes samples throughout the day (Mahler *et al.*, 1997), but the advantage of this marker is that it is inexpensive and does not require sophisticated analytical techniques.

Markers used in the prediction technique of estimating digestibility

The most commonly used marker in this technique is nitrogen, although a range of other markers have been investigated as well.

Nitrogen

Minson (1990) noted that some of the first equations for predicting DMD from the N concentration in faeces were published by Gallup and Briggs (1948). When regressions were based on a wide range of temperate forages, the residual standard deviation varied between ±0.029 and ±0.057 (Minson, 1990).

However, the regression equations developed from forage cut at different times of the year are very different (Minson, 1990), because of the higher concentration of N in autumn forage and the difference in regressions for leaf and stem fractions. There is some evidence that the application of N as fertilizer may affect the relationship, as the N content of the pasture may be increased without affecting its digestibility (Omed, 1986). In other circumstances, if animals are grazed on sparse pasture, they may ingest large amounts of soil, which causes an increased secretion of metabolic N. High concentrations of faecal N are therefore recorded and so predicted digestibility increases, although the actual digestibility may be depressed (Omed, 1986). The major disadvantage of this technique, however, is that there is no single general equation that is suitable for a wide range of forages under different conditions. Many equations have been reported, but each one is valid only for the particular set of circumstances under which it was produced (Omed, 1986). Some of the variation in these relationships is illustrated in Fig. 6.2. Despite these drawbacks, the technique can produce a reasonably accurate estimation of digestibility, and Omed (1986) noted that it had been observed that 93% of the variation in *in vivo* digestibility could be explained by the concentration of faecal N.

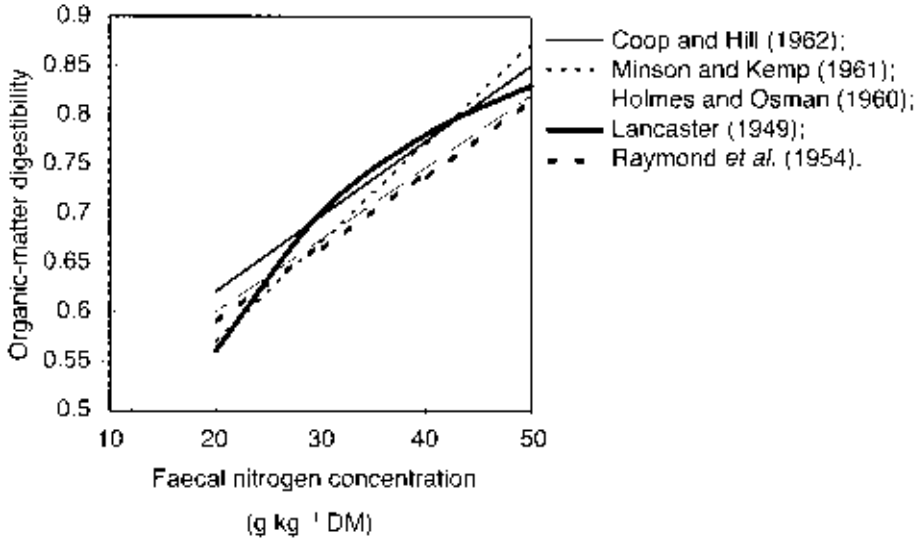

Fig. 6.2. Observed relationships between organic-matter digestibility and faecal nitrogen concentration for temperate forages (from Minson, 1990).

Chromogen

A prediction equation may also be established between faecal chromogen concentration and digestibility. However, with temperate forages in the UK and New Zealand, it was observed that the residual standard deviation of this relationship was greater than regressions based on faecal N (Minson, 1990). The regressions have a seasonal bias similar to that observed with faecal N, and it is now unusual for faecal chromogen to be used to predict digestibility (Minson, 1990).

Fibre

The digestibility of temperate forages is negatively related to the concentration of crude fibre in the faeces. The regression has a residual standard deviation only slightly greater than that of faecal N (Minson, 1990). However, as feed intake is increased, the faecal fibre concentration decreases as more nutrients are excreted and dilute the fibre. The decreased faecal fibre concentration results in a predicted increase in digestibility, although digestibility has in fact decreased. This results in a large bias in the predicted digestibility of grazed forage (Minson, 1990). However, faecal NDF was observed to be well related to the digestibility of both dry matter ($r = 0.93$) and organic matter ($r = 0.87$) in the dromedary camel (Abdouli *et al.*, 1992).

Methoxyl

The lignin molecule contains methoxyl groups, and it was noted by Minson (1990) that the faecal methoxyl content was significantly and negatively related to DMD. However, faecal methoxyl concentration does not appear to be used for routine forage evaluation (Minson, 1990).

Preparation of samples for analysis

It is important that samples of feeds and faeces are dried quickly to get an accurate measure of DMD, as slow drying increases the loss of dry matter (Raymond and Harris, 1954). However, if they are dried at too high a temperature, then volatile compounds will be lost, and this will also lead to inaccuracies in the estimation of dry matter. Silages are particularly at risk from loss of volatiles by oven-drying. Drying at too high a temperature may also affect the estimation of fibre fractions in the feed and faeces, and this will affect the estimation of fibre digestibility. Usually, feeds and faeces are oven-dried at 60°C and, following the estimation of dry and organic matter, they may be ground through a 1 mm screen pending further analysis. However, the N and volatile fatty acid content of silage needs to be estimated using a fresh sample, and the gross energy content of forages should be determined using samples of fresh forage that have been chopped under liquid nitrogen.

Accuracy of Estimating Digestibility

The estimation of digestibility *in vivo* is quite accurate, with a standard error of only 0.015 units for most feedstuffs, although this increases to 0.030 for straw (Cottyn *et al.*, 1989).

However, it is important that digestibility trials are conducted with care. For example, if lambs are fed 800 g day^{-1} hay (850 g DM kg^{-1}), and excrete 500 g day^{-1} faeces (400 g DM kg^{-1}), the DMD of hay is 0.706. A 10% error in the weighing out of the feed or in the estimation of its DM content would result in a change in the estimated digestibility to either 0.673 or 0.733. A similar error in the weighing of faeces or its estimation of DM content would change the estimated digestibility to either 0.735 or 0.676. In experiments where the differences between treatments are small, this error would result in no significant difference between treatments being observed.

Relevance of *In Vivo* Measures of Digestibility

The estimation of the DMD of forage does give some measure of the availability of energy and other nutrients, although it ignores the loss of energy in the form of methane. As a means of evaluating forages, it is labour-intensive and time-consuming and requires large quantities of the forage concerned. It also relies on the use of animals. It is therefore not appropriate as a means of routinely evaluating forages. However, it is needed as a technique to validate many of the *in vitro* procedures that have been developed, although it provides no information on the site of digestion, the kinetics of digestion or the supply of nutrients to the animal.

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7 Faeces as a Source of Microbial Enzymes for Estimating Digestibility

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The Need for an Alternative to Rumen Fluid

Waentig and Gierisch (1919), cited by Hungate (1966), suggested that *in vivo* digestibility could be predicted from *in vitro* procedures that re-created the conditions of the rumen and abomasum. Of the attempts to achieve this, the most significant was the development of a gravimetric two-stage microbial digestibility assay by Tilley and Terry (1963).

In this method, strained rumen liquor, artificial saliva (McDougall, 1948) and dried milled forage samples are combined in digestion tubes. The tubes are flushed with carbon dioxide (CO_2), closed with Bunsen gas-release valves and incubated for 48 h at 38°C. The samples are agitated and the pH checked at intervals. The acidity is maintained between pH 6.7 and 6.9 to optimize conditions for microbial growth and development. After 48 h of incubation, mercuric chloride ($HgCl_2$) and sodium carbonate (Na_2CO_3) are added to inhibit further microbial activity and aid sedimentation. The supernatant is discarded following filtration or centrifugation. Acid pepsin is added to the residue and the mixture reincubated for 48 h at 38°C. The sample is recentrifuged, the supernatant discarded and the residue washed. This undigested residue is dried to a constant weight. Digestibility is calculated by dividing the weight loss of the sample by its initial weight, after correction for the blank weight of the diluted rumen liquor.

The Tilley and Terry (1963) method has been widely tested and used, and sometimes modified in its detail (e.g. Alexander and McGowan, 1966). Estimates of dry-matter digestibility obtained have shown good correlations with *in vivo* digestibilities in sheep, for a variety of fibrous foods. Marten and Barnes (1980) noted that two-stage *in vitro* fermentations had become universally recognized as methods of choice to predict digestibility of all types of forages, and McLeod and Minson (1980) stated that they were often the standards of excellence against which other procedures were compared. A valuable feature of the method is that *in vitro* estimates not only are closely correlated with *in vivo* values, but also closely

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approximate to them (Tilley and Terry, 1963; Pigden, 1969). This makes results easy to interpret and minimizes the reliance on standards.

Surprisingly, there are no data available to show if this *in vitro* procedure and sheep digest similar fractions of the forage, or whether the close agreement in overall apparent dry-matter digestibility is purely fortuitous. The Tilley and Terry (1963) first stage does not closely reflect the complexity of ruminant digestion. Bacterial colonies are known to be difficult to maintain unchanged *in vitro* without complex apparatus (Czerkawski, 1976). Fermentation in glass tubes is not regulated by rumination, salivation, absorption of products or fractional outflow rates. The diverse processes of digestion in the hind-gut of the ruminant are not duplicated by second-stage acid-pepsin treatment (Tilley and Terry, 1963). The justification of the method is not the re-creation of ruminant digestive functions, but that it works. The prime function of the rumen liquor is to provide a rich source of fibrolytic organisms, which leaves open the possibility that these might be more readily obtained elsewhere.

Many investigators (e.g. Reid *et al.*, 1964; Milne, 1977) have found that the activity of rumen inocula is affected by the diet of the donor. A more serious practical disadvantage is the need for fistulated donor ruminants to provide the liquor (Abonge, 1988; Omed *et al.*, 1989a). These are subject to restrictive legislation in many countries and are costly to prepare and maintain. Removal of the need for these could make the procedure more widely available. These concerns led to consideration of sheep faeces as an alternative source of fibrolytic organisms.

The use of faecal organisms for the estimation of the digestibility of forages was first explored, using liquor derived from sheep faeces, by Balfe (1985), as an undergraduate project. She used a filtered homogenate of sheep faeces in artificial saliva, instead of rumen liquor, in the modification by Rogers and Whitmore (1966) of the Tilley and Terry (1963) method. Balfe (1985) first showed that filter-paper was digested by faecal liquor, and then obtained a correlation of 0.93 between *in vitro* estimates of dry-matter digestion of three grass samples and one hay sample and their determined *in vivo* dry-matter digestibility. Her method was not convenient for batch operation, as the faecal liquor rapidly blocked the sintered filters, a feature confirmed by El Shaer *et al.* (1987) and Akhter (1994), and the undigested residues were recovered by centrifugation after transfer of the liquor from the fermentation tubes. Later, Williams (1986) demonstrated that increasing the concentration of faeces in faecal liquor increased its activity, and Puw (1986) showed that faeces from horses and cattle also contained activity appropriate for the method.

A procedure using sheep faeces, suitable for batch operation, was subsequently tested by El Shaer *et al.* (1987). The methodology of Balfe (1985) was modified in that fermentation took place in McCartney bottles fitted with wine fermentation locks. Transfer losses were avoided by centrifuging residues within the same bottles, which made it possible to minimize sample size. Omed *et al.* (1989a, b) found that the procedure could be carried out satisfactorily in sealed bottles, dispensing with the fermentation locks. The sample size was reduced to 180 mg and the faecal liquor volume scaled down correspondingly to 18 ml, with the resultant airspace at the top of the McCartney bottles providing sufficient space for gas accumulation. Fermentation gases were released by gently loosening the caps twice during the incubation period.

Despite some conflicting results concerning dietary effect on faecal liquor activity (e.g. El Shaer *et al.*, 1987; Aiple *et al.*, 1992; Akhter, 1994), the impact of this appears to be less than that observed when using rumen liquor. Thus faecal liquor is useful when donor material must be obtained from free-ranging animals.

Rumen and Faecal Fluids as Source of Microbial Enzymes

Physical degradation of ingested forages increases its surface area, resulting in rapid colonization by fibrolytic organisms and rapid solubilization of non-structural carbohydrates (McDonald *et al.*, 1995). Saliva buffers the rumen liquor. This is very important, as cellulolytic bacteria cannot grow at pH values below 6.0 (Stewart, 1977).

The anaerobic environment, fatty acids and rumen fluid rapidly destroy the plasmalemma and much of the cytoplasmic structure of the plant cells (Cheng *et al.*, 1980). Internal solubles leach out, presumably via natural openings, such as stomata, and sites of extensive cell damage (Cheng *et al.*, 1980; Halliday, 1985; McAllister *et al.*, 1994), allowing microbes direct access to the internal structures of the leaf. Bacteria attracted to these sites by the concentration of nutrients (Cheng *et al.*, 1980) bypass the resistant cuticle (Akin, 1979; Bauchop, 1980; McAllister *et al.*, 1990). Large populations of cellulolytic bacteria quickly establish around such openings (Cheng *et al.*, 1980, 1984; Craig *et al.*, 1987). Within 5 min of food ingestion, intimate bonds between substrate particles, bacteria and protozoa have been observed (Bonhomme, 1990). Thus digestion of insoluble feed materials occurs from the inside out (Chesson and Forsberg, 1988; Cheng *et al.*, 1991; McAllister *et al.*, 1994).

Intimate bacterial and forage association is a prerequisite for efficient forage digestion. Between 70 and 80% of all microbial matter in the rumen has been reported to be bonded to forages (Craig *et al.*, 1987); Czerkawski (1986) believed this figure to be nearer 95%. Hemicellulase and cellulase activities are also notably higher in the particulate fraction than in the fluid (Williams and Strachan, 1984), leaving no doubt that particulate-associated microbial populations are responsible for the majority of feed digestion in the rumen. Different internal structures themselves have differing susceptibilities to attachment by rumen microorganisms; high-lignin-content structures, such as vascular and sclerenchymal cells, have a high resistance to attachment, whilst the mesophyll and phloem are easily colonized and rapidly digested (Akin, 1979). Wilson (1990) suggested that the proportion of uncolonizable tissue often dictates the extent to which a forage within the rumen is digested.

Microbes, by attaching themselves to forage particles, not only increase their ability to deliver enzymes but also are able to extend their residence time within the rumen by avoiding passage through the reticulorumen orifice. As a consequence, high population levels can be maintained at low growth rates (Mackie and White, 1990). Four spatially distinct domains coexist and interact within the rumen (Czerkawski, 1986), enabling rapid colonization of ingested forage. These are shown in Table 7.1.

Rumen microbial species differ in their abilities to survive passage through the

Table 7.1. The spatial organization of the rumen microbes according to the compartment model of Czerkawski (1986).

Compartment	Definition	Function
1	Rumen liquor	Transport medium carrying feed particles and microbes. Soluble sugar fermentation
2	Loosely adherent to feed particles	Shuttle compartment between 1 and 3
3	Tightly adherent to feed particles	Breakdown of solid feeds
4	Adherent to rumen wall	O_2 scavenging, urea hydrolysis

O_2, oxygen.

abomasum and small intestines to the large intestine (Pounden *et al.*, 1950; Bergen *et al.*, 1967; Van Soest, 1982; Wallace, 1983). Although isolated microbes are readily killed by acidic environments, the cuticle and other lignin structures of plant particles may insulate embedded microbes from the hostile environment of the abomasum. Bhat *et al.* (1990) found that significant cellulolytic bacteria attachment to substrate occurred down to a pH of 4.0, even though they were unable to grow at a pH lower than 6.0 (Stewart, 1977).

Susceptibility of bacteria to proteolytic digestion ranges between extremes of complete destruction in the abomasum to passage through the entire digestive tract of the host (Baker and Harris, 1947; Bergen *et al.*, 1967; Wallace, 1983; Allison, 1984). Wallace (1983) established that Gram-negative species were more thoroughly digested than Gram-positive ones. Viability was not tested. By combining the findings of Bergen *et al.* (1967), Wallace (1983) and Stewart and Bryant (1988) on the degradability of bacteria exposed to acid pepsin (as shown in Table 7.2), it is possible to speculate that the large intestine will recruit a higher proportion of Gram-positive than Gram-negative bacteria from the rumen. Gram-positive bacteria, however, usually form only a minor component of the rumen microbial population (Hungate, 1966).

It has been suggested from numerical data that *Bacteroides succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Bacteroides fibrisolvens* were the most important cellulolytic bacteria. *Bacteroides fibrisolvens* is one of the most vigorous digesters of cellulose (Hungate, 1966). *Ruminococcus* and *Bacteroides* species have important roles in cellulose degradation (Cheng *et al.*, 1980). With the exceptions of *R. flavefaciens* and *B. succinogenes*, these have higher than average digestibility values when exposed to an acid-pepsin/pancreatin solution.

While this evidence suggests that most rumen organisms fail to survive into the large intestine, it is likely that the bacterial community there is self-sustaining. The bacterial population of the large intestine and faeces of ruminants includes numerous bacterial species also present in the rumen. These have mostly been identified at the family level and include *Bacteroides*, *Fusobacterium*, *Streptococcus*, *Eubacterium*, *Ruminococcus* and *Lactobacillus* (Allison, 1984). The dwell time permits multiplication, so species depleted by proteolytic activity have a chance to recover, provided niches are available. The hind-gut of the faeces donor needs

Table 7.2. The digestibility of bacteria when exposed to acid pepsin and pancreatin, their substrate and response to Gram stain (data from Bergen *et al.,* 1967; Wallace, 1983; Stewart and Bryant, 1988).

Bacteria	Strain	Substrate	Gram +/-	Digestibility
Bacteroides amylophilus	70	NC	Negative	67.7
Bacteroides fibrisolvens	H10b	C	Negative	84.7
Bacteroides ruminocola	H8a	NC	Negative	73.3
Bacteroides ruminocola	223	NC	Negative	94.0
Bacteroides succinogenes	A3c	C	Negative	62.0
Bacteroides succinogenes	S85	C	Negative	68.6
Bacteroides succinogenes	B21a	C	Negative	67.6
Butyrivibrio fibrisolvens	NOR37	C	Negative	90.0
Butyrivibrio fibrisolvens	B834	C	Negative	91.0
Eubacterium ruminantium	2388	NC	Positive/negative	73.0
Lactobacillus casei	LB17	NC	Positive	58.0
Megasphaera elsdenii	J1	NC	Negative	83.0
Peptostreptococcus elsdenii	H8a	NC	Negative	72.1
Ruminococcus albus	7	C	Positive/negative	80.4
Ruminococcus albus	SY3	C	Negative	96.0
Ruminococcus flavefaciens	B1a	C	Positive	44.2
Ruminococcus flavefaciens	B34b	C	Positive	85.9
Ruminococcus flavefaciens	C1a	C	Positive	63.5
Ruminococcus flavefaciens	C94	C	Positive	80.3
Selenomonas ruminantium	WPL 151/1	NC	Negative	92.0
Selenomonas ruminantium	GA192	NC	Negative	83.4
Staphylococcus	3588/2	NC	Positive	33.0
Streptococcus bovis	no. 26	NC	Positive	19.0
Streptococcus bovis	45S1	NC	Positive	37.0
Streptococcus faecium	SFDC	NC	Positive	48.0
Succinivibrio dextrinosolvens	24	NC	Negative	93.1
Veillonella alcalescens	692	C	Negative	88.0

NC, non-cellulolytic; C, cellulolytic.

slowly fermentable nutrients to stimulate multiplication of cellulolytic digesters (Aiple *et al.*, 1992). An appropriate diet will provide such nutrients for the hindgut, whilst at the same time increasing the residence time of the feedstuff. Both factors should increase the numerical presence of cellulolytic bacteria, which explains the higher *in vitro* digestibility values obtained by Pilling (1997) when diets for donor animals included hay or straw, rather than grass.

Hidayat *et al.* (1993), when investigating digestibility of forages by isolated mixed bacterial populations, established a maximal digestion rate above which the addition of more bacteria or protozoa failed to stimulate further digestion. The same was true when bacteria were added to a 100% protozoa population. This suggests that both populations are highly cellulolytic and are capable of occupying all niches within the rumen environment and presumably also in the large intestine.

Incidences of lower *in vitro* digestibilities obtained using faecal liquor when compared with rumen inocula (Manyuchi *et al.*, 1991; Akhter, 1994; Gonçalves

and Borba, 1996) could be due to differences in the microflora present. It is unlikely that lower bacterial numbers in the large intestine are responsible, as the bacterial density there is similar to that in the rumen (10^{10}–10^{11} g^{-1}) (Allison, 1984). This suggests that the difference is due to fewer cellulolytic species being extracted into the liquor. It may be compounded by the supply of nutrients obtained from the rumen fluid. Corbett (1981) found that nutrient deficiencies within test forages were not detected when using rumen inocula *in vitro*, as the liquid contained sufficient trace elements and micronutrients. Faecal fluid may lack some of these, reinforcing the effect of forage deficiencies on digestibility. Few soluble carbohydrates will be available; consequently, non-cellulolytic microbial growth will be inhibited until solubilization occurs under the actions of the cellulolytic microbial population.

The liquor compartment in the rumen is large and can readily be sampled. If the nutritional state of the animal is closely controlled, this fluid should be relatively constant in composition, as should be the activity of its microbial population. Rumination and ruminal mixing continously resuspend particle-bound bacteria in the liquid fraction. There is no equivalent of this compartment in faeces as a source of fibrolytic organisms; the liquor component is too small to be accessible. Fibrolysis in faecal liquor is believed to be due to particle-bound organisms that have been detached and suspended in liquid during the preparation process. Omed *et al.* (1989b) recommended the use of 60 g fresh faeces in each litre of faeces liquor. Thus, each 18 ml aliquot of liquor applied to 180 mg forage is derived from approximately 360 mg faecal dry matter. To seed all the niches in the forage to the level ocurring in the large intestine, the extraction of fibrolytic faecal bacteria must be about 50% efficient. Effective extraction of faeces with artificial saliva is therefore necessary to make an active suspension.

Validation of the Faecal Liquor Method

The only satisfactory proof of this method would be to show that it gives estimates which agree closely and consistently with digestibilities determined *in vivo*. This should be done as a blind test on a range of forage types and on a sufficient scale to allow for the uncertainties in the *in vivo* data, as these too are subject to error (Omed *et al.*, 1989a). Table 7.3 includes data that fall short of these requirements but which are sufficient to justify a properly organized large-scale trial. The table also illustrates a problem that must be addressed before such a trial.

The time course of solubilization of dry matter from forage samples suspended in the rumen follows a well-established curve (Mehrez and Ørskov, 1977). This approaches an asymptote, taken to represent the potential degradability of the sample. Under the *in vitro* conditions of the rumen liquor or faecal liquor methods, fibre degradation follows a similar course (Akhter, 1994; Solangi, 1997). If the liquor is of high activity, degradation of most forages will be substantially completed within the prescribed 48 h, yielding results in which *in vitro* weight loss is close to the *in vivo* apparent digestibility. Unfortunately, it is not easy to gauge if fibre degradation is complete when the prescribed time is reached. Liquor of low activity may need longer than 48 h to achieve the degradation potential. At the cut-off time of 48 h,

Table 7.3. Relationship between *in vitro* dry-matter digestion % (x), estimated using faecal liquor from different donor species, and apparent dry-matter digestibility % (y), obtained using the specified standard procedure.

Species of faeces donor	Variance accounted for (r^2)	Forage type	Line of best fit	Reference
Ovine	0.93	Grasses and hay	*$y = 7.9 + 0.83x$	Balfe (1985)
	0.98	Variety of feeds	*$y = 1.003x$	El Shaer *et al.* (1987)
	RSD 2.75	Variety of forages	*$y = 0.7 + 1.01x$	Omed *et al.* (1989a)
	0.91	Grasses and straws	*$y = -3.1 + 0.99x$	Faza (1991)
	0.33	Fresh and conserved feeds	*$y = 35.4 + 0.33x$	Borba and Riberiro (1996)
	0.85 (gas)	Tropical feeds	†$y = -20 + 1.26x$	Nsahlai and Umunna (1996)
	0.95	Variety of forages	*$y = 4.0 + 0.94x$	Solangi (1997)
	0.97	Tropical feeds	†$y = 33.8 + 0.55x$	Aboud *et al.* (1999)
Bovine	0.97	Variety of forages	†$y = 17.3 + 0.98x$	Akhter (1994)
(frozen)	0.97	Variety of forages	†$y = 17.2 + 0.97x$	Akhter (1994)
	0.98†	Variety of forages	†$y = 21.4 + 0.83x$	Akhter *et al.* (1995)
	0.88	Variety of forages	*$y = 19.6 + 1.31x$	Akhter *et al.* (1995)
(freeze-dried)	0.83	Variety of forages	*$y = 26.9 + 1.15x$	Akhter *et al.* (1995)
(freeze-dried)	0.92	Variety of forages	*$y = 17.7 + 1.5x$	Akhter *et al.* (1995)
(frozen)	0.80	Variety of forages	*$y = 24.2 + 1.19x$	Akhter *et al.* (1995)
(frozen)	0.77	Variety of forages	*$y = 25.7 + 0.99x$	Akhter *et al.* (1995)
	0.94	Variety of concentrates	†not available	Harris *et al.* (1995)
	0.95	Tropical feeds	†not available	Sileshi *et al.* (1996)
	0.98	Tropical feeds	†$y = -5.8 + 1.04x$	Jones and Barnes (1996)
Equine and	0.90	Tropical forages	†not available	Sileshi *et al.* (1996)
donkey		Tropical feeds	†$y = 29.7 + 0.78x$	Aboud *et al.* (1998)
Goat	0.96	Variety of forages	‡$y = 6.7 + 0.88x$	O'Donovan (1995)
	0.97	and straws	‡$y = 5.0 + 0.86x$§	O'Donovan (1995)
Rabbit	0.96	Variety of forages	‡$y = 11.2 + 0.81x$	O'Donovan (1995)
	0.88	and straws	‡$y = -0.4 + 0.69x$§	O'Donovan (1995)

* *In vivo* apparent digestibility in sheep.
† Rumen liquor *in vitro*.
‡ Ovine faecal liquor *in vitro*.
§ Phosphate buffer used.
RSD, residual standard deviation.

digestion of fibre in some forages may be incomplete and still progressing. No stable, repeatable end-point is reached. Weight losses from standard forages may still show significant correlations with their *in vivo* apparent digestibilities, as indeed they might if merely extracted with water, but the data will reflect variations in the rates of digestion both within and between samples; and forages with highly digestible components may be overvalued. For maximum confidence in the results of a digestibility estimation, the constant *a* in the following equation should be very small and the coefficient *b* should approximate to 1, indicating that digestion has reached its natural limits:

In vivo apparent digestibility = $a + b \times$ *in vitro* digestion

Balfe (1985), El Shaer *et al.* (1987) and Omed *et al.* (1989a) all sought to achieve conditions where *in vitro* weight loss equalled *in vivo* digestibility. This particularly involved thorough extraction of the faeces with artificial saliva to suspend the maximum number of the fibrolytic organisms believed to be entrenched in plant debris. It is possible that some later workers have expected the method to be more robust and easily applied than it really is.

To achieve complete digestion when investigating low-digestibility hays and straws, it may be necessary to increase the liquor/sample ratio or to increase the duration of incubation. El Shaer *et al.* (1987) confirmed completion of the fermentation stage after 48 h by noting cessation of gas release from the fermentation locks. Solangi (1997) and Abonge (1998) collected fermentation gases over acidified water in graduated centrifuge tubes. Using the end of gas emission to indicate completion of digestion, Solangi (1997) obtained a close relationship between *in vitro* and *in vivo* digestibilities of a variety of fibrous feeds. Alternatively, routines could be developed that produce potent faecal liquor that rapidly occupies all appropriate niches in the sample (Hidayat *et al.*, 1993) for maximum rate of digestion. Fibrolytic activity could be monitored by including cellulose samples in each batch, as the digestion of pure cellulose is progressive and without asymptote.

Faeces liquor has recently been used in the investigation of the dynamics of gas production (e.g. Altaf *et al.*, 1998). This situation too may be complicated by the variability of faeces liquor preparations. Fibrous carbohydrate is slowly solubilized, at a rate dependent on the activity of the particular liquor preparation, and its conversion becomes rate-limiting on gas production when initial solubles have been used up. Thus, while the total volume of gas should be independent of the quality of the inoculum, the rate of gas production will be characteristic of the particular liquor preparation, as well as of the feedstuff.

Therefore, all potential applications need high-activity faeces liquor.

Factors Affecting the Activity of Faecal Liquor

Source of faeces

Odo (1990), Aiple *et al.* (1992), Akhter (1994) and Aboud *et al.* (1998a) found that gathering naturally voided faecal matter was inconvenient, and collected faeces directly from the anus. Comparisons were not made, but the collection method had no apparent effect.

As ovine faecal output is small (Akhter, 1994) and sheep are not universally available (O'Donovan, 1995; Sileshi *et al.*, 1996), the use of faeces from other species has been tested (Puw, 1986; Akhter, 1994; O'Donovan, 1995; Aboud *et al.*, 1998, 1999; Table 7.3).

Inocula made from bovine faeces have been investigated (Akhter, 1994; Akhter *et al.*, 1994, 1995; Harris *et al.*, 1995; O'Donovan, 1995; Jones and Barnes, 1996; Sileshi *et al.*, 1996); and it has been established that bovine faeces could be successfully utilized in forage evaluation studies. O'Donovan (1995) noted that the percentage of ryegrass digested during 48 h of fermentation with bovine liquor was less than when ovine faeces were used.

Sileshi *et al.* (1996) and Aboud *et al.* (1998, 1999) found a strong relationship between *in vitro* results using rumen liquor and equine faecal liquor, although the extent of overall digestion using equine faeces was lower than when using ruminant faeces. Similarly, O'Donovan (1995) found that faecal liquor made from horse faeces digested 8% to 16% less, respectively, from straw and ryegrass samples than did that made from sheep faeces.

In his study of six different faecal sources, O'Donovan (1995) found that goat and rabbit faeces (in phosphate buffer) produced strong correlations with *in vitro* results using ovine faecal liquor, although total digestibility was lower (Table 7.3). Guinea-pig faeces produced the lowest *in vitro* digestibility of the investigation, possibly because the pellets failed to provide a sufficiently stable internal environment for sufficient numbers of cellulolytic microorganisms to survive. All six faecal sources did, however, rank the forages in the same order of digestibility, suggesting that any of them could be utilized, providing suitable standards were included in the batch.

Where comparison has been made between liquors made from different faecal donor species, sheep faeces have been consistently shown to produce the greatest fibrolytic activity. Accordingly, sheep must be considered to be the preferred faecal source.

Effect of diet

Akhter (1994) found no significant differences between *in vitro* digestibility assays when bovine faecal liquor was prepared from concentrate-, concentrate-and-hay- or hay-fed donors, although the lowest mean results were obtained from the concentrate (barley beef) diets. This agrees with the findings of El Shaer *et al.* (1987), who noted that faeces obtained from sheep fed hay or equal weights of hay and concentrate produced indistinguishable results. Food residues reaching the large intestine are thoroughly leached, leaving mainly residual roughage. Providing sufficient fibrous residue is present in the hind-gut to sustain an active cellulolytic bacterial population, concentrate within the diet would not be expected to influence *in vitro* digestibility results.

Gas-production studies using a variety of feeds have been conducted with liquor from faeces obtained from animals fed different basal diets (Aiple *et al.*, 1992). The results are shown in Table 7.4. Small but significant effects were identified. It was also noted that inocula from hay and concentrate faeces were more

Table 7.4. Effect of diet of the donor sheep on gas production (ml 200 mg^{-1} DM) after 48 h of incubation in 1 : 30 faeces buffer dilution (from Aiple *et al.,* 1992).

Substrate/ diet	Maize : grass pellets : hay 35 : 15 : 50 (%)	Concentrate* : hay 40 : 60 (%)	Hay 1† 100 (%)	Hay 2‡ 100 (%)
Concentrate – standard	65.0^{a}	65.5^{a}	66.1^{ab}	67.4^{b}
Hay – standard	44.3^{a}	46.3^{b}	47.2^{b}	46.5^{b}
Barley	75.6^{a}	77.8^{b}	77.5^{b}	76.7^{ab}
Soybean extract meal	53.6^{a}	56.0^{b}	55.3^{ab}	57.3^{bc}
Hay	50.9^{ac}	49.4^{a}	54.2^{b}	52.3^{c}
Straw	35.4^{a}	34.1^{a}	41.2^{b}	38.6^{c}

* Concentrate, on dry-matter % basis, was wheat (25) : barley (25) : oats (20) : sugar-beet pulp (20) : soybean extract meal (10).
† 8.75 MJ ME kg^{-1} DM.
‡ 7.91 MJ ME kg^{-1} DM.
Means with different subscripts in the same line differ significantly ($P < 0.01$).
ME, metabolizable energy; DM, dry matter.

stable when stored under aerobic conditions than those obtained from donors fed on a pure hay diet.

H.M. Omed (unpublished observation) obtained low digestibilities using faeces from sheep on bare spring pastures. The faeces were greasy and without body, and the resulting liquor was of high colour and high blank weight. All these data suggest that a high fibre dietary component is necessary for donor animals.

Age of faeces

Initial studies on faecal liquor (El Shaer *et al.*, 1987; Omed *et al.*, 1989a, b) used ovine faecal pellets within 1 h of voiding, on the assumption that the pellets would only briefly maintain a suitable environment for cellulolyic bacteria. However, it was noted that faeces voided up to 6 h previously were equally effective for forage digestibility (El Shaer *et al.*, 1987). This confirmed the preliminary results of Balfe (1985), who found no difference in the digestive efficiency of fresh or old ovine faeces when applied to hay. More recently, Pilling (1997) established that ovine faeces maintained their cellulolytic capabilities for up to 24 h when stored either at room temperature or at 5°C, under anaerobic conditions. Storage for 24 h at room temperature did, however, result in a significant decline in activity. Akhter (1994), using bovine faecal liquor, noted a delay of 5 h before digestibility was reduced. In contrast, Aiple *et al.* (1992) found that gas production from hay, but not from concentrates, by faecal liquor was reduced following storage of faeces for 1 h under aerobic conditions at room temperature (Table 7.5), indicating the greater sensitivity of cellulolytic organisms than non-cellulolytic ones to storage conditions. Although total dry-matter disappearance was reduced when preserved faecal material was used, the relationship between faeces liquor digestibility and that of rumen liquor was still maintained (Akhter, 1994). Faecal pellets originating from donor

Table 7.5. Effect of storage of fresh ovine faeces under aerobic conditions and room temperature on gas production after 48 h of incubation (ml 200 mg^{-1} DM) (from Aiple *et al.,* 1992).

		Hours of storage before incubation				
Substrate	Control	1 h	2 h	4 h	12 h	24 h
Concentrate	65.8^{a}	64.9^{a}	63.9^{ab}	64.4^{ab}	61.7^{b}	54.5^{c}
Hay	46.5^{a}	41.0^{b}	39.6^{b}	40.8^{b}	34.7^{c}	33.0^{c}

Means with different superscripts in the same line differ significantly ($P < 0.01$).

sheep fed a mixed diet were observed to provide a more stable environment than those fed roughage only (Aiple *et al.*, 1992).

Most recent investigations have used freshly voided faeces (e.g. Miguel, 1994; Borba and Riberiro, 1996; Buwembo, 1996; Jones and Barnes, 1996). Nsahlai and Umunna (1996) found that faeces voided up to 2 h prior to inoculum preparation produced a significant correlation of *in vitro* faecal liquor digestibility ($r^2 = 0.85$) with *in vitro* rumen liquor, but this was lower than for other investigations using fresh faeces.

Akhter (1994) and Akhter *et al.* (1994, 1995) found that preserved bovine faeces obtained by rectal grab could also be used for digestibility assays. Both freeze-dried and deep-frozen faeces produced inocula with similar digestibility characteristics to those of fresh rumen liquor. The results obtained were highly repeatable.

It can be concluded that the method does not depend on fresh faeces, although these are most effective.

Choice of buffer

El Shaer *et al.* (1987) successfully used artificial saliva (McDougall, 1948), a bicarbonate buffer. Comparable results were obtained by Akhter *et al.* (1995), Jones and Barnes (1996), Sileshi *et al.* (1996) and Aboud *et al.* (1998, 1999) when compared with rumen liquor *in vitro* digestibility. Bicarbonate buffer (McDougall, 1948), the most common choice, when dissolved has a natural pH of 8.23. Thorough gassing with CO_2 is needed to achieve the pH of 6.8 favoured by cellulolytic bacteria (Stewart, 1977). This is a time-consuming procedure. An additional disadvantage associated with this buffer is that direct acidification following completion of the fermentation stage causes extensive frothing. It is therefore necessary to physically separate the residue from the inoculum, prior to second-stage acid digestion.

To overcome these limitations, Menke's medium (Menke *et al.*, 1979) or a modified version of it has been used when investigating gas production (Aiple *et al.*, 1992; Gonçalves and Borba, 1996), or a phosphate buffer (Pigden, 1969) when investigating *in vitro* dry-matter digestibility (Faza, 1991; O'Donovan, 1995; Omed *et al.*, 1998) or for gas-production studies (Morris, 1994; Buwembo, 1996; Solangi, 1997). Gas production is higher when bicarbonate buffer is used than that obtained

with phosphate buffer (Morris, 1994; Buwembo, 1996; Solangi, 1997), since bicarbonate buffer reacts with the acid products of fermentation to release CO_2 (Solangi, 1997).

Faza (1991) reported an interaction between buffer type and the bacteria separation method on the *in vitro* digestion of straw. This was greater in bicarbonate buffer when the inoculum was prepared in a food blender than when a pestle and mortar were used. When the bicarbonate buffer was replaced by a phosphate buffer, the opposite was true. O'Donovan (1995) found that sheep and goat faeces were equally effective as agents for *in vitro* digestibility in either a phosphate or a bicarbonate buffer. Rabbit faeces, however, gave lower digestibility when used in a phosphate buffer than in a bicarbonate buffer.

Separation of bacteria

Effective faecal inocula cannot be produced from ovine faeces simply by washing (H.M. Omed, unpublished observation), as the cellulolytic organisms form strong bonds with their substrates. Various techniques have been used to break these bonds (Table 7.6) but few comparisons have been made between these methods.

Solangi (1997) found that the percentage of cellulose samples digested during 48 h incubation was greater when the faeces and artificial saliva were pummelled together in a plastic bag (75%) or ground together in a pestle and mortar (70%) than when shaken with glass beads in a bottle (67%) or homogenized in a food processor (65%). He also found that activity was increased when large sieve sizes (300–800 μm) rather than small sizes (212 μm or doubled muslin) were used, indicating that some bacteria were still particle-bound.

Cattle faeces directly strained through muslin cloth have been used by Akhter (1994) and Sileshi *et al.* (1996). Lower *in vitro* digestibility values, when compared with alternative inocula preparation methods, were obtained, perhaps due to the lack of physical disruption to which the faecal matter was subjected.

Faeces-to-buffer ratio

El Shaer *et al.* (1987) and Omed *et al.* (1989a) used 60 g of faeces 300 ml^{-1} of artificial saliva (McDougall, 1948), although later Omed *et al.* (1989b) found 60 g l^{-1} produced a more active inoculum. Bacterial separation is presumably more efficient when a higher buffer-to-faeces ratio is used.

The effect of faeces dilution on both *in vitro* digestibility systems (Akhter, 1994; Solangi, 1997) and gas production (Aiple *et al.*, 1992; Solangi, 1997) has been studied. Larger concentrations of faeces (up to 120 g l^{-1} of buffer) were found to result in increased *in vitro* digestion and/or rates of gas production (Akhter, 1994; Solangi, 1997). The digestibility was found not to increase directly in proportion to the increase in faeces utilized (Solangi, 1997). Aiple *et al.* (1992) noted that dilutions of dry matter between 1 : 20 and 1 : 50 produced similar gas volumes; above or below this, gas production from fibrous substrates was depressed.

A trade-off appears to exist between the mass of faeces used and the potency of

Table 7.6. The effectiveness of different methods of separating the cellulolytic bacteria from the faeces substrate.

Method	Species	Digestibility/ gas production	Correlation coefficient	Reference
Pestle and mortar	Ovine	Digestibility	0.98*	El Shaer *et al.* (1987)
	Ovine	Digestibility	0.88†	Nsahlai and Umunna (1996)
	Ovine	Gas production	0.85†	Nsahlai and Umunna (1996)
Homogenization in food processer	Donkey	Digestibility	0.97†	Aboud *et al.* (1998)
	Ovine		0.97†	
	Ovine	Gas production	0.93*	Aiple *et al.* (1992)
Plastic bag	Rabbit	Digestibility	0.96‡	O'Donovan (1995)
	Goat		0.96‡	
Strained through muslin	Bovine	Digestibility	0.9†	Akhter (1994)
	Bovine	Digestibility	0.982†	Jones and Barnes (1996)
	Bovine Donkey	Digestibility	0.9†	Sileshi *et al.* (1996)

* Correlation with *in vivo* digestibility.
† Correlation with rumen liquor *in vitro*.
‡ Correlation with ovine faecal liquor *in vitro*.

the inoculum. Too small a faeces mass contains insufficient cellulolytic bacteria, while too large a mass of faeces inhibits separation of bacteria (Solangi, 1997).

Addition of a nitrogen supplement

The earliest studies employing faecal liquor (Balfe, 1985; Williams, 1986) did not include a nitrogen supplement. El Shaer *et al.* (1987) found that the inclusion of urea in the buffer improved correlations with *in vivo* digestibility for all assays involving grasses. As lucernes already provided a good correlation, it was assumed that the nitrogen supply was a limiting factor. The addition of urea has now become standard to all buffer solutions utilized in Bangor (e.g. Awemo, 1988; Omed *et al.*, 1989a, b; Faza, 1991; O'Donovan, 1995). Nsahlai and Umunna (1996) and Aboud *et al.* (1998, 1999) replaced urea with ammonium sulphate and found this to be equally successful.

Manyuchi *et al.* (1991) used no nitrogen supplement and obtained low *in vitro* results when digesting tropical grasses with faecal liquor. It was for this reason that Jones and Barnes (1996), whilst digesting tropical browse, added 0.6 ml of a freshly prepared solution of urea (30 g l^{-1}) to every sample and extended the incubation period to 72 h. This produced the best combination when digesting tannin-containing forages, agreeing with earlier work on the rumen inoculum by Drew (1966).

Mixing of the sample and faeces liquor

The liquor must be mixed intimately with the forage sample. Dried and ground forage samples may clump when the inoculum is added, reducing the surface area available for colonization (Miller and Hobbs, 1994). Peel and Schofield (1993) reported a coefficient of variation between gas volumes produced by identical samples of 2.1% for samples stirred with a rod, compared with 4.2% for unstirred samples. A system of agitation during incubation therefore appears necessary.

Samples are normally shaken for 30 s to suspend the solids, after adding faeces liquor and sealing the digestion tubes (e.g. Abonge, 1988; Omed *et al.*, 1989b; Odo, 1990; Miguel, 1994). O'Donovan (1995) shook samples for 30 s. Following this initial suspension, three different forms of agitation have been used: a shaking water-bath (El Shaer *et al.*, 1987; Buwembo, 1996; Solangi, 1997), a rotor contained within an incubator (Faza, 1991; Gonçalves and Borba, 1996) or manual shaking at regular intervals (e.g. Omed *et al.*, 1989b; Odo, 1990; Morris, 1994; O'Donovan, 1995). All aim to ensure that the intimate mixing between inoculum and substrate continues. Only Faza (1991) has made a direct comparison between mixing methods. He found that manual shaking four times daily produced a value for digestibility four percentage units higher for straw than did rotating the samples at 2 r.p.m. inside an incubator. It is possible that periodical suspension allows bacteria to settle and colonize virgin substrate, whereas constant mixing prevents this.

Duration of incubation

The colonization of a fresh forage by microbes is rapid and concentrates around sites of mechanical damage and stomata (Cheng *et al.*, 1980; Halliday, 1985), where leaching of soluble carbohydrates is thought to attract bacteria (Cheng *et al.*, 1980). It is reasonable to assume that, until the internal cells are rehydrated, colonization will be haphazard and slow, leading to a lag in digestion (Spallinger, 1985).

Tilley and Terry (1963) reported that cellulolytic digestion was complete following a 48 h incubation with rumen fluid. El Shaer *et al.* (1987) and Omed *et al.* (1989a, b) showed similarly that 48 h incubations in faecal liquor were sufficient for most forages. However, for highly digestible forages, accurate predictions of digestibility (residual standard deviation (RSD) 0.74) could be obtained with a reduced incubation period of 36 h (Omed *et al.*, 1989b), while 72 h incubations were needed to provide a good correlation with *in vivo* data for straws (El Shaer *et al.*, 1987; Faza, 1991). Solangi (1997) found that at least 96 h incubation was necessary before digestion of straw reached its asymptote. Tannin-containing forages also require extended incubations when using either rumen inocula (Drew, 1966) or faecal fluid (Jones and Barnes, 1996).

Long incubations must be treated with caution as microbial lysis may occur, but variability between duplicate samples may be reduced by extending the incubation time in either rumen inocula (Barnes, 1967) or faecal fluid (Solangi, 1997). The forage being tested may be of unknown quality, preventing prediction of an

appropriate incubation period. A possible way of detecting completeness of fibre digestion is to monitor gas evolution from the sample.

Factors Affecting the Second-stage Incubation

In the Tilley and Terry (1963) procedure, the residue remaining after fermentation is further digested in a second incubation, using acid pepsin to solubilize protein and protein-bound materials, before the undigested residue is dried and weighed. This is also necessary in the faecal liquor method. The residue is usually separated from the inoculum before acidification, to avoid frothing of the bicarbonate buffer. However, Alexander and McGowan (1961), using rumen inoculum in a bicarbonate buffer, achieved acidification using a syringe pushed through the cap of the fermentation tube and found this method more convenient and quicker than centrifugation. Abonge (1988), using this procedure with faecal fluid in a bicarbonate buffer, also found it convenient, but noted that great care was needed to minimize frothing.

Centrifugation of faecal liquor at 2000 g for 20 min was recommended by El Shaer *et al.* (1987) and later used by Omed *et al.* (1989a, b). O'Donovan (1995) encountered problems with centrifugation, for, upon completion, some of the forage samples failed to settle, requiring supernatant removal by pipette.

Filtration has been less satisfactory (Tilley and Terry, 1963). Filtration through sintered glass has been reported to be problematic (El Shaer *et al.*, 1987; Ahkter, 1994), as the faecal residue rapidly blocked the filter. Ahkter (1994), however, persevered with the method. Miguel (1994) attempted to utilize filter crucibles when investigating degradability, but consistently obtained low digestibility values. Filtration following secondary digestion with acid pepsin is more rapid and easier (Akhter, 1994).

Phosphate buffer does not need extensive gassing with CO_2 and allows direct acidification without frothing (Marten and Barnes, 1979; Omed *et al.*, 1998).

The acid pepsin treatment used by El Shaer *et al.* (1987) was copied from Tilley and Terry (1963), but it was recommended that the concentration of pepsin be raised from 2 g to 4 g l^{-1}, as 2 g l^{-1} produced lower-than-expected digestibilites. The correlation between *in vitro* estimates and *in vivo* digestibility remained unchanged when the length of incubation with pepsin was reduced to 36 h (Omed *et al.*, 1989b) and later to just 8 h (Solangi, 1997). These results agree with the findings of Pounden *et al.* (1950), Bergen *et al.* (1967) and Wallace (1983) that bacteria which are susceptible to proteolytic agents will be rapidly degraded. Continued solubilization will occur only at a slow rate, for the remaining bacteria population is composed of either very slowly degradable or insoluble bacteria. Short incubations, as reported by Solangi (1997), save time.

An alternative proteolytic agent, biological washing liquid (Persil) at a rate of 0.2–1 ml sample^{-1}, has been tested (Faza, 1991; Morris, 1994; Solangi, 1997; Omed *et al.*, 1998). An advantage of this agent is that acidification is not required, so frothing is avoided. Also, the final separation of the supernatant can be achieved by cautious aspiration following sedimentation, as washing liquid promotes sedimentation. Omed *et al.* (1998) reported an r^2 value of 0.953 for *in vitro* compared

with *in vivo* digestibility for a combination of biological washing liquid (to solubilize the protein) and aspiration (to remove the supernatant). This compared with an r^2 of 0.944 for centrifugation followed by acid pepsin when digesting straws for 72 h. The washing liquid increased the sedimentation rate of the sample. Morris (1994) found that the use of biological washing liquid and separation by aspiration increased *in vitro* digestibility. Solangi (1997) found that replacing acid pepsin by biological washing liquid produced digestibilities very close to the known *in vivo* digestibilities for a variety of grasses, legumes and hays after 24 h of second-stage incubation.

Future of the Faecal Liquor Technique

The main strength of the faecal liquor technique is its simplicity, combined with its minimal cost when compared with the methods based upon rumen inocula. It uses freely available waste products from farm animals managed under normal commercial conditions, saving the cost and inconvenience of maintaining fistulated ruminants. The materials needed are intact sheep for faeces, bottles, cheap chemicals, carbon dioxide, a 39°C incubator, a centrifuge and a balance (Omed *et al.*, 1989a). The operator needs few chemist's skills. There is also evidence that the centrifuge and carbon dioxide can be dispensed with (Omed *et al.*, 1998). The other requirements are of insignificant cost, apart from the major items of laboratory equipment. If animal warmth could be used to replace the incubators and the preliminary findings of Omed *et al.* (1998) are confirmed, the irreducible need will be for a sensitive weighing-machine. In view of the potential use of this technique, this needs further investigation.

In technically advanced countries, the faster, cleaner method of near-infrared reflectance spectroscopy (NIRS) is more attractive for estimating digestibility, although faecal liquor may find application in studies on the dynamics of digestion. The faeces liquor procedure would therefore seem to be particularly suitable where potential food sources need to be screened using limited equipment. This type of application would be facilitated if the method could be made consistently direct reading, with digestibility being equal to *in vitro* solubility, as discussed above. This would also give it an advantage over NIRS, which always needs a calibration set of standards.

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8 Enzyme Techniques for Estimating Digestibility

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The development and application of laboratory methods for predicting the nutritive quality of forages have been an active and successful area of research during the latter half of this century. An important development has been the introduction of biological methods, using a rumen microbial inoculum as a digestion medium in place of the chemical analysis for constituents such as fibre, cellulose and lignin. Although methods using microbial inocula have been widely and successfully used for a range of different forages, there are inherent problems in their use. Attention has, therefore, been focused more recently on the replacement of microbial inoculum methods by enzyme techniques which use crude cellulase preparations.

The Need for Alternatives to Rumen Fluid

Methods of predicting *in vivo* digestibility using rumen microbial inoculum have become well established, particularly the two-stage *in vitro* technique of Tilley and Terry (1963). It has been shown that their method gives results well related to *in vivo* digestibility for a range of forages and under a variety of growing conditions. There are, however, inherent problems in the use of microorganisms in rumen fluid for forage analysis.

1. The activity of the rumen microorganisms is lost or altered by freeze-drying or other methods of preservation. Fresh rumen fluid must therefore be readily accessible, which entails having fistulated sheep or cattle available as donor animals.
2. The donor animals need to be maintained on a standard feeding regime for long periods to minimize variability in inoculum potency. There is therefore a high and costly labour input involved in the feeding and care of animals.
3. There are analytical difficulties in using rumen fluid, e.g. the need to maintain anaerobic conditions, the high viscosity of digesta precluding filtration, unpleasant smells and the need to observe strict hygiene to avoid possible risk of infection from pathogens.

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4. Increasingly, there is concern over the use of surgically modified animals in experimentation, and alternative methodologies need to be used where appropriate.

The main disadvantage of using rumen microbial inoculum in routine analytical procedures, however, is the variability that is found in the potency of the inoculum from week to week. The factors influencing the potency of inoculum have been widely studied, particularly in relation to the Tilley and Terry (1963) technique. Significant variation in the activity of inoculum has been shown to occur between different donor animals and for the same animal on different days (Drew, 1966; Yates and Allden, 1966). Troelsen and Hanel (1966) suggest that the consumption of water by sheep at varying intervals prior to collecting inoculum may explain much of the variation in the potency of inoculum; different methods of processing inoculum have been proposed in attempts to reduce variability (Barnes, 1967). McLeod and Minson (1969) have stressed the need for proper calibration when using microbial inoculum methods and regard the inclusion of appropriate standard forages of known *in vivo* digestibility within each analytical batch to be essential. The inclusion of standards allows correction of the results relative to standard values; in practice, it is often difficult to make a valid correction unless a large number of standard samples are included in each analytical batch.

The use of cellulolytic enzymes as alternatives to rumen fluid is clearly an attractive prospect if their use gives results comparable to those of inoculum methods. The use of enzymes eliminates the need for fistulated animals and anaerobic procedures, thereby simplifying analytical methodology and eliminating the variability in activity between analytical batches.

Characteristics and Activities Required for Enzyme Techniques

The chemical constituents of forages can be divided into those making up the structure of the plant (cell-wall constituents) and those contained within the cell (cell contents). This partition can be conveniently defined analytically by the van Soest (1963a, b) neutral- and acid-detergent procedures. The cell contents are essentially completely digestible *in vivo* and comprise soluble carbohydrates, starch (in some forages), protein, organic acids, lipids and soluble minerals. The cell-wall constituents (essentially cellulose, hemicellulose and lignin) vary in digestibility depending on their polymeric configuration, degree of crystallinity and degree of lignification. Lignin itself is largely indigestible under anaerobic conditions and its presence exerts a profound effect on the digestibility of the cell-wall fractions associated with it. The *in vivo* digestibility of the cellulose and hemicellulose therefore varies from completely digested when unlignified to completely undigested when highly lignified; the cuticle and surface waxes are also indigestible to a large extent.

The enzyme activities sought for analytical procedures to predict *in vivo* digestibility need to reflect the digestive processes in the ruminant. The analysis needs to remove the soluble constituents (cell contents) and to solubilize unlignified and even moderately lignified cell wall to a significant extent. The extent of solubilization does not necessarily need to be as complete as *in vivo* but needs to reflect the effects of lignification in a comparable and correlated way.

Soluble constituents are readily removed in the pretreatments with acid pepsin or neutral detergent, which form part of most of the proposed enzyme methods. The presence of starch in certain forage crops (maize, cereals) needs to be taken into account, since it is not removed by acid pepsin, neutral detergent or incubation with cell-wall-degrading enzymes. An additional treatment with amyloglucosidase or mild acid hydrolysis is therefore required in the analysis of forages containing starch.

The cell-wall-degrading enzyme used must be sufficiently active to achieve the second objective, i.e. the solubilization of a significant amount of the cell wall. Crude 'cellulase' preparations are readily available in liquid or solid form; all appear to hydrolyse both cellulose and hemicelluloses (Table 8.1). There is a wide disparity in the activity of cellulases from different fungal sources (Table 8.2) and it is essential to establish the activity of the cellulase by reference to plant cell-wall material or cellulose paper and not to soluble derivatives, such as carboxymethylcellulose. Crude cellulases from *Trichoderma* species have generally been found to be the most reliable sources of crude cellulase, but the activity of enzymes from different suppliers should always be checked before use.

Sources of Cellulolytic Enzymes

Fungi and other microorganisms producing enzymes able to digest plant fibre are very widely distributed in nature, although those able to degrade crystalline cellulose are less common. The microbial degradation of cellulose and its related polysaccharides in soil is a vital process in the maintenance of the carbon cycle, enabling the decay of vegetation and the recycling of nutrients. Microorganisms in the gut of herbivores enable these animals to survive on diets largely composed of plant fibre; vast areas of natural and improved grasslands are thereby utilized for the production of food.

There is, therefore, a wide choice of potential sources of cellulases for use in forage analysis. Ideally, the enzyme system used to predict *in vivo* digestibility would be derived from rumen microorganisms. Cell-free extracts from disrupted mixed rumen bacteria have been shown to rapidly hydrolyse plant hemicelluloses (Bailey and McRae, 1970); these extracts, however, have little or no activity against purified crystalline cellulose, although they do attack the amorphous polymer to varying degrees. Attempts to extract active cellulase preparations from rumen

Table 8.1. Hydrolysis (%) by *Trichoderma* cellulase of cellulose and hemicellulose isolated from ryegrass (from Jones and Hayward, 1973).

	Hours' incubation			
	3	6	24	48
Cellulose	42	66	98	101
Hemicellulose	39	59	73	77

microorganisms for use in enzyme assays have been largely unsuccessful in comparison with aerobic fungi and gave poor yields. This is undoubtedly related to the anaerobic growth habit of microorganisms in the rumen, which means that the yield of extracellular hydrolases is poor in comparison with that of their aerobic counterparts. Other significant factors in enzyme production include the fact that rumen bacterial cellulases are not extracellular but are often produced in a large complex, the cellulosome, which is tightly associated with the bacterial cell or bound to the substrate that is being degraded. The hydrolytic enzymes produced by the anaerobic gut fungi are an exception, however, and culture filtrates of these organisms do have considerable extracellular hydrolase activity, probably because they have very high specific activities. Nevertheless, the ability to produce significant quantities of gut fungal hydrolases is limited by the fact that these organisms are obligate anaerobes and large-scale industrial fermentation systems do not exist for their exploitation.

In view of the difficulties in obtaining cellulolytic enzymes from rumen sources, attention has been directed towards the aerobic cellulolytic fungi. The cellulase and associated enzyme systems of these fungi are extracellular and are therefore readily recovered from the culture fluid in which the organism is grown. Culture solutions comprise a mixture of salts, nutrients and cellulose at the requisite pH. After a growth period of 7–10 days under aerobic conditions, the culture fluid is removed by filtration and freeze-dried or an acetone powder is prepared. Suitable conditions for the preparation of an active cellulase–hemicellulase from *Trichoderma* species have been described by Reese and Mandels (1963).

Trichoderma cellulases have generally been found to be more active than those of other fungi, when measured using native cellulose or plant cell walls as substrates. Extracts from *Trichoderma* have therefore been extensively studied to determine the nature of the cellulase complex. It is evident that the complex comprises several enzymes necessary for attack of highly orientated forms of cellulose. This includes exocellulase and endocellulases, which attack amorphous regions of the cellulose polymer, producing shorter-chain oligomers, and a cellobiohydrolase, which hydrolyses cellobiose to glucose. All components apparently need to be present for the efficient solubilization of cotton cellulose with complicated synergistic effects existing between the different enzymes. It is clearly essential that cellulases used for forage analysis retain all the components of the cellulase complex. Attempts by suppliers to purify or concentrate crude cellulase extracts may be responsible for the poor activity noted in cellulases from some fungal sources (Table 8.2).

Table 8.2. Activity of cellulases from different fungal sources (from Jones and Hayward, 1975).

	% dry matter solubilized	
	Herbage	Cellulose paper
Trichoderma spp.	57	69
Basidiomycete	48	20
Rhizopus spp.	35	7
Aspergillus niger	45	10
Buffer only	37	5

The Development and Validation of Cellulase-based Techniques

Relationship of solubility in cellulase to in vivo *digestibility*

The earliest attempt to replace rumen inoculum as a digestion medium for the laboratory estimation of forage digestibility appears to be that of Donefer *et al.* (1963). These workers found the solubility of forage in cellulase, or cellulase with pepsin, to be no better related to *in vivo* digestibility than solubility in buffer solution without enzyme; solubility in cellulase was better related to voluntary intake than to digestibility. It should be noted that the cellulase used by Donefer *et al.* appears to have a low activity and that the concentration of cellulase used was much lower than that used by subsequent workers.

The first successful use of cellulase as a replacement for inoculum was reported by Jarrige *et al.* (1970), using a crude cellulase derived from a soil basidiomycete. Jarrige *et al.* found solubility in the cellulase to be highly correlated ($r = 0.92$) with *in vivo* digestibility for grass and legume forages. Cellulase solubility gave a better prediction of digestibility than the Tilley and Terry (1963) inoculum method and was highly reproducible, with only a third of the standard error of the inoculum method. There was no significant week-to-week variation using cellulase but variability using inoculum was highly significant between weeks. It was shown that the crude cellulase also contained an active hemicellulase and protease.

Jones and Hayward (1973) found the solubility of dried grasses in *Trichoderma* cellulase to be highly correlated ($r = 0.92$) with *in vivo* digestibility. The crude cellulase used contained 71 g kg^{-1} protein and 640 g kg^{-1} of water-soluble carbohydrate, half in the form of reducing sugars. The enzyme preparation contained active cellulases, hemicellulases and proteases, and completely hydrolysed isolated cellulose and 70% of isolated hemicellulose from ryegrass in 24 h (see Table 8.1); the cellulase removed about half the total protein in the grasses in 24 h.

The amount of dry matter solubilized by the enzyme preparations used by Jarrige *et al.* (1970) and Jones and Hayward (1973) was 15–25 units lower than *in vivo* or *in vitro* (inoculum) values; a regression equation was therefore required to relate cellulase solubility to *in vivo* digestibility. It has been well established that cellulases from different fungal sources or from different suppliers can vary greatly in their capacity to solubilize native cellulose or plant cell walls (Jones and Hayward, 1975; Tinnimit and Thomas, 1976; Rexen, 1977). Cellulases from *Trichoderma* have generally been found to be the most effective and consistent. Some cellulases have no greater solubilizing activity than simple buffer solutions (Table 8.2).

The effectiveness of *Trichoderma* cellulase is confirmed by other studies, showing simple solubility in cellulase to be well related to *in vitro* (inoculum) digestibility of grasses and legumes (Pulli, 1976) and to be applicable to the evaluation of fresh sorghum leaf (Tarumoto and Masaoka, 1978) and treated wood products (Moore *et al.*, 1972).

Other workers have studied the effects of combining cellulase digestion with subsequent treatment with other enzymes, notably proteases. Guggolz *et al.* (1971) found solubility in cellulase followed by pronase to be well related to *in vivo* digestibility for a range of forages, cob residues, straw, etc. Different regression

equations were required for the different materials assayed, but, subject to this provision, cellulase solubility was considered a suitable alternative to inoculum methods. *Trichoderma* cellulase was found by McQueen and van Soest (1975) to digest forage cell walls more efficiently than *Aspergillus* cellulase; incubation with acid pepsin following cellulase treatment increased the amount of dry matter solubilized. Cellulase–pepsin solubility was highly correlated (r = 0.87) with *in vivo* digestibility, but different regression equations were required for grasses and legumes. *In vitro* (inoculum) digestibility predicted *in vivo* digestibility more precisely (r = 0.95) and was less influenced by species differences. Similarly, Tinnimit and Thomas (1976) concluded that *in vitro* (inoculum) digestion gave a better prediction of *in vivo* digestibility for tropical grasses and legumes than an enzyme method involving successive incubations with cellulase, amylase and pepsin.

The effect of different pretreatments on cellulase digestion

Incubation with acid pepsin

Incubation with pepsin in 0.1 mol l^{-1} hydrochloric acid prior to cellulase treatment was shown by Jones and Hayward (1975) to increase the amount of dry matter solubilized and to significantly improve the correlation of enzyme solubility with the *in vivo* digestibility of grasses (Table 8.3).

Incubation with pepsin after cellulase only slightly improved the correlation with *in vivo*, while pretreatment with 0.1 mol l^{-1} hydrochloric acid had no effect on the correlation. It was concluded that acid–pepsin treatment improved the accessibility of the cell-wall polysaccharides to the cell-wall-degrading enzymes. Pepsin–cellulase solubility was also found to be highly correlated with *in vitro* (inoculum) digestibility for grasses (r = 0.96) and clover (r = 0.94), with very similar regression equations derived from the different species. The pepsin–cellulase method was found to be highly reproducible, with a coefficient of variation of 1.09% for successive batches of analyses. The technique has been in routine use at the Institute of Grassland and Environmental Research for the past 20 years. The only modifications to the original method described by Jones and Hayward (1975) are: (i) the reduction of incubation time in cellulase from 48 h to 24 h; and (ii) the use of a liquid *Trichoderma* cellulase (Celluclast 1.5 L from Novo Nordisk 4/5, 2880 Bagsvaerd, Denmark). The liquid concentrate is diluted 10 ml l^{-1} with the acetate buffer previously described and 20 ml used 200 mg^{-1} of dried forage. The enzyme concentrate is purchased in 5 l quantities and each batch calibrated against a set of

Table 8.3. The effect of pepsin pretreatment on the relationship of cellulase solubility with *in vivo* digestibility (from Jones and Hayward, 1975).

Method	Correlation coefficient (r)	Residual standard deviation
Cellulase only	0.87	3.74
Pepsin + cellulase	0.93	3.17
In vitro (inoculum)	0.91	3.42

samples of known *in vivo* digestibility. The regression equations used to predict digestibility are then adjusted, if necessary; changes in the equation over many years have been minor and would have little effect on predicted values.

Many workers have shown the pepsin–cellulase technique, as described by Jones and Hayward (1975) or with minor modifications, to predict *in vivo* digestibility with an accuracy comparable to inoculum methods. The method has been successfully applied to a variety of materials, including tropical grasses and legumes (Adegbola and Paladines, 1977), grass silages (Downman and Collins, 1977), hays (Adamson and Terry, 1980), treated straw (Rexen, 1977) and kale cell walls (McCluskey *et al.*, 1984). Goto and Minson (1977) found pepsin–cellulase solubility to be highly correlated ($r = 0.94$) with *in vivo* digestibility for both tropical and temperate grasses over a range of 40–76% *in vivo* digestibility. Using separate regressions for different grass species reduced the standard error of prediction. It was concluded that the enzyme method could accurately predict *in vivo* digestibility, provided samples of known *in vivo* digestibility similar to those being tested were included as standards in each run.

Bugge (1980) showed a simple digestion with cellulase to predict the digestibility of grasses as precisely as the pepsin–cellulase method; predicted digestibility for clover and high-protein feeds (peas, beans, fish-meal), however, was much improved by pepsin pretreatment. Clark and Beard (1977) found pepsin pretreatment to improve correlations of cellulase solubility with *in vivo* digestibility for grain diets and forages; differences in substrate solubility by *Aspergillus* compared with *Trichoderma* cellulases were far less marked if samples were pepsin-treated. A cellulase from *Aspergillus niger* appeared to contain an amylase and was better suited for the analysis of cereal feeds than *Trichoderma* cellulase. Allison and Borzucki (1978) claimed that increasing the acid concentration for the pepsin stage and the incubation temperature to 50°C gave pepsin–cellulase solubility values comparable to *in vitro* digestibility. Jones and Hayward (1982) compared this and other suggested modifications, but could find no improvement over the original procedure.

Terry *et al.* (1978) compared the pepsin–cellulase technique with the two-stage inoculum method (Tilley and Terry, 1963) for predicting *in vivo* digestibility for grasses, lucerne, red clover and sanfoin. They found the enzyme method to be very repeatable and only slightly less accurate than inoculum for predicting the digestibility of grasses (Table 8.4). Enzyme solubility was less accurate than the inoculum method, however, for predicting the digestibility of the legumes. For the inoculum method, a single regression equation was permissible for both grasses and legumes. Separate regressions were required for the grasses and for each legume species when the enzyme technique was used.

Aufrere and Michalet-Doreau (1988) have suggested an interesting variation of the pepsin–cellulase technique to cater for feeds containing starch. The method involves three stages: after the initial acid–pepsin incubation at 40°C, the temperature is raised to 80°C for 30 min to hydrolyse starch; the final stage is an incubation with cellulase after removal of the acid pepsin. The results using this technique were highly correlated ($r = 0.98$) with *in vivo* digestibility for a range of forage, straw and mixed diets, containing varying preparations of concentrates and by-products. A similar procedure with a longer heating time at 80°C has been described by de Boever *et al.* (1988) (see below, 'Hot-acid extraction').

Table 8.4. Relationship between *in vivo* digestibility and (a) *in vitro* digestibility (inoculum) and (b) pepsin–cellulase solubility (derived from Terry *et al.,* 1978).

	Grasses	Lucerne	Red clover	Sainfoin
No. of samples	48	8	8	9
(a) *In vitro* (inoculum)				
r	0.97	0.99	0.89	0.99
RSD	1.46	0.74	2.77	0.70
Slope	1.01	1.03	0.99	0.99
Intercept	0.52	−2.56	3.19	2.00
(b) Pepsin–cellulase				
r	0.96	0.98	0.86	0.99
RSD	1.80	1.49	3.14	0.93
Slope	0.56	1.05	0.77	0.97
Intercept	34.72	−9.25	14.33	2.21

RSD, residual standard deviation.

Neutral-detergent extraction

Neutral-detergent extraction, under reflux or at 100°C, has been widely used as a pretreatment before incubation with cellulase (Abe *et al.*, 1972a, b; Hartley *et al.*, 1974; Jones and Bailey, 1974; Roughan and Holland, 1977; Downman and Collins, 1982).

Jones and Bailey (1974) showed cellulase digestion with neutral-detergent residues from grasses to be highly correlated with the voluntary intake of grasses by sheep. The procedure was considered appropriate for breeding programmes aimed at improving both digestibility and intake. Difficulties were encountered in routine use, however, due to the inhibition of cellulase digestion by traces of lauryl sulphate from the neutral-detergent reagent. Hartley *et al.* (1974) found that the optical density (324 nm) of cellulase extracts of neutral-detergent residues from grasses was highly correlated with *in vivo* digestibility; the ultraviolet (UV) absorption was attributed to the release of carbohydrate esters of phenolic acids by the cellulase.

Roughan and Holland (1977) prepared their own potent crude cellulase from a mutant strain of *Trichoderma*, which readily attacked plant cell wall isolated by neutral-detergent extraction. They proposed a method involving hot neutral detergent, followed by exhaustive digestion with two successive batches of cellulase. Values obtained using this technique were highly correlated ($r = 0.98$) with *in vivo* digestibility of legumes and grasses. Downman and Collins (1982) compared cellulase solubility, using varying concentrations of cellulase, after either pepsin or neutral-detergent pretreatment, with *in vitro* (inoculum) and *in vivo* digestibility for a range of forages, silages, cereals and complete diets. An additional digestion with amyloglucosidase was included in the enzyme methods for materials containing starch. Some of Downman and Collins' (1982) results are summarized in Table 8.5 and show the enzyme techniques to be at least as precise as *in vitro* inoculum for predicting the *in vivo* digestibility of most forages; there was little difference found between the two enzyme methods. The authors point out that the pepsin–cellulase method was easier to manipulate and less time-consuming than the detergent

Table 8.5. Correlation (*r*) of *in vivo* digestibility with cellulase solubility* after pepsin or neutral-detergent pretreatment and with *in vitro* (inoculum) digestibility (derived from Downman and Collins, 1982).

	Pepsin–cellulase	Detergent–cellulase	*In vitro* (inoculum)
Grass silage (n = 16)	0.87	0.84	0.81
Maize silage (n = 16)	0.72	0.72	0.68
Dried grass (n = 16)	0.84	0.70	0.51
Hay (n = 28)	0.81	0.88	0.93

* *Trichoderma* cellulase (20 g l^{-1}).

method; an advantage of the latter was that samples could be analysed in fewer working days. It was concluded that both cellulase methods could be regarded as acceptable alternatives to the *in vitro* inoculum method for the routine evaluation of forages. A routine procedure for the estimation of neutral detergent–cellulase digestibility (NCD) has been described by Alderman (1985); Morgan *et al.* (1989) has described a variation of the method using the Fibertec digestion system.

More recently, Downman (1993) has proposed a modified detergent–cellulase method to accommodate the galactomannans found in palm-kernel oil. These polysaccharides are not solubilized by cellulase but can be solubilized by the inclusion of an additional carbohydrase, gamanase, in the buffered cellulase and an increase in digestion time to 40 h. The modified method significantly improved the correlation with *in vivo* metabolizable energy values; the inclusion of gamanase did not affect predicted energy values for feedingstuffs not containing palm-kernel oil. It was concluded that the extra expense of the modified method was justified for compound feedingstuffs containing 10% or more of palm-kernel oil; for samples known to be free of palm oil, the original detergent–cellulase method would remain valid. Downman (1993) points out that new commodities introduced into the feed trade in the future will need to be monitored to decide on the appropriate analytical methodology required. A routine procedure for the determination of neutral detergent cellulase–gamanase solubility (NCGD) has been described by the Ministry of Agriculture, Fisheries and Food (MAFF)-sponsored working party on the prediction of energy values of compound feeds (MAFF, 1993). Appropriate test kits have been prepared for the technique (Biotal Ltd., Cardiff) and the working party specify that kits from this source must be used in support of their prediction equation for the metabolizable energy content of ruminant compound feeds.

McLeod and Minson (1982) compared the accuracy of the detergent and pepsin–cellulase methods for predicting the *in vivo* digestibility of a range of grass and legumes. Both cellulase methods were highly correlated with *in vivo* digestibility values (Table 8.6); pepsin pretreatment appeared to be more precise for grasses and detergent pretreatments for legumes. It was concluded that pretreatment with detergent increased analytical error and that the method failed to produce an overall improvement in the accuracy of predicting *in vivo* digestibility; any advantage gained from a shorter analysis time was offset by an increase in analytical error. Similarly, Coehlo *et al.* (1988) found no advantage of detergent, compared with

Table 8.6. Relation of *in vivo* digestibility to DM solubility in cellulase after either pepsin or neutral-detergent pretreatment (derived from McLeod and Minson, 1982).

	r value	RSD	Analytical error (±)
Pepsin–cellulase			
Grasses (*n* = 50)	0.94	2.6	0.37
Legumes (*n* = 30)	0.92	2.9	0.18
All (*n* = 80)	0.92	2.9	0.29
Detergent–cellulase			
Grasses (*n* = 50)	0.90	3.3	0.90
Legumes (*n* = 30)	0.94	2.7	1.06
All (*n* = 80)	0.89	3.4	0.98

RSD, residual standard deviation.

pepsin, pretreatment for predicting *in vivo* digestibility for lucerne (alfalfa), a range of grasses and ryegrass–lucerne mixtures. The addition of *Aspergillus* hemicellulase to *Trichoderma* cellulase improved the correlation with *in vivo* values for both pepsin and detergent methods. The use of this enzyme combination following pepsin pretreatment predicted *in vivo* digestibility as precisely as the two-stage *in vitro* (inoculum) method studied (r^2 = 0.88).

Givens *et al.* (1990a) compared the accuracy of different laboratory techniques in predicting the *in vivo* digestibility of 173 spring-grown grasses in England and Wales. Samples were taken from commercial farms at different locations and at different growth stages over a 4-year period; the swards varied in age and in the component species and varieties. The relationship of *in vitro* digestibility (inoculum) and fibre content to *in vivo* digestibility was significantly affected by year of harvest, location, predominant variety and age of ley. The detergent–cellulase and pepsin–cellulase methods were less affected by these factors; both, however, were significantly affected by predominant variety. Overall, the enzyme methods were better related to *in vivo* values than either *in vitro* (inoculum) or fibre estimations (Table 8.7); there was a slight advantage in precision indicated for the detergent– compared with the pepsin–cellulase method. It was concluded that the considerable benefits of the cellulase methods, including increased accuracy of prediction and relative freedom from external effects, should ensure that they replace fibre estimations for the routine evaluation of fresh herbage. The same conclusions were shown to be valid for predicting the metabolizable energy content of fresh grass (Givens *et al.*, 1990b).

In a comparison of spring- and autumn-harvested pasture herbage, Givens *et al.* (1993a) found both detergent– and pepsin–cellulase methods to be highly correlated (74.6–76.7% of accountable variance) with the *in vivo* digestibility of spring grass; both enzyme methods were superior to *in vitro* (inoculum) and modified acid-detergent (MAD) fibre methods. Regression equations for predicting *in vivo* digestibility were significantly different, however, from those established in the previous study (Givens *et al.*, 1990a) for all methods except pepsin–cellulase. The difference for detergent–cellulase, though statistically significant, was numerically

Table 8.7. Relationship of *in vivo* digestibility of grasses to enzyme solubility and *in vitro* digestibility (inoculum).

Reference	Method	Accountable variance (%)	SEP
Givens *et al.* (1990a)	Detergent–cellulase	76.6	27.1
	Pepsin–cellulase	75.9	28.8
	In vitro (inoculum)	67.0	33.2
	MAD fibre	66.9	33.3
Givens *et al.* (1993a)	Detergent–cellulase	53.3	29.3
	Pepsin–cellulase	63.4	25.9
	In vitro (inoculum)	40.5	33.1
	MAD fibre	49.4	30.5

SEP, standard error of prediction.

small and would have little effect on predicted values. Both enzyme techniques and *in vitro* (inoculum) were poorly related to the *in vivo* digestibility of autumn pasture (13–20% of accountable variance) and inferior to MAD fibre; the relationships found for the autumn differed significantly in slope and intercept from those for spring herbage for all methods. The best overall (spring and autumn combined) relationship with *in vivo* digestibility was with pepsin–cellulase (Table 8.7); in this study, the relationship for *in vitro* (inoculum) accounted for less of the variance in *in vivo* values than MAD fibre. It was suggested that the poor relationships found for autumn grass could be largely attributable to the small range in *in vivo* values for autumn grass. Other suggested explanations were that environmental factors changed the relationship between plant cell-wall structure and *in vivo* digestibility and changes in species composition, particularly clover, between spring and autumn. It was concluded that cellulase-based techniques, particularly pepsin–cellulase, were more strongly related to digestibility and metabolizable energy than the inoculum or fibre methods, particularly for spring primary growths of grass. The accuracy of prediction, however, could be improved by developing separate regression equations for spring and autumn crops.

When the same enzyme and inoculum techniques were applied to dried samples of 124 clamp silages from farms (Givens *et al.*, 1989), the best single predictor of silage *in vivo* digestibility was the *in vitro* inoculum method (Table 8.8). Of the enzyme methods, pepsin–cellulase was better related to digestibility than detergent–cellulase; all the biological methods were superior to MAD fibre content. The relationships of the laboratory techniques to *in vivo* metabolizable energy were much poorer than for digestibility, the best method (*in vitro* inoculum) only accounting for 24% of the variance. This was increased to 77% by inclusion of a gross energy term in a bivariate equation, and it was concluded that inclusion of this or a related term was essential for predicting silage metabolizable-energy content with acceptable accuracy.

Givens *et al.* (1993b) found the detergent–cellulase method to be the best predictor of *in vivo* digestibility for big-bale silage (Table 8.8). Both enzyme methods, however, gave prediction equations differing significantly in both slope and intercept

Table 8.8. Relationship of the *in vivo* digestibility of clamp and big-bale silages to enzyme and inoculum laboratory techniques.

	Method	Accountable variance (%)	SEP
Clamp silage (Givens *et al.,* 1989)	Detergent–cellulase	58.8	39.7
	Pepsin–cellulase	68.0	32.3
	In vitro (inoculum)	74.1	33.6
Bale silage (Givens *et al.,* 1993b)	Detergent–cellulase	84.0	25.3
	Pepsin–cellulase	75.6	25.7
	In vitro (inoculum)	74.5	32.9

from those previously established for clamp silage; equations for *in vitro* (inoculum) did not differ significantly between clamp and bale. Using the enzyme methods, different prediction equations would therefore be required for clamp and bale silages to predict *in vivo* digestibility with acceptable accuracy. Similarly, Barber *et al.* (1990) found the slope and intercept of the relationship between pepsin–cellulase solubility and *in vivo* digestibility to vary significantly for silages from different centres. Within centres, pepsin–cellulase was as well related to *in vivo* digestibility (r^2 = 0.53 to 0.82) as was *in vitro* (inoculum) digestibility (r^2 = 0.48 to 0.83). The inoculum method, nylon-bag digestion and near-infrared reflectance (NIR) relationships with *in vivo* digestibility were not significantly different between centres. A good relationship was found by Givens *et al.* (1995) between detergent–cellulase solubility and the *in vivo* digestibility of maize silages from different centres; one population of silages, however, gave a regression equation significantly different from that of the others. It was suggested that environmental factors or interactions due to drying may have contributed to the differing equations. In a comparison of field-dried and barn-dried hays, Moss and Givens (1990) found a significant effect of curing on the relationship of detergent–cellulase and *in vitro* (inoculum) with *in vivo* digestibility; relationships for pepsin–cellulase and MAD fibre were not significantly affected by curing and were deemed to be the only methods giving acceptable prediction equations for hay.

Bughrara and Sleeper (1986) found a simple digestion in buffered cellulase to be highly correlated (r = 0.91) with the *in vivo* digestibility of a range of temperate forage species. The correlation with *in vivo* was improved, however, if the forage was pretreated with neutral detergent (r = 0.97) or acid pepsin (r = 0.94) prior to cellulase digestion. They concluded that the one-stage cellulase technique would be adequate as a rapid, low-cost, method for screening in plant-breeding programmes. More precise estimation required pretreatment with detergent or pepsin; both enzyme methods were considered to be more rapid and convenient and less costly methods of predicting *in vivo* digestibility than the two-stage *in vitro* inoculum method of Tilley and Terry (1963). Subsequent work (Bughrara *et al.*, 1989) showed similar results for lucerne. Again, simple digestion in cellulase was well related to *in vivo* digestibility (r = 0.91), but the relationship was improved by pretreatment with detergent (r = 0.94) or acid pepsin (r = 0.96). Similarly, Stakelum *et al.* (1988) found both detergent– and pepsin–cellulase methods to be as precise

as *in vitro* inoculum for predicting the *in vivo* digestibility of fresh grass for dairy cows.

Hot-acid extraction

More drastic pretreatments than pepsin or neutral detergent have been proposed in efforts to increase the amount of dry matter solubilized by cellulase to levels comparable with *in vivo* digestion. Kirchgessner and Kellner (1977) found refluxing with 2 mol l^{-1} hydrochloric acid for 30 min, followed by digestion with cellulase and then pepsin, to give values numerically similar to *in vivo* digestion for a range of forages (grasses, legumes and cereal straw). Results obtained with this method were highly correlated (r^2 = 0.85) with *in vivo* digestibility and the method was concluded to be a viable alternative to inoculum methods.

Few comparisons appear to have been made between the acid–reflux method of Kirchgessner and Kellner (1977) and the detergent–cellulase or pepsin–cellulase procedures. Pace *et al.* (1984) found *in vitro* (inoculum) values for grasses and legumes to be highly correlated with pepsin–cellulase solubility (r = 0.96) and the reflux–acid method (r = 0.91). Different regression equations were needed to predict *in vitro* values of grasses and legumes for pepsin–cellulase but not for the acid–reflux procedure. Highly significant week-to-week differences were found between different analytical runs for the inoculum procedure, but not for the enzyme methods. Bughrara *et al.* (1989) found that the reflux–acid method compared favourably with the detergent and pepsin methods when applied to lucerne forages.

De Boever *et al.* (1988) compared the accuracy of different enzyme techniques and *in vitro* inoculum for predicting the *in vivo* digestibility of maize silage, grass silage and grass hays. The enzyme techniques included: (i) the 2 mol l^{-1} acid-pepsin–cellulase procedure; (ii) pepsin–cellulase solubility with varying incubation times for the pepsin and cellulase stages; and (iii) a variant of the pepsin–cellulase method involving three stages, namely: incubation in acid pepsin, heating the acid pepsin solution at 80°C for 45 min to remove starch and finally incubation with cellulase. The detergent–cellulase method was not evaluated, as this method had proved more difficult to manipulate than pepsin–cellulase, resulting in a lower repeatability. The *in vitro* inoculum method was the best predictor of *in vivo* digestibility for grass silage and hay, but the enzyme methods were generally superior for maize silage, particularly methods involving a hot-acid pretreatment (Table 8.9). No one enzyme method was best for all forages, but the pepsin–cellulase-based methods were more consistent than those involving acid–reflux. A similar method to that proposed by De Boever *et al.* (1988), but with a shorter heating time at 80°C, has been suggested by Aufrere and Michalet-Doreau (1988). The method was found to be more precise than the *in vitro* (inoculum) procedure for predicting the *in vivo* digestibility of a diverse range of feeds, including cereals, by-products and dried forages.

Table 8.9. Relationship of the *in vivo* digestibility of silage and hay to enzyme solubility and *in vitro* (inoculum) digestibility (derived from de Boever *et al.,* 1988).

	Correlation coefficient (*r*)		
Procedure	Maize silage	Grass silage	Grass hay
(a) 2 mol l^{-1} HCl (reflux) Cellulase (40°C) Acid pepsin 40°C)	0.89	0.45	0.91
(b) Acid pepsin (40°C) Cellulase (40°C)	0.84	0.77–0.82	0.92–0.93
(c) Acid pepsin (40°C) Acid pepsin (80°C) Cellulase (40°C)	0.88	0.76–0.78	0.90
(d) *In vitro* (inoculum)	0.84	0.90	0.96

(a) Kirchgessner and Kellner (1977); (b) Jones and Hayward (1973); (c) de Boever *et al.* (1988); (d) Tilley and Terry (1963).

Comparative Assessment of Cellulase Techniques, Their Limitations and the Need for Further Work

The experimental work reviewed shows that cellulase-based techniques predict *in vivo* digestibility more precisely than chemical methods, such as MAD fibre, and are at least comparable in accuracy to inoculum digestion methods. Provided an active cellulase (usually from *Trichoderma*) is used, a simple one-stage digestion in cellulase permits a rapid, reliable and low-cost estimate of digestibility, which may be adequate for screening in plant-breeding programmes (Jones and Hayward, 1973; Bugge, 1980).

It has also been shown that the precision of predicting *in vivo* digestibility is significantly improved if the forage is pretreated with acid pepsin, neutral detergent or 2 mol l^{-1} hydrochloric acid prior to the cellulase digestion. The results do not show a clear and consistent advantage for any of the pretreatments, although the drastic 2 mol l^{-1} acid pretreatment has sometimes given poor results (de Boever *et al.*, 1988). This is not surprising, since this treatment would remove hemicelluloses, generally shown in *in vivo* studies to be the least digestible fraction of the cell-wall polysaccharides. This method does, however, have the advantage of removing starch, while the detergent and pepsin methods require an additional amyloglucosidase digestion or acid hydrolysis for its removal.

In nearly all the work reviewed, the relationships between enzyme solubility and *in vivo* digestibility have been examined in one population of forages and predictive equations derived for that population. For a method to have any real value in forage evaluation, it is essential that predictive equations are applicable to other populations, at least of the same or similar forages. Comparisons made by Givens *et al.* (1990a, 1993a) suggest that the pepsin– and detergent–cellulase methods are more robust and less variable than the *in vitro* (inoculum) method of Tilley and

Terry (1963) for predicting the *in vivo* digestibility of fresh grass; the enzyme methods were less affected by factors such as year of harvest, age of crop and location, but were affected by species differences. Equations for predicting *in vivo* digestibility differed significantly between spring and autumn grass for both the enzyme and the inoculum methods. Significantly different predictive equations for *in vivo* digestibility have also been reported for the enzyme methods between clamp and bale silages (Givens *et al.*, 1993b) and between different populations of grass silage (Barber *et al.*, 1990) and maize silage (Givens *et al.*, 1995); predictive equations based on *in vitro* (inoculum) were not significantly affected.

A further complication in deriving general equations for predicting *in vivo* digestibility for pasture crops containing clover is the extent to which enzyme solubility is influenced by species. Jones and Hayward (1975) found no differences in the relationship of *in vitro* (inoculum) digestibility to pepsin–cellulase solubility between grasses and clovers. Terry *et al.* (1978), however, found marked differences in the relationship of pepsin–cellulase solubility to *in vivo* digestibility for arable legumes compared with grasses. Other studies (Goto and Minson, 1977; McLeod and Minson, 1982) have shown the relationship of enzyme solubility to *in vivo* digestibility to be improved if the species are considered separately. There is evidence that species specificity is reduced if the $2\ \mathrm{mol\ l^{-1}}$ acid method of Kirchgessner and Kellner (1977) is used (Pace *et al.*, 1984).

It is, perhaps, not surprising that the relationships between *in vivo* digestibility and enzyme solubility for different crops should prove variable under different environmental and other conditions. It is known that environmental factors can influence both the content and degree of lignification of the cell wall. Enzyme methods solubilize less of the cell wall than *in vivo* digestion and are more influenced by the degree of lignification; it is likely, therefore, that environmental effects on cell-wall structure will influence enzyme solubility differently from *in vivo* digestion. Differences in botanical composition may also be significant, since it appears that the cell walls of legumes are more readily solubilized by cellulase than those of grasses. A further consideration is that *in vivo* digestion can be influenced by a variety of factors that do not affect cellulase solubility, e.g. level of feeding, degree of chop or the nutrient status of the feed. Rees and Minson (1979), for example, found that pepsin–cellulase solubility gave a biased estimate of *in vivo* digestibility in sheep whose digestive efficiency was decreased by inadequate sulphur levels. Additional factors that could influence predictive equations for silages are differential losses of volatiles during sample drying or the influence of the degree and type of fermentation on rumen function and digestibility.

There is clearly a need for further *in vivo* and analytical studies to determine the extent to which predictive equations derived from enzyme solubility apply to different environmental and other conditions. Such studies need to take account of the increased use of grass–clover pastures and the introduction of alternative crops, such as forage maize, kale, red clover and whole-crop cereals, into livestock systems. The current interest in bicropping of protein- and energy-rich crops may also require additional analytical studies on the suitability of different enzymic procedures.

Of the cellulase methods currently in use, the pepsin–cellulase method has been found to be least time-consuming and easiest to manipulate (Downman and

Collins, 1982; de Boever *et al.*, 1988); it also uses inexpensive glassware and chemicals and a higher output per person can be achieved than with methods involving refluxing with detergent or strong acid. Samples containing starch can be accommodated with little extra manipulation if the acid hydrolysis method of de Boever *et al.* (1988) is used. The pepsin–cellulase method has been shown to have a higher analytical accuracy than the detergent technique (McLeod and Minson, 1982). The gamanase modification of the detergent–cellulase procedure (NCGD) was developed by Downman (1993) specifically to cater for polysaccharides in palm-kernel oil not hydrolysed by cellulase. This procedure, however, is being widely used for forages, in addition to concentrates, presumably to avoid a proliferation of methods and to simplify laboratory procedures. It can be argued that, in consequence, an unnecessarily complicated and costly procedure is being applied when simpler methods, such as pepsin–cellulase or detergent–cellulase, would give analytical results for forages of at least equal accuracy and validity and at less cost. A danger in attempting to develop a 'universal' method for all forages and all concentrates is that the method will inevitably become increasingly complicated. Downman (1993) anticipated such a development and emphasized the need to carefully monitor new raw materials being introduced to the feed trade so that appropriate analytical procedures could be devised.

Whatever the merits or otherwise of particular techniques, their usefulness in forage evaluation will ultimately depend on the reliability and consistency of the predictive equations derived for *in vivo* digestibility. Further work is clearly needed to determine the causes of variability in predictive equations and the significance of environmental and other factors.

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9 The *In Situ* Technique for the Estimation of Forage Degradability in Ruminants

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In the title, the technique to be discussed is termed the *in situ* technique. However, this is identical to the *in sacco* technique or the Terylene (Dacron)- or nylon-bag techniques. The procedure is as follows. Samples of dried and milled feed (to pass a 3 mm screen) or wet minced samples are placed in nylon bags (usually 10 × 17 cm). About 2 to 5 g, depending on density, are weighed precisely into each bag. The tied-up bags are incubated in the rumen of sheep or cattle on an appropriate diet by suspending them from a rumen cannula. They are then withdrawn after various intervals of time, washed and dried. Degradability of dry matter, nitrogen, energy, etc., can thus be measured against time. The pore size of the bags, about 40–60 μm, is such that few particles can escape, and yet microorganisms can enter the bag. Thus, fermentation rate inside the bag is similar to that in the rumen. The amount of soluble material in the sample is measured by washing and reweighing an unincubated bag. See also the recent review by Huntington and Givens (1995).

The development of the *in situ* technique was first intended to provide a dynamic assessment of the degradation of protein (Mehrez and Ørskov, 1977; Ørskov and McDonald, 1979). The characteristics of the degradation curve are described by the equation:

$$P = a + b\,(1 - e^{-ct})$$

where P is degradation at time t, a and b are constants and c is the rate constant of b. For protein, the intercept, a, is similar to the soluble fraction (washing loss); b represents the potential degradability.

The *in situ* technique for estimating degradability in the rumen is not new. It was first described by Quin *et al.* (1938), who used silk bags, and it was again employed by McAnally (1942). However, standardization and validation have only occurred recently. Incubation often proceeded for a maximum period of 48 h, and choice of this time was probably influenced by the *in vitro* digestibility method of

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Tilley and Terry (1963), who used this interval to reflect *in vivo* retention time for fibre and the solid fractional outflow rate of rumen solids, about 0.025 to 0.030 h^{-1}.

The Use of Dynamic *In Situ* Techniques to Evaluate Forages

When the technique was used for estimating degradation of roughages, it was soon found that the equation $P = a + b\ (1 - e^{-ct})$ had to be reassessed. This was first done by McDonald (1981), who defined the concept of a lag phase.

Figure 9.1 illustrates the degradation of a typical roughage. It can be seen that the intercept *a* is no longer similar to the solubility; in fact, it is most often negative, as there is a lag-phase period, when no net disappearance of substrate occurs. This is because the microbes take time to adhere to the substrate, so that, at first, there is no net loss in dry matter; in fact, there may be a small increase. While it is possible to describe a separate curve for the soluble fraction, there is always a problem. Increasing mathematical sophistication must be seen in relation to the accuracy required. Is the additional information generated as a result of increased mathematical complexity and is the additional sampling required necessary and cost-effective for describing feed degradation characteristics accurately enough for practical use?

In order to describe the feeding value of roughages, therefore, Ørskov and Ryle (1990) developed another system, in which the soluble fraction of the sample is determined in the laboratory. The method can use the neutral-detergent fibre (NDF) soluble fraction, the loss after washing with buffer solutions or the washing loss from a nylon bag containing substrate that was not incubated in the rumen.

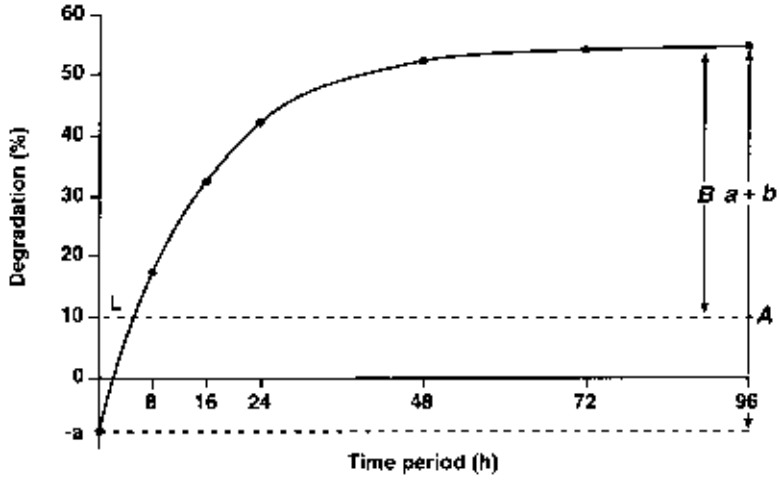

Fig. 9.1. Degradation of a typical roughage diet expressed by the formula $p = a + b\ (1 - e^{-ct})$. Due to a lag phase (L), *a* is negative. *A* is the soluble fraction, *B* the insoluble but potentially fermentable fraction ($B = (a + b) - A$) and *c* the rate constant of degradation.

Since A represents the soluble fraction, the insoluble but fermentable fraction *B* is worked out as $(a + b) - A$, with *c* as the rate constant (see Fig. 9.1). Particular attention must be given to the choice of incubation times. Ideally, no sample should be taken out of the rumen before the lag phase has ended, normally not before 8 h of incubation. If bags are withdrawn earlier, the curve is distorted and residual standard deviation (RSD) increases. The other rule is that the asymptote must be clearly identified. This is necessary for accurate description of both *B* and *c*. This is achieved by ensuring that the differences in degradability between the last two incubation times is small (less than 10% of the second to last incubation), i.e. 40 and 44%. Sometimes, bags have to be incubated for up to 120 h to describe the asymptote clearly, but usually 72 or 96 h are sufficient.

Some standardization of the microbial fermentation in the animal used for incubation has to be achieved in order to ensure that rumen conditions are optimal for cellulolysis. This is best achieved by feeding the animals on a high-quality roughage diet with about 25 g ntirogen (N) kg^{-1} digestible dry matter (Ørskov, 1992), which means that, if the digestibility is 60%, the diet contains 15 g N or 90–100 g crude protein kg^{-1}, and with rumen pH above 6.2 (Mould and Ørskov, 1984).

Use of the *In Situ* Technique for Evaluation of Roughages

The failure of all feed evaluation systems, including those based on net energy, such as the starch-equivalent systems and the Scandinavian feed-unit systems, or digestible energy, such as the total digestible nutrient (TDN) systems and the metabolizable-energy systems, is that they do not predict intake. While this is not serious as far as concentrate feed is concerned or when roughages are given in restricted amount, it is serious when, as is commonly practised, roughages or forages are grazed or stall-fed *ad libitum*. Ideally, feed evaluations should provide farmers with an exchange rate of one feed compared with another. They should allow planners of livestock production to assess the possible production and predict export or necessity for import of feed, etc. The present systems provide no such guidance and, as a result, farmers who feed mainly or exclusively roughages to their ruminants have no good feed-evaluation system. Digestibility and metabolizability, while positively correlated with feed intake, can generally only account for about a half of the variation in feed intake. While a variety of animal factors have been described which may limit intake of roughages (see Tolkamp and Ketelars, 1994), it does seem that we can advance most readily in practice by giving attention to the physical restriction to intake. This view is supported by the fact that ruminants with a large rumen volume can consume more roughage than animals with a low rumen volume and yet retain it long enough for thorough fermentation, e.g. indigenous vs. Holstein cows in Bangladesh (Mould *et al.*, 1982) and indigenous vs. upgraded sheep (Weyreter *et al.*, 1987).

If we characterize feeds in such a way that potential feed intake is predicted, we may begin to make progress in roughage evaluation. While intake is also influenced by interactions between feed and animal, especially those that affect ruminal retention of fibre, such as reduction of long to small particles, outflow of small particles

and, indeed, rumen volume, a feed evaluation system must characterize the feeds themselves, as the animal interaction cannot be determined in each instance. Also, because of the length of time it takes, it would be very expensive and impractical to determine intake and utilization of each feedstuff by feeding trials. Evaluation by chemical determination of feed ingredients, such as by the Weende system for proximate analysis and the more specific system of neutral- and acid-detergent fibre, has been practised for many years. However, these systems suffer from the same problem: they cannot predict feed intake accurately. While the level of acid-detergent fibre in the feed is correlated with feed intake, it accounts for only a half of feed-intake variation (Ørskov *et al.*, 1988). If, for instance, roughages are upgraded by alkali treatment, feed intake is increased, sometimes by as much as 50%, although the fibre content is not decreased or is decreased very little.

In many experiments, the characteristics of feed degradability have been described by the soluble fraction *A*, the insoluble but degradable fraction *B* and *c*, the speed at which the *B* fraction is degraded. These rate constants have been used in an attempt to develop a system that can predict not only feed nutritive value but consumption as well.

In a first trial (Ørskov *et al.*, 1988), five varieties of cereals were studied whose straw showed a range of different degradation characteristics. These straws were examined in their untreated state and after treatment with ammonia, so that ten different feeds were used. The results are seen in Table 9.1. All the straws were then offered *ad libitum* to groups of beef cattle consuming also 1.5 kg of concentrate daily, with sufficient protein to ensure that fermentation rate of untreated straw was not inhibited by low rumen-ammonia concentrations. The range of feed intakes and daily body-weight gains are also presented. It can be seen that the straws varied in quality, so that there were large differences in growth rate. However, the main purpose was to see if feed intake could be predicted from the multiple regression, using *A*, *B* and *C* as explanatory (independent) variables. All of these characteristics have an influence on the volume of ingesta in the rumen. The soluble material, denoted *A*, arises from cell contents and takes up little space. Generally, this fraction is almost 100% fermentable. The potentially degradable fraction, *B*, takes up space for as long as it remains in the rumen undegraded, and this depends on *C*. The undegradable fraction, $100 - (A + B)$, of course, takes up space until it is all eliminated by outflow from the rumen.

In order to see if intake and digestibility could be predicted from values for *A*, *B* and *C*, multiple regressions were tested on the data (Table 9.2). Here it can be seen that the multiple regressions of using *A*, *B* and *C* separately gave the best estimates of intake, because the correlations between *A*, *B* and *C* were only weak. This was further corroborated in an experiment with feeding of browse species to goats by Kibon and Ørskov (1993) in Nigeria (Table 9.3). In Tanzania, Shem *et al.* (1995) conducted similar work with steers receiving 17 different feeds from the slopes of Kilimanjaro (Table 9.4), while Khazaal *et al.* (1993), using ten leguminous herbages fed to sheep, showed essentially the same accuracy of prediction of dry-matter intake, digestibility and digestible dry-matter intake (Table 9.5). It is clear that, if digestible dry-matter intake can be predicted accurately, then, given sufficient length of time, growth rate can also be predicted accurately within an experiment.

Table 9.1. The effect of type and variety of straw and of feeding it untreated (U) or treated with ammonia (AM) on growth rate and dry-matter (DM) intake by steers and on *in vivo* digestibility by sheep, together with degradation characteristics (*A*, *B* and *C*).

Type	Variety	Treatment	Growth rate (g day^{-1})	Intake of straw (kg DM day^{-1})	Dry matter digestibility of straw (g kg^{-1})	*A*	*B*	*C*
Winter barley	Gerbel	U	106	3.43	409	12.5	26.3	0.0359
		AM	359	4.70	487	16.0	46.3	0.0257
	Igri	U	126	3.56	412	13.6	29.2	0.0309
		AM	332	4.82	455	15.9	37.3	0.0350
Spring barley	Corgi	U	400	5.16	484	16.0	36.1	0.0481
		AM	608	5.86	596	19.0	47.7	0.0457
	Golden Promise	U	198	4.43	452	15.0	40.5	0.0304
		AM	602	4.93	506	20.1	41.2	0.0377
Winter wheat	Norman	U	273	4.57	343	19.3	29.4	0.0343
		AM	516	5.81	484	24.5	37.5	0.0364
SE			40	0.17	14			

SE, standard error.

Table 9.2. Multiple correlation coefficients (r^2) between factors of the degradation equation and digestibility, dry-matter intake, digestible dry-matter intake and growth rate of steers (from Ørskov and Ryle, 1990).

Factors used in multiple regression analysis	Digestibility	Dry-matter intake	Digestible dry-matter intake	Growth rate
$(A + B)$	0.70	0.83	0.86	0.84
$(A + B) + c$	0.85	0.89	0.96	0.91
$A + B + c$	0.90	0.93	0.96	0.95
Index value	0.74	0.95	0.94	0.96

Table 9.3. Multiple correlation coefficients (r^2) between factors of the exponential equation and digestibility, dry-matter intake, digestible dry-matter intake and growth rate of goats (from Kibon and Ørskov, 1993).

Factors used in multiple regression analysis	Digestibility	Dry-matter intake	Digestible dry-matter intake	Growth rate
$(A + B)$	0.65	0.57	0.15	0.41
$A + B + c$	0.88	0.99	0.92	0.99
Index value	0.75	0.90	0.88	0.81

Table 9.4. Multiple correction coefficients (r) between feed-degradation characteristics and digestibility, dry-matter intake, digestible dry-matter intake and growth rate of steers.

Factors used in multiple regression analysis	Digestibility	Dry-matter intake	Digestible dry-matter intake	Growth rate
$(A + B)$	0.85	0.83	0.84	0.80
$(A + B) + c$	0.95	0.84	0.88	0.90
$A + B + c$	0.98	0.90	0.93	0.93
Index value	0.95	0.90	0.92	0.89

Although A, B and C can all positively influence intake, the intake of diets of similar digestibility can be different. In general, if leguminous herbages are compared with graminaceous herbages of the same digestibility, the intake of leguminous herbages is greater, largely due to a greater C value (Ishida *et al*., 1996). This implies that digestion of leguminous herbages more rapidly approaches completion in the rumen and therefore is not greatly affected by level of feeding, while differ-

Table 9.5. Multiple correlation coefficients (r^2) between degradation characteristics and leguminous forages and digestible and intake of hay by sheep (from Khazaal *et al.,* 1993).

Factors	Digestibility	Dry-matter intake
(*A* + *B*)	0.82	0.77
(*A* + *B*) + *c*	0.86	0.88
A + *B* + *c*	0.95	0.88

ences in feeding level and thus rumen fractional outflow can reduce the digestibility of graminaceous herbages (Ishida *et al.*, 1996).

For practical application and advice, it is necessary to generate a simple value, which has been termed feed potential, i.e. the ability of a feed to provide a given level of digestible nutrient intake. This was done in the work of both Ørskov *et al.* (1988) and Shem *et al.* (1995), with quite similar results. Let us assume that intake = $\chi_1 A + \chi_2 B + \chi_3 C$ and simplify by dividing each coefficient by χ_1, thus:

$$\left(\frac{\chi_1}{\chi_1}\right)A + \left(\frac{\chi_2}{\chi_1}\right)B + \left(\frac{\chi_3}{\chi_1}\right)C$$

Then the coefficients of *A*, *B* and C were 1, 0.4 and 200 respectively from the work referred to. Thus, if a feed – for instance, straw – has values of *A* = 12, *B* = 40 and C = 0.03, the index value would be 12 + 16 + 6 = 34. This index or feed-potential value has no biological meaning, but in the work of Ørskov *et al.* (1988) an index value of about 30 enabled the animals to consume sufficient for energy maintenance. It is likely that different breeds of animals and animals in different physiological states will require feeds of slightly different index values to provide for maintenance, but the ranking of feeds is likely to be similar. Much more work is needed to provide a strong database. It is likely that the coefficients will be similar, since essentially they rely on dietary microbial interactions and only to a limited extent on host animal (see later), except when the rumen environment is less than optimal. The degradation characteristics of a number of roughages are compiled in Table 9.6.

If this concept can be confirmed to be useful, then it may serve as a basis for a new global roughage evaluation system, which could perhaps also be applied to rangeland in different seasons. It may be possible to provide feed-potential inventories in different regions and in different seasons, from which one could match animal potential to feed-resource potential. It may also be possible to estimate the extent to which feed should be imported or upgraded to meet an animal potential greater than the present feed potential can realize and thus help to avoid the many mistaken ways in which animal potential and feed potential have been mismatched. A particular example of this is the introduction of animals with a potential far above the potential of the local feed supply, e.g. the introduction of so-called exotic cattle into areas where only crop residues are available.

Table 9.6. Degradation characteristics of selected roughages expressed as *A*, *B*, and *C* and index values representing a measure of feed potential (from E.R. Ørskov and W. Shand, unpublished).

Forage	*A*	*B*	*C*	Index value
Spring barley straw	10.6	45.3	0.0622	41.1
Barley leaf blade	15.6	70.2	0.0672	57.1
Barley stem	13.5	36.4	0.0406	36.2
Oat straw	11.4	38.2	0.0240	31.4
Oat leaf blade	10.4	50.9	0.0348	37.9
Oat stem	10.7	25.9	0.0196	25.0
Wheat straw	10.9	42.1	0.0369	27.6
Wheat leaf blade	8.9	58.4	0.0526	42.8
Wheat stem	11.2	30.6	0.0274	28.9
Maize stover	10.0	44.3	0.0330	34.3
Maize leaf	19.7	38.0	0.0410	43.1
Maize stem	14.1	37.0	0.0320	35.3
Rape-seed straw	8.6	21.3	0.0697	23.5
Rice straw	17.1	35.9	0.0399	39.5
Rice leaf	14.6	35.0	0.0374	36.1
Rice stem	22.7	32.5	0.0548	46.7
Ryegrass hay	20.9	44.7	0.0485	48.5
Teff (Ethiopia)	20.0	54.3	0.0390	49.6
Leucaena (Ghana)	31.7	52.7	0.0209	56.9
Eicus (Ghana)	11.6	79.9	0.0333	50.2
Lupinus albus straw	13.9	37.5	0.0357	35.9
Vicia bengalensis hay	19.5	34.4	0.0627	51.5

Use of the *In Situ* Technique to Evaluate the Rumen Environment

When this technique is used to evaluate feed, the rumen environment should be optimal, so that the maximum degradability of the feed is expressed. However, the rumen environment is not always optimal; this may be due to acid conditions, to negative interactions between feeds or to deficiencies in the diets, e.g. of N or sulphur (S).

The *in situ* technique can be effectively used for such studies, using a standard feed substrate and varying the rumen environment. A typical example is given in Fig. 9.2. In almost all instances, the rumen environment acts by influencing cellulolysis. From the discussion on degradation characteristics earlier, it is clear that the solubility, *A*, can scarcely ever by affected by rumen environment. Fermentation of soluble sugars is not affected by rumen pH. It is also logical that *B* is not affected since (*A* + *B*) represents the asymptote of potential degradability and, given a sufficient length of time, an asymptote will be reached. The study of rumen environment is centralized in changes in the rate constant, C. In order to make this study more sensitive, it is often an advantage to prepare a large amount of a substrate in which the cellulosic material is highly digestible, such as good-quality hay. This can be made easier by washing away most of the solubles, so that the test material

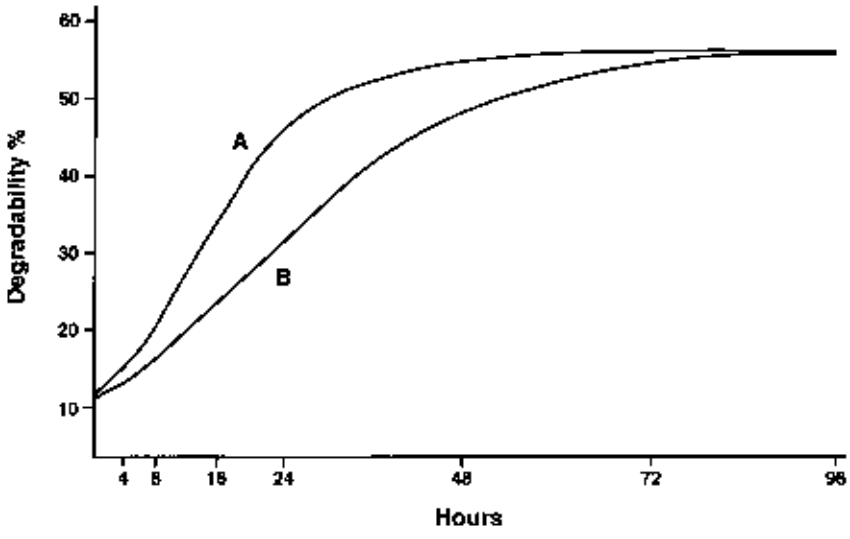

Fig. 9.2. Degradation of a roughage diet in a rumen in which the rumen microbial environment is optimal (A) and suboptimal (B). Note intercept and asymptote are similar.

consists of highly digestible cellulosic roughage. In this way, the feeding regime in question can be studied and the optimal or most cost-effective rumen environment identified. The method was effectively used to identify optimal grain processing for sheep and cattle when grain was used to supplement roughage-based diets (Ørskov and Greenhalgh, 1977). While *in situ* feed evaluation will give similar results for a feedstuff when carried out in different laboratories, since the rumen environment must be optimal, rumen-environment studies can only be made using the feeds, etc., that are to be used locally and these may not be easily transported to a distant laboratory in the quantity needed for feeding.

The method can be used to find the optimal addition of N to a low-N diet and to identify the optimal content of easily fermentable fibre in the diet (Silva and Ørskov, 1988). It is also possible to identify the season at which supplements to pasture-based diets are likely to be cost-effective. Thus, rumen-cannulated cattle or sheep can graze together with the main herd and, every 2 weeks or so, the degradation-rate constant of a standard substrate determined. It would then be possible to identify periods of grazing or seasons in which the degradation-rate constant is reduced. Here, the correct supplement can be used not only as a supplement in its own right, but also to improve intake and digestion of the pasture as well.

Determination of Degradability of Chemical and Botanical Plant Fractions

It is, of course, possible to analyse both botanical and chemical fractions of a feed separately (Ramanzin *et al.*, 1986). One can, for example, determine degradability of NDF in the cell-wall fraction. But, before undertaking such refinements, it is essential to consider the accuracy needed. Are the results going to be used for advice on feeding? If so, it may be difficult to justify more than determination of dry-matter degradability. On the other hand, research to identify varietal differences in fibre degradability may justify such in-depth studies.

Design and Replications

Practical considerations

In earlier studies using the *in situ* technique, it was usual to place all the bags together in the rumen and withdraw them at different intervals of time. However, it is generally more practical to put the bags in at different times and withdraw them all at once. This means that all the bags can be treated and washed together. It is immaterial, on the whole, which system is used. Often, the procedure has to be matched with what is practicable, as it may not be possible to insert bags during the night. Flexibility of incubation times is also possible, since the usual 8, 16, 24 h, etc., are only guidelines; a modified scatter of times is acceptable provided the actual incubation times are entered in the calculations. While the general objective for sample preparation is to imitate chewing, this is obviously not possible and, generally, hammer-milling of dry feed through 2.5–3 mm screens is used. For wet materials, e.g. green forage and silage, a mincing machine is most appropriate, with up to 5 mm screen size. The objective is to avoid small particles leaving the bags as much as possible, and drying silages also leads to loss of volatile materials.

Replications

It is important to recognize that the replication adopted will depend on the purpose or objective of the work. If a feed is evaluated and the results are to be used in diet formulation or to estimate rumen environmental conditions, then the values have to be applicable to the animal population. Mehrez and Ørskov (1977) observed that the greatest source of variation was between-animal variation. The variations within animals and between days within animals were small in comparison with between-animal variation. They came to the conclusion that, for such purposes, three cannulated animals had to be used and that replication within animals and between days was not worthwhile and made little difference to the total variance.

However, if the purpose or objective is to rank the feed potential of forages, which may be the case for plant breeders at early stages of selection for forage or straw quality, then only one animal needs to be used. In fact, perhaps only one sample is required. Here a word of caution. Many research workers use 48 h degradability,

as it is close to *in vivo* digestibility. However, if the objective is to rank them, some indication of *A*, *B* and C is required. This is best obtained if, for instance, 24 h is used, rather than 48 h. Degradability values at 48 h tend to blur differences in rate of degradation, the C value.

Future Research

With respect to using the *in situ* technique for forage or roughage evaluation, there is a need for a better and more comprehensive database. There will undoubtedly be feeds whose intake cannot be predicted readily from their *A*, *B* and C values. Banana pseudostem is one feed that is consumed in smaller amounts than predicted (Shem *et al.*, 1995). This contains 95% water and the fibre is very tough; perhaps in such feeds the breakdown time of long to small particles needs to be taken into account.

There is also a problem relating to small-particle loss, such as starch in maize silage. These particles will appear in the model as soluble particles if they are small enough to pass through the pores of the nylon bag. Many of these aspects have been discussed in some detail by Huntington and Givens (1995), together with possible mathematical solutions. Small starch particles could escape rumen fermentation and be digested in the lower gut (see Ørskov and Ryle, 1992). More production data upon intake of silages, in particular maize silage, are called for.

There is also a need to consider more carefully the measuring of the lag phase. It must be understood that the lag phase refers to net disappearance. There is an increase in dry matter due to microbial biomass adhesion but at the same time some degradation of substrate. In the suggested model, lag phase is not taken into account, because the limited database did not allow improvement in prediction of intake, but with an extended database this may well be important.

Another problem occurs if the feed contains antinutritive factors. Here the *in situ* technique is not ideal, because the very small amount of antinutritive factors in the sample contained in the bag will have little or no effect on the environment of the rumen and so will not affect the sample's degradation characteristics. There are also feeds that cannot be assessed using the nylon-bag technique. Soluble feeds, such as molasses, and small-particle feeds, such as blood meal, single-cell protein, sago meal and cassava meal, cannot be retained in the nylon bag. Here *in vitro* techniques are superior.

Another question is: will feed potential be additive? While an animal can consume twice as much feed with an index value 60 as one with 30, does this mean that animal production will be proportional? Probably not. The total energy cost of eating and rumination may well be similar whether the index value is 30 or 60, and as a result the former provides proportionally more energy for production. This is probably one of the reasons why the metabolizable energy from more concentrated diets is utilized better than that from roughages, as has been known for many years, since proportionally less energy is expended in eating and rumination and standing up to eat.

Since the characteristics of *A*, *B* and C are generally a result of microbe–plant interaction, it is quite likely that evaluation systems based upon them can be

globally important for assessment of roughage quality, but there is room for further improvement. The association of *B* and *C* is so firm that perhaps it would be as good to combine them as $B \times C$ as to treat them separately. There are some roughages that have a very low *B* value and a high *C* value, in which case the importance of *C* may be exaggerated.

The *in situ* technique does require use of fistulated animals, which may pose difficulties in some laboratories. The next best technique may be the Menke gas-test technique (Menke and Steingass, 1988), which describes fermentation dynamics by the rate constant of gas production from a feed sample incubated *in vitro* with a rumen inoculum. The rumen inoculum required may be obtained by stomach pump rather than by withdrawal from a rumen fistula or, in some instances, faeces may be used. However, *in vitro* techniques are discussed in detail in another chapter in this book. Blummel and Ørskov (1993) compared results obtained by nylon bag and gas production and found closely similar results. For feeds containing antinutritive factors affecting microbial activity, the gas-production technique is superior, in that the inoculum causes much less dilution than occurs when nylon bags are incubated in the rumen. This can be further refined by the selective removal of particular groups of antinutritive factors (Khazaal *et al.*, 1994; Makkar *et al.*, 1995).

Another intriguing aspect to analyse is whether the feed potential can also predict microbial protein production. The microbial protein is dependent on the extent of fermentation of carbohydrate and also influenced by rate of outflow (see Chen *et al.*, 1992). It is thus possible that both intake and a good estimate of microbial protein supply can be obtained from the concept outlined, but here a new research area must be opened up to provide the evidence for that.

How can chemical analysis of the feed complement results obtained by the *in situ* technique? There is no doubt that determination of N content is essential in order to see if N supplementation is needed to ensure optimal digestion. For some feeds, it may be useful to determine organic-matter rather than dry-matter degradation. Further refinements, such as determination of cell-wall degradation characteristics, may be useful for research purposes but will probably not be valuable for forage evaluation in addition to the characteristics for dry matter, inasmuch as the potential degradability (*B*) of dry matter closely reflects that of the fibre fractions.

For the past 15–20 years, the *in situ* technique has proved to be an extremely useful and robust technique for estimating the feeding value of forages and the quality of the rumen environment. Without that technique, progress would have been much less rapid. In particular, it has been useful in many developing countries, where constancy of electricity supply and consequently of incubation temperature, is difficult to maintain. Without constant temperature, *in vitro* techniques are totally unreliable. The *in situ* technique has no such problems and generally it allows better prediction of feed intake.

Cost-effectiveness

Farmers and the feed industry require reliable information rapidly and, if possible, 'upfront', i.e. before the feed is used. The *in situ* technique can achieve this, whereas measurement of digestibility by feeding trials *in vivo* is slow and laborious

and feed evaluation using respiration calorimetry is still more difficult and costly. While the last method has given very valuable information regarding general categories of feedstuffs, it would be prohibitively costly to use routinely for numerous individual batches of feed. When the digestibility of feeds is measured *in vivo*, in the UK, even when sheep are used, the cost is about £1000 per feed; when it is done in respiration chambers, the cost is more like £2000. Estimation of dry-matter degradability using nylon bags costs around £100 per feed. When digestibility or metabolizability is determined *in vivo* at only one feeding level, usually maintenance, only one point on the degradability curve is obtained. The problem, then, is that since the extent to which feeding level influences digestibility depends on the degradation rate (and on retention time in the rumen), intake cannot be predicted accurately from a static measure of *in vivo* digestibility or metabolizability, nor can digestibility account for more than about 50% of variation in intake. The value of estimates *in situ* of degradation characteristics is therefore considerably more than the value of measurements *in vivo* of digestibility or metabolizability at a single feeding level. In relation to *in vivo* measurement, therefore, description of degradation characteristics is very cost-effective. However, as mentioned earlier, gas-production measurements *in vitro* may provide a sufficiently accurate and cheap alternative. It is possible that NIR measurements (spectroscopic measurements in the near-infrared) may also be sufficiently accurate to predict *A*, *B* and *C* values of roughages. This would provide a rapid upfront measurement of degradation characteristics, once it is adequately calibrated in relation to *in situ* technique. For assessment of the rumen environment, however, there is, as yet, no adequate cost-effective substitute for the *in situ* technique.

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10 Cumulative Gas-production Techniques for Forage Evaluation

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Introduction

The quest for a simple, cheap and reliable laboratory method to evaluate the nutritive value of forages for ruminant animals resembles the search for the philosopher's stone during the Middle Ages in Europe. There is an urgent need for such a technique. Not only are cost and animal-welfare considerations making the use of animals less desirable, but also the increasing human population and demand for animal products create a need for new animal feedstuffs and improved varieties of traditional ones. However, like the philosopher's stone, a reliable and accurate technique to predict the nutritive value of all possible feeds is probably impossible to find. The microbial degradation of forages in the rumen is a complex process, involving complex interactions, both between the microorganisms present (bacteria, protozoa and fungi), and between the microbial population and the host (Czerkawski, 1986). To develop such a method, the findings of rumen microbiologists published for the last 40 years provide an important source of information (e.g. Hungate, 1966; Hobson and Wallace, 1982). For example, factors that must be considered when trying to simulate the rumen environment include temperature, pH, buffering capacity, anaerobiosis and nitrogen source.

Existing laboratory techniques concentrate on estimating rumen degradation. The rationale for this is that forage consists largely of carbohydrate in the form of fibre, and this can only be digested by microorganisms in the rumen. However, the effective degradability (the digestibility that can be expected *in vivo*) also depends on the rate of degradation, the potential degradability and the fractional outflow rate from the rumen (Hvelplund *et al.*, 1995). At the moment, there is no *in vitro* technique that can estimate the lattermost aspect of *in vivo* digestibility. Existing techniques measure the potential degradability (Tilley and Terry, 1963) or both the degradability and rate of degradation (*in situ*, and cumulative gas production).

The *in situ* technique has been widely used for the last 50 years. Whatever it is called (*in sacco*, nylon bag, Dacron bag, cloth bag), it requires the incubation of

 Forage Evaluation in Ruminant Nutrition (eds D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed)

substrates in bags within the rumen of fistulated animals. These bags are removed at regular intervals, the weight loss is plotted (usually as dry matter (DM)) and a curve is fitted to the resultant data points (see Chapter 12). Van Soest (1994) has listed the advantages and disadvantages of this kind of technique.

While by no means a new concept, the measurement of gas production to evaluate forages has come to prominence in the last 10 years. There are several methodologies in current use, and the differences and similarities between them will be described, along with current research into the fundamentals of what the technique can predict and how best to carry it out. The general stoichiometry of gas related to volatile fatty acid (VFA) production is described for the rumen fermentation process. The use to which the technique is being put and potential future developments are then discussed in relation to feed evaluation.

A History of Gas-production Techniques for Forage Evaluation

Early techniques

Gas (carbon dioxide (CO_2) and methane (CH_4)) is a waste product of fermentation. In 1943, Quin incubated feedstuffs in gas-tight flasks and measured gas production manometrically. By the 1960s, the measurement of gas using liquid displacement systems was suggested as a technique to evaluate ruminant feedstuffs (Johnson, 1963; Trei *et al.*, 1970) and this is still used today (Waghorn and Stafford, 1993).

In order to simplify measurement of gas by manometry, O'Hara *et al.* (1974) used a technique described by El-Shazly and Hungate (1965), whereby gas production was measured by use of a syringe attached to a flask in which the substrate was fermented. They found characteristic patterns of change in rates of gas production according to the substrate used.

The Hohenheim gas test

The group at Hohenheim University (Germany) were the first to conduct the fermentation process within large glass syringes, so that the gas produced pushed the plunger outwards, and the total volume produced was recorded after 24 h (Menke and Ehrensvard, 1974; Fig. 10.1). This evaluation system combines the total gas volume after 24 h with the concentration of crude protein (CP), crude fat, crude fibre and ash of the feed to predict metabolizable energy (ME). In Germany, the Hohenheim *in vitro* gas test is now widely used for the estimation of *in vivo* digestibility and ME for ruminants (Menke and Steingass, 1988).

Recently, Blümmel and Ørskov (1993) have adapted the Hohenheim gas test by recording the change in gas production in the glass syringes at regular time intervals, so that a measure of the kinetics of fermentation is obtained, as well as the end-point value.

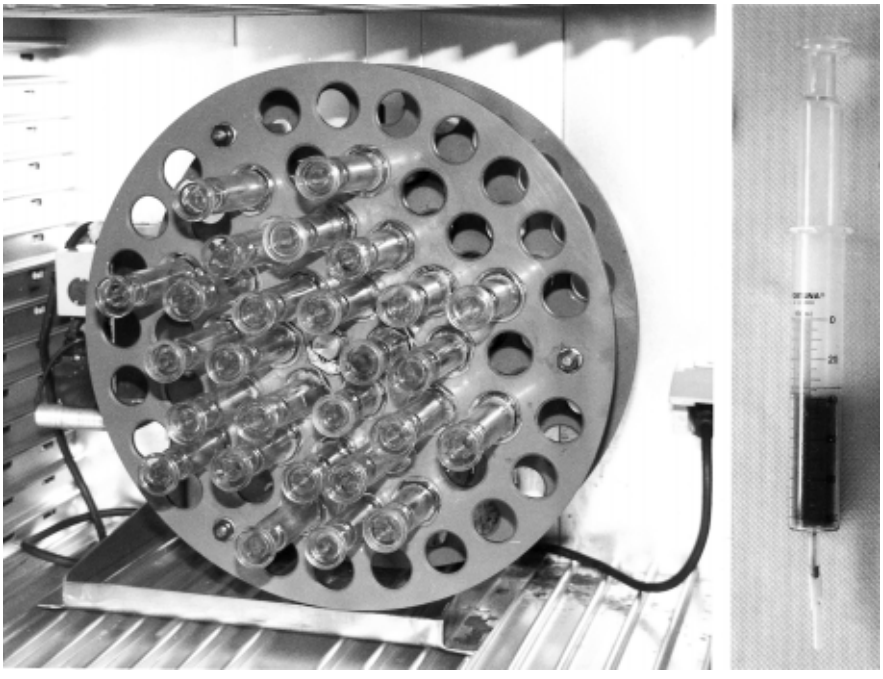

Fig. 10.1. Equipment for the Hohenheim gas test (photo courtesy of Klaus Aiple and Klaus Becker, University of Hohenheim).

Manual transducer technique

A new method, using a pressure transducer to measure head-space gases in sealed serum bottles, was first described in 1991 by Theodorou *et al.* from the Institute of Grassland and Environmental Research (IGER) in the UK (Fig. 10.2). The pressure and volume were recorded at regular intervals: approximately every 3–4 h for the first 24 h, and then less frequently to the end of fermentation at about 120 or 144 h. The head-space gas is removed after each reading. The cumulative gas profile represents the kinetics of the fermentation process of individual feedstuffs. An example of such a profile comparing wheat straw and ryegrass is shown in Fig. 10.3.

Automated systems

The earliest reported automated device was developed by Beuvink *et al.* (1992) at the Institute for Animal Science and Health, Lelystad, the Netherlands. This system was based on the weight of fluid displaced by fermentation gas for 24 individual bottles. The change in weight was recorded and a calculation made to convert the weight to gas volume, which was then registered by a data logger.

Pell and Schofield (1993), from Cornell University in the USA, first described the use of computerized pressure sensors to monitor gas production. They investigated

Fig. 10.2. The pressure transducer, complete with serum bottle, connectors and computer, as used for the manual transducer technique.

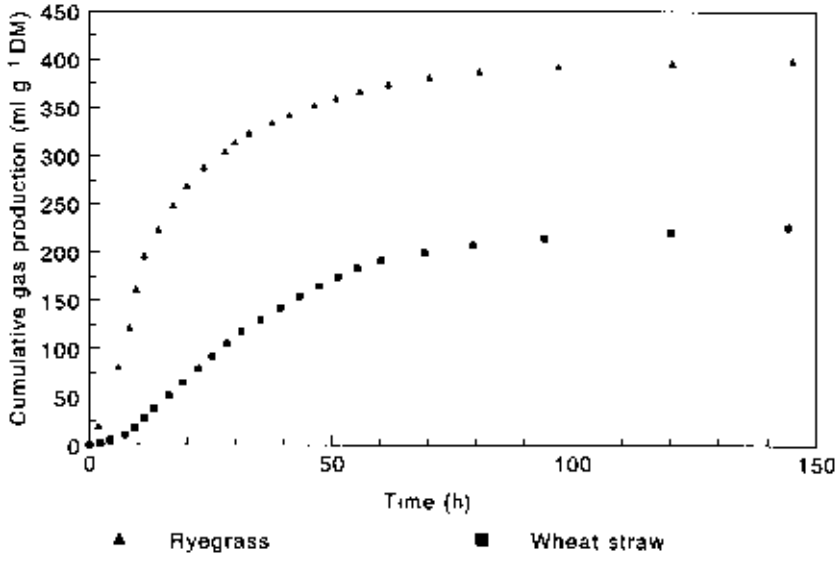

Fig. 10.3. Example of cumulative gas-production profiles generated using the manual transducer technique, showing the differences between the fermentation of wheat straw and ryegrass.

the use of several variables, including sample size, inoculum size, vessel size and type of pressure sensor, and reported the ranges within which gas production could be accurately measured. For the Cornell technique, gas is not vented during the fermentation but the head space of the bottles is much larger than for the IGER technique and the sample much smaller (Fig. 10.4).

An automated version of the manual transducer technique (called the automated pressure evaluation system (APES)), developed at IGER, was first reported by Davies *et al.* (1995). The system allowed for 50 gas-tight culture bottles, each of which was fitted with a pressure sensor and solenoid valve linked to a computer, for continuous monitoring of all bottles (Fig. 10.5). During fermentation, each solenoid valve opens to release the accumulated gas when the pressure sensor registers a pre-set gas pressure. The number of vents and time between each vent for each bottle are recorded automatically by a computer and are then used to plot cumulative gas profiles. This system is now also in place at Wageningen Agricultural University, and was used to produce the profiles for wheat straw and ryegrass shown in Fig. 10.6. The large number of data points produce very detailed fermentation profiles.

Cone *et al.* (1996) described what was called 'a new fully automated time-related gas-production apparatus', developed at ID-DLO, Lelystad, in the Netherlands. This apparatus uses a combination of pressure transducer and an

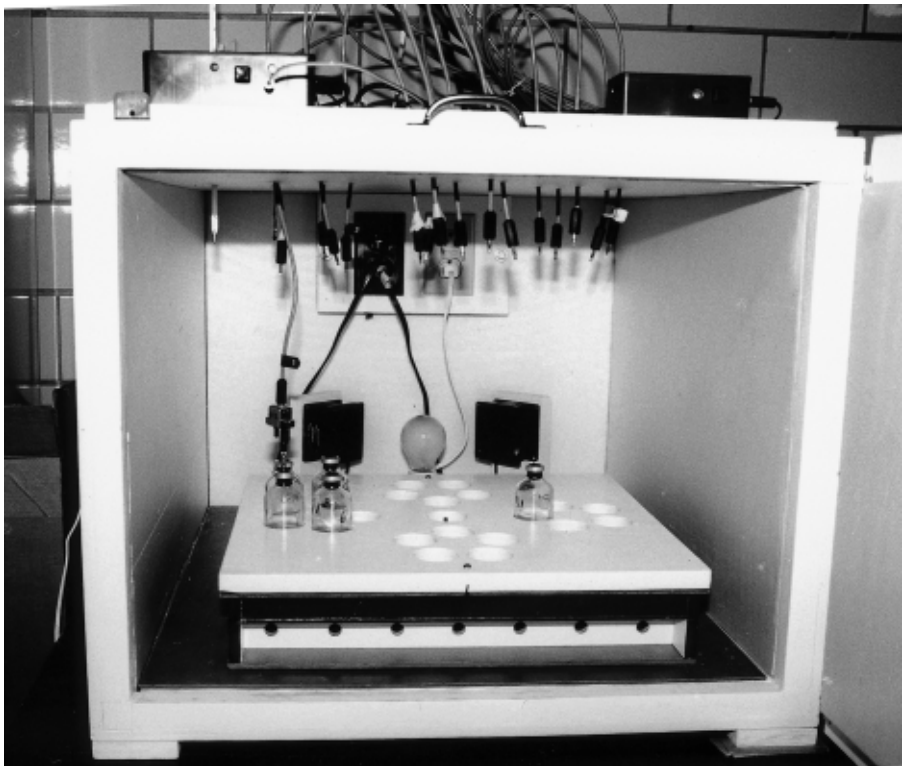

Fig. 10.4. Equipment used for the automated gas system at Cornell University (photo courtesy of Alice Pell, Cornell University).

Fig. 10.5. The IGER automated pressure evaluation system at Wageningen University, The Netherlands.

electric valve for every bottle. Each valve opening represented a fixed amount of gas and the fermentation kinetics were determined by recording the time of each valve opening on a data logger. A diagrammatic representation of the apparatus was published as part of the methods paper.

A General Description of the Method

Broadly speaking, the general principles of what the different techniques measure are the same. The technique is similar to other *in vitro* digestibility procedures which use milled substrates, an anaerobic medium and an inoculum of a mixed microbial population from the rumen. The pre-weighed substrate is suspended in a medium, the mixture warmed to ~39°C and a freshly collected sample of rumen fluid added as inoculum. From that moment, the production of gas resulting from fermentation is recorded, either at the end of the fermentation or at regular time intervals for those methods that measure fermentation kinetics. A cumulative gas profile is plotted from the results at the end of fermentation. This curve records the

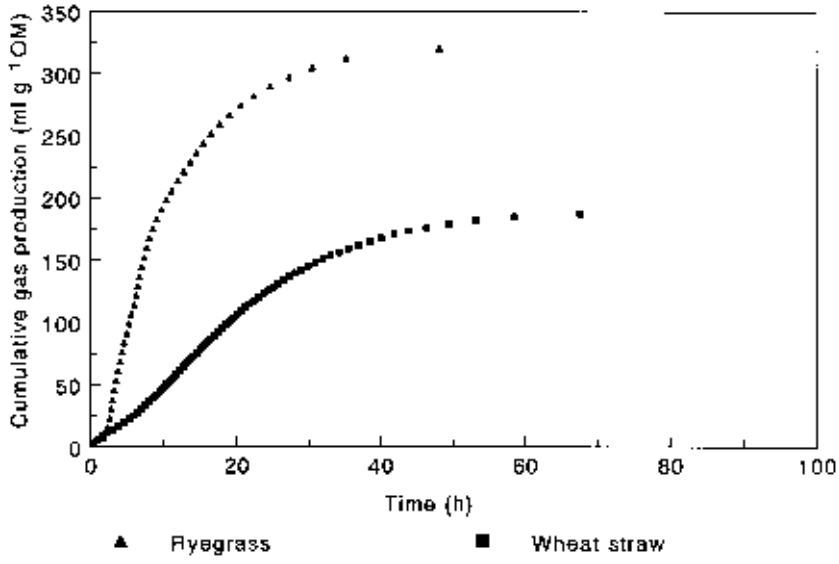

Fig. 10.6. Example of cumulative gas-production profiles generated using the automated pressure evaluation system, showing the differences between the fermentation kinetics of wheat straw and ryegrass (from Pablo Chilibroste, unpublished data). OM, organic matter.

production of a waste product by the microorganisms, and so reflects the kinetics of microbial activity within the bottle.

Sample preparation

As with all laboratory techniques, the preparation of a forage sample is fraught with many factors to be considered, which have been dealt with exhaustively for other *in vitro* methods (Goering and Van Soest, 1970; Huntington and Givens, 1995). It is generally recognized that a smaller particle size increases the surface area available for microbial degradation. Consequently, for routine gas-production analysis most authors mill the test substrates through a 1 mm screen (Menke *et al.*, 1979; Pell and Schofield, 1993; Theodorou *et al.*, 1994), in keeping with other *in vitro* methods (Tilley and Terry, 1963; Goering and Van Soest, 1970).

The method and temperature of drying a substrate were reviewed for the *in situ* technique by Huntington and Givens (1995). Most groups either freeze-dry samples (which, in the case of silages, can lead to the loss of volatile components) or oven-dry at a low temperature, either 60°C (Pell and Schofield, 1993) or 70°C (Beauvink *et al.*, 1992). Cone *et al.* (1994) reported a comparison of different drying conditions on the fermentation kinetics of grass and maize stems, and concluded that freeze-drying was better than oven-drying (at 30, 50, 70 and 105°C).

Media

The living rumen provides an ideal environment for its anaerobic microorganisms in terms of pH, temperature, buffering capacity and nitrogen in the form of urea, while the microorganisms behave symbiotically by providing each other with micronutrients, such as branched-chain fatty acids and vitamins. There are also likely to be other unknown factors present in the rumen milieu, which cannot yet be replicated *in vitro*. In the past, quite a number of media used to characterize rumen microorganisms overcame this problem by addition of a large proportion of sterile rumen fluid to ensure the presence of all possible factors for microbial growth.

All the media in current use have the following in common: bicarbonate and phosphate buffers, a reducing agent, a source of nitrogen, various minerals and resazurin as an indicator of redox potential. In all cases, CO_2 is used during the preparation of the medium to ensure a low redox potential at the time of inoculation. A low redox potential is very important for the fermentation of very fibrous forages, or even for those rich in starch. Leedle and Hespell (1983) showed that the absence of strict anaerobiosis resulted in partial losses of cellulolytic and amylolytic bacterial groups from rumen fluid. Therefore a low redox potential is very important, not only for the fermentation of fibrous forages, but also for those rich in starch. Grant and Mertens (1992) recommended that all *in vitro* systems used to assess fibre digestion kinetics must use continuous CO_2 gassing and reducing agents to promote both the rapid initiation and the progression of fermentation. Minimizing the limitations of the *in vitro* system means that differences between substrates will be more readily detected.

A modified version of medium B (Williams *et al.*, 1995b) is used most commonly in Wageningen; it is a complex semi-defined medium, which, in principle, should provide most of the rumen microorganisms with all of their requirements (Lowe *et al.*, 1985), except for energy, which is provided by the test substrate. This medium, while suitable for a wide range of the rumen microorganisms, comprises several component solutions and so is time-consuming and complicated to use for routine analyses. Not surprisingly, several other media have been developed, most of which seem to originate either from the medium of Menke and Ehrensvard (1974) or from that of Goering and Van Soest (1970).

Research continues to develop media that are appropriate for different situations. The composition and strength of the buffer are still a focus of interest (Pell and Schofield, 1993; Nsahlai *et al.*, 1995). Measurement of pH at the end of fermentation is to be recommended to ensure that sufficient buffering capacity was available for novel or concentrate substrates.

Inoculum

Conditions for the keeping of donor animals, their feeding, and the timing of rumen fluid collection, so as to obtain the most consistent result between runs for *in vitro* techniques was reviewed for *in vitro* techniques by Alexander and McGowan (1966). It was recommended that a sample should be taken after overnight fasting from at least three animals, all of which were fed the same diet of medium-quality hay with a small amount of concentrate.

Animal species

Sheep (usually wethers) are most often used as a routine rumen fluid donor for *in vitro* techniques (Beuvink *et al.*, 1992; Khazaal *et al.*, 1994; Theodorou *et al.*, 1994; Bonsi *et al.*, 1995; Williams *et al.*, 1995b). They are chosen mostly for their size, which makes them cheaper to feed and house for an extended period. However, cattle are also used as inoculum donor (Grant and Mertens, 1992; Pell and Schofield, 1993; Pienaar, 1994; Makkar *et al.*, 1995; Sileshi *et al.*, 1996). Differences in the activity of microorganisms from rumens of different species or of the same species but different diets mean that all descriptions of *in vitro* gas-production evaluations should describe the conditions of the donor animal (species and breed, diet, time of collection in relation to feeding, etc.).

Time of collection

Rumen fluid taken after overnight fasting is less active than that taken 2 h after feeding, but is more consistent in its composition and activity. As a rule, therefore, it has been recommended (Menke and Steingass, 1988) that rumen fluid be collected before feeding, which is the routine followed by many groups (Blümmel and Ørskov, 1993; Pell and Schofield, 1993; Theodorou *et al.*, 1994; Williams *et al.*, 1995b).

Preparation of inoculum

Another important consideration is that of homogenization of the rumen fluid before inoculation. It has been shown that blending increases the number of adherent bacteria (which are most often the cellulolytic species) in the inoculum, but it can also increase the number of small feed particles present (Pell and Schofield, 1993), which can lead to higher gas production in the blank bottles. The blending procedure can also increase the risk of oxygen exposure if the stream of CO_2 is not forceful enough.

Proportion of inoculum to medium

The proportion of inoculum to medium has been investigated by Pell and Schofield (1993), who recommended an inoculum of 20% of a total volume of 10 ml. However, the amount of added inoculum varies widely between groups. Hidayat *et al.* (1993) found increased rates of gas production from forages, in response to increased bacterial density.

What does the Technique Measure?

In vitro gas production is related solely to fermentation in the rumen, rather than to digestibility in the whole digestive tract, which also includes enzymatic digestion, absorption and hind-gut fermentation. Rumen fermentation is mainly the result of the activity of the bacteria, protozoa and fungi that inhabit it. Some species from each of these groups possess cellulolytic and hemicellulolytic activity (Hungate, 1966; Orpin 1983/4; Williams and Coleman, 1988), though the relative contribution of each to fermentation is still not completely understood.

Stoichiometry of rumen fermentation

Energy for the growth of rumen microorganisms is derived from the fermentation of carbohydrates, particularly starch and cell walls, which are first broken down into hexoses and pentoses, from which the main end-products are acetate, propionate and butyrate (VFA), CO_2 and CH_4 (Wolin, 1975), but which can also supply essential carbon skeletons for the synthesis of microbial biomass (Beever, 1993b). The VFA are absorbed across the rumen mucosa and provide energy for the mucosal tissue, as well as for the host animal. The gases produced from VFA production and from the bicarbonate buffer in the rumen are eructated to the atmosphere. It is this gas that is being measured *in vitro*. The stoichiometric reactions of fermentation of cell-wall hexoses were described by Hungate (1966):

$$1 \text{ mol Hexose} + 2H_2O \rightarrow 2 \text{ Acetate} + 2CO_2 + 4H_2 \quad (1)$$

$$1 \text{ mol Hexose} + 2H_2 \rightarrow 2 \text{ Propionate} + 2H_2O \quad (2)$$

$$1 \text{ mol Hexose} \rightarrow 1 \text{ Butyrate} + 2CO_2 + 2H_2 \quad (3)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (4)$$

Formation of propionic acid is the only reaction that requires the uptake of hydrogen (H_2) (Van Soest, 1994). Any remaining H_2 is usually converted in the rumen to methane by methanogenic bacteria. Also, the production of propionate does not involve the generation of CO_2. Therefore, to compare gas data between different forages, it is also useful to examine differences in the acetate:propionate ratio at the end of fermentation (Stefanon *et al.*, 1996; Groot *et al.*, 1998).

The complete pathway of VFA production for the hexose equivalents from high-forage diets was summarized by Beever (1993b) as:

1 mol Hexose → 1.34 mol acetate + 0.45 mol propionate + 0.11 mol butyrate + 0.61 mol methane + 4.62 mol adenosine triphosphate (ATP)

All techniques, except for the Hohenheim gas test, rely on the measurement of pressure in a receptacle of fixed volume. The empirical gas laws of Boyle and Gay-Lussac, from which the volume of gas can be calculated are:

$$PV = nRT \quad (5)$$

where:

P = pressure of gas (atm)
V = volume of gas (l)
n = number of moles of gas
R = the molar gas constant at 0°C and 760 mm Hg
T = temperature (°K)

In most studies, it is assumed that the pressure of gas is at 1 atm, so 1 mmol of gas occupies 25.62 ml at 39°C (Weast, 1968). However, if laboratories are located in areas where the atmospheric pressure is not 1 atm, an adjustment must be made accordingly.

Microbial biomass production

It has been shown that an inverse relationship can exist between gas volume (or VFA) and microbial biomass production (Naga and Harmeyer, 1975; Leng, 1993). Krishnamoorthy *et al.* (1991) indicated that the type of carbohydrate and its rate of fermentation could influence total synthesis of microbial protein per unit volume of gas produced. On the one hand, it has been suggested that the efficiency of microbial growth can vary depending on feeding conditions (Leng, 1993). Yet it has also been pointed out that, if there are no deficiencies of microbial growth factors, both the source of carbohydrate (whether sugar, starch or fibre) and the flow rate of liquor from the rumen have little effect on microbial growth yield. The methodology used to measure cumulative gas production can easily be adapted to investigate the effect of microbial growth factors and sources of carbohydrates under controlled conditions, which could help to answer some of these questions.

Little work has been done to examine the relation between gas production and microbial growth, though Blümmel *et al.* (1997b) have done some work in that direction, using the Hohenheim system. They demonstrated that some substrates with proportionally high gas volumes had comparatively low biomass yields. Gas-volume measurements were complemented by a determination of the truly degraded substrate *in vitro* to avoid selection of substrates with proportionally high VFA production and low microbial biomass yield (Blümmel *et al.*, 1997a), which is relevant for forages with a low nitrogen content. It has been pointed out by Leng (1993) that one of the factors affecting microbial growth efficiency is the relative amounts of carbohydrate and protein that are fermented. For example, a high protein-to-carbohydrate ratio could lead to a relatively low microbial protein-to-VFA ratio. These relationships could be examined more closely using cumulative gas-production techniques, particularly if other parameters, such as VFA, ammonia (NH_3) and microbial protein production are also measured, using a medium that is known to contain all possible microbial growth factors, or, contrarily, is deficient in one or more of them.

Data Processing and Interpretation of Results

Conversion of raw data to cumulative volumes

Gas volumes recorded at each time interval are added together to give a cumulative gas profile. In the case of the measurement of head-space pressure and volume, a correction is made for irregularities in head-space volume between bottles, by a regression of pressure against volume for each bottle. A correction is also made to cumulative volumes for the starting DM/organic matter (OM) weight of the substrate. Ideally, if remaining substrates are not required for further analysis, OM data should be used, since ash makes no significant contribution to gas or VFA production and can vary significantly between substrates. The yield of gas or VFA (Y_G or Y_{VFA}: the amount of gas or VFA produced per gram material disappeared) can also be calculated (as DM or OM) if the substrate losses at the end of fermentation are measured.

Correction for blanks

A series of blank bottles containing medium and inoculum but no substrate is routinely included in each run. The total gas recorded for the blanks is subtracted from the total gas produced from the test substrates to obtain the total gas derived from fermentation of the substrate. However, the blank profile is not subtracted from the substrate profiles prior to curve fitting, particularly in the case of the multiphasic curves, as the phases present in the blank may not be relevant to what occurs in the presence of substrate (Fig. 10.7).

Curve fitting

Fitting cumulative gas profiles to an appropriate equation allows one to summarize the kinetic information therein. Several equations have been developed for this purpose (Beuvink and Kogut, 1993; France *et al.*, 1993; Groot *et al.*, 1996).

The choice of equation to fit gas profiles must take into account not only mathematical considerations (such as the statistical fit) but also the biological meaning of the parameters. Increasingly frequent readings for gas production lead to very detailed profiles, and considerations of mathematical fit (particularly very low Durbin–Watson values) have led to the development of a multiphasic model (Groot *et al.*, 1996), which was based on the multiphasic growth model of Koops (1986). The potential value of this model is that it separates the test substrate into the more rapidly and more slowly fermentable fractions (Oostdam *et al.*, 1995; Groot *et al.*, 1998).

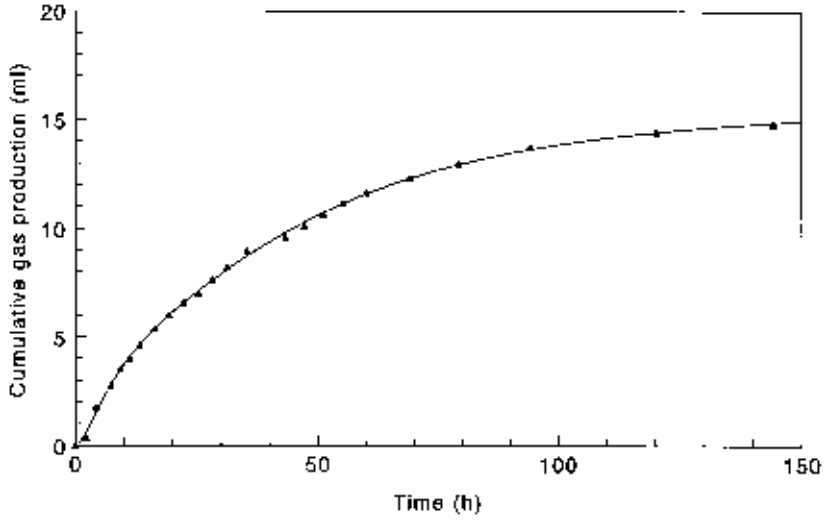

Fig. 10.7. Cumulative gas-production profile of a blank bottle, containing medium B and sheep rumen fluid as the inoculum.

France *et al.* (2000) have compared the different equations available in mathematical terms, while Dhanoa *et al.* (2000) have compared the same equations, using a large series of data sets from widely different forages, and have analysed the results from the point of view of the use of the equations in practice.

Applications of the Technique

A wide range of forages has been investigated using cumulative gas-production techniques, of which a selection is shown in Table 10.1. The largest proportion of materials are those which are fed as a source of energy, i.e. carbohydrates. It is important to realize that a nitrogen-rich medium should provide ample nitrogen for microorganisms, so a proteinaceous feed fermented in a nitrogen-rich medium would actually assess the potential energy, not the protein, content of that feed.

Grass, hay and silage

Aspects of grass, hay and silage quality that have been investigated include the detailed differences between substrates due to plant maturity (Stefanon *et al.*, 1996; Williams *et al.*, 1999) and conditions of growth. Gas-production kinetics are also used to rank different species or cultivars (Piva *et al.*, 1988).

Table 10.1. Cumulative gas production characteristics after 144 h of fermentation of a range of substrates inoculated with sheep rumen fluid.

Substrate	DMCV* (ml g^{-1} DM)	$t_{\frac{1}{2}}$ (h)	t_{RM}† (h)	RM† (ml h^{-1})	DM loss (%)
Glucose	392	19.7	7.5	0.032	100
Starch	425	23.6	22.2	0.037	99.3
Crop residues					
Sorghum stem	231	44.4	18.7	0.014	68.4
Ammoniated wheat straw	232	34.0	31.0	0.025	60.3
Wheat	223	36.2	30.7	0.021	52.3
Red clover	207	22.6	18.8	0.034	79.6
Ryegrass hay‡	352	23.2	14.4	0.028	52.6
Silage					
Grass§	283	17.4	14.0	0.050	67.9
Maize‖	258	22.1	24.2	0.060	67.6

* DMCV is the measured total gas production at 144 h g^{-1} dry matter of the substrate.
† RM (fractional rate of substrate digestion) and t_{RM} (time at which the fractional rate occurs) were both calculated according to the monophasic version of the model reported in Groot *et al.* (1996).
‡ From Groot *et al.* (1998).
§ From Calabrò and Williams (1997).
‖ From Williams *et al.* (1995b).

Crop residues

Cumulative gas production has been used to determine differences in fermentability between different chemical (urea: Prasad *et al.*, 1994) and physical (steam: Castro *et al.*, 1994; steam explosion: Williams *et al.*, 1995b; extrusion: Williams *et al.*, 1997) pretreatments of straw. The fermentabilities of crop residues of different cultivars (Hermanto *et al.*, 1991) or those grown under differing environmental conditions (Williams *et al.*, 1996) have also been examined. The technique is also increasingly being used to investigate the effect of addition of supplements on the fermentability of straws (Prasad *et al.*, 1994).

Browses

The technique has been used to rank different browse trees as potential feed supplements for poorly degradable roughages (Topps, 1992). Many browses contain antinutritive factors (ANFs), as well as significant amounts of protein, which complicates *in vitro* assessment of their nutritive value. The effect of such ANFs on gas production and on DM and protein degradability depends on an array of factors, which includes their molecular structure and reactivity (Hagerman *et al.*, 1992; Kaitho *et al.*, 1998). Newbold *et al.* (1997) reported that some ANFs could be quite selective in their effects on certain microorganisms, while others only affect the host animal.

Some phenolic compounds from browses have been shown to be negatively related to gas production (Siaw *et al.*, 1993; Khazaal *et al.*, 1994). The latter compared gas production with the *in situ* technique and concluded that gas production was more efficient for the assessment of feeds containing ANFs. They suggested that, while physical binding of polyphenols to substrate (protein or carbohydrate) could be detected in a nylon bag incubated in a large environment (i.e. rumen), other effects, such as toxicity to microbes or binding to their enzymes, would be diluted and would therefore be more difficult to detect.

Given that there are several possible mechanisms by which browse ANFs could affect microbial activity, the technique has been used to pinpoint which mechanism is responsible for changes to fermentation. Several variations, combining different inocula (Nsahlai *et al.*, 1995), the use of tannin-binding agents (Makkar *et al.*, 1995; Wood and Plumb, 1995) or supplementation of crop residues, have been used to determine the exact effect of specific ANFs.

Feed components

Protein (legumes, browses and grasses) and starch (maize and whole-wheat silages) are often important components of forages, and their fermentation can provide insight into the fermentation of the whole forage.

Starch

Trei *et al.* (1970) reported that gas production was highly correlated with *in vitro* DM disappearance, total VFA production and starch digestion. More recently,

Opatpatanakit *et al.* (1994) found that gas production from fermentation of several starch-based substrates proved to be an accurate index of VFA production and change in pH.

Protein

Raab *et al.* (1983) described a method for the determination of protein degradation based on measurements of NH_3 concentration and gas production. NH_3 liberated during incubation is partly used for microbial protein synthesis, while the production of CO_2 and CH_4 is a measure of the energy available for protein synthesis. The ratio gas production : incorporation of NH_3 nitrogen was estimated by addition of starch to the substrate.

Until now, gas production has most commonly been used to assess protein (particularly browses) as a supplement to determine whether supplementation with a concentrate or browse legume could enhance the fermentation of poor-quality crop residues, such as teff and wheat straw (Prasad *et al.*, 1994; Bonsi *et al.*, 1995). Investigations have started in Wageningen to determine whether the cumulative gas-production technique can be used as an indicator of the relationship between carbohydrate and protein on *in vitro* fermentation. For example, the form of nitrogen supply to microorganisms can affect the total gas production of carbohydrate, as shown in Fig. 10.8 (Debersaques *et al.*, 1998).

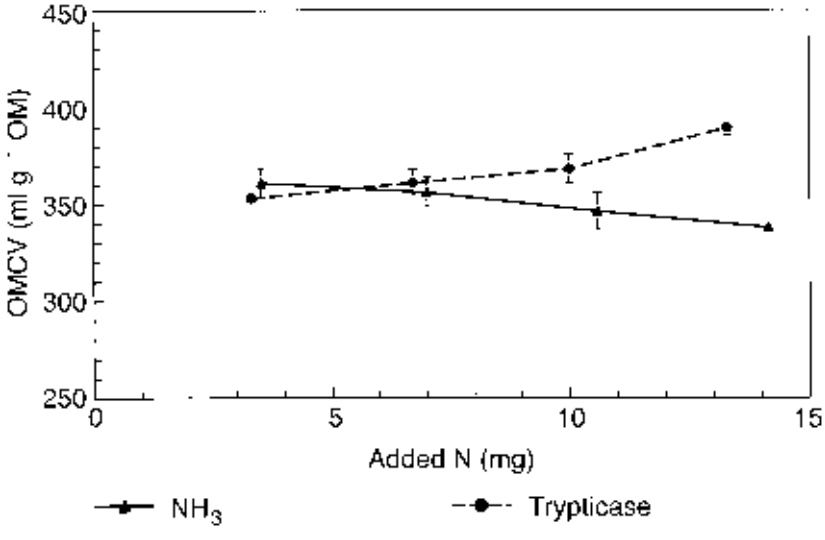

Fig. 10.8. Total gas production from citrus pulp as affected by increasing amounts of nitrogen originating either from trypticase or from ammonia (from Debersaques *et al.*, 1998). OMCV, organic matter cumulative volume.

Relation of Fermentation Data to *In Vivo* Parameters

All *in vitro* techniques have been developed to provide a cheaper, easier and faster technique to predict the availability of certain nutrients for an animal. Comparing *in vitro* techniques with each other is pointless, unless they are both being related to the same set of *in vivo* data and one can determine which technique is a better predictor of the *in vivo* parameters.

Voluntary intake of roughages is considered to be an essential factor in quality assessment (Minson, 1990). The relation between gas production and feed intake is interesting, as being of immediate practical relevance. However, a correlation between the *in vitro* relationship of a given microbial population and a specific substrate cannot take into account important elements of animal behaviour, including appetite, preference and the unmeasurable (until now) factor of taste. Uden and Van Soest (1984) noted that because there was a balance between digestion and passage in the rumen *in vivo*, *in vitro* batch systems could be expected to give higher digestibilities than *in vivo* values. However, substrates that are not readily digested in the rumen but are readily digested elsewhere (such as herbage protein) could have higher *in vivo* digestibilities than *in vitro* (Prasad *et al.*, 1994).

Several groups have compared *in vivo* digestibility and/or intake with results of *in vitro* gas production. Results of 400 digestibility trials and corresponding total gas production were used to calculate the regression equations between *in vivo* digestibility and total gas, and results of 300 sets of data were then used to check these equations (Menke and Steingass, 1988). There was a close correlation between gas production and *in vivo* digestibility, with the exception of succulent feeds. They concluded that the inclusion of CP content for concentrate feeds and CP and ash content for succulent feeds improved the accuracy of prediction.

Blümmel and Ørskov (1993) correlated total gas production from ten straws with DM intake (r = 0.88) and digestible DM intake (r = 0.93) of the straw and growth rate (r = 0.95) of steers. In another experiment, Blümmel and Becker (1997) reported that 75% of the variation in intake of whole roughage was accounted for by *in vitro* gas production after 8 h of incubation, while the average gas production from neutral-detergent fibre (NDF) measured from 24–96 h accounted for 81% of the variation in intake.

However, given that cumulative gas production can provide kinetic fermentation data, it seems a pity to compare it with end-point values, such as *in vivo* intake, or even apparent or true digestibility, though this is the comparison made most commonly at the moment, as these are the parameters most commonly measured. Models to predict intake usually include parameters related to feedstuff characteristics, particularly to carbohydrate degradability (Fisher, 1996), and, from this point of view, a kinetic parameter related to fermentability could prove a valuble contribution to the model as a whole, rather as the rate of degradation measured using the *in situ* techniques is used now (Forbes, 1993).

Advantages and Limitations of the Technique

Cumulative gas-production techniques have several major advantages in terms of animal welfare, sample size and cost and, most importantly, in the fact that they describe the kinetics of microbial activity in response to a given substrate.

Animal welfare

While the use of animals is far less than is required for *in vivo* digestibility trials, gas-production techniques still require animals as a source of rumen fluid. To eliminate this need for fistulated animals, some investigations have been carried out to determine whether or not faeces could be used as an alternative inoculum (Aiple, 1993). A comparison between rumen fluid and faeces from cows fed a silage diet (Williams *et al.*, 1995a) showed that the faecal inoculum resulted in significantly lower total gas, DM loss and VFA production for all substrates (wheat straw, rye grass, soybean hulls, wheat, maize and soybean meal). Consequently, it is not yet clear whether faeces would be an ideal replacement for rumen fluid for this method.

Sample size

The amount of material required to assess the fermentation kinetics of substrates ranges from 0.1 to 1 g. More is required than is actually fermented, in order to ensure a representative sample, but these are still small amounts compared with other techniques, making it suitable for materials available in limited quantities (e.g. from plant-breeding trials, plants grown under special conditions, etc.).

Costs

The costs of laboratory tests are usually much lower than those required to maintain animals. Whether the manual or automated systems are cheaper to run depends, to a large extent, on the cost of labour in the country where the laboratory is located. The initial equipment required can be quite expensive for the automated systems, but, as the hours for measuring are significantly reduced, it is a matter of balancing the capital costs of the equipment with the labour costs.

Information obtained

Cumulative gas production measures the kinetics of fermentation (i.e. microbial activity), rather than a reduction in particle size (Williams *et al.*, 1997). Reduction of particle size is an indication of substrate degradation, but does not necessarily indicate the availability of material to microorganisms. However, gas production remains an indicator of what occurs in the rumen, rather than in the whole animal.

Limitations

The limitations of the gas-production technique rest, for the most part, with the expectations of those using the technique. If someone hopes that the technique can be used to predict all aspects of animal health and behaviour, he/she will be disappointed. However, it does measure the kinetics of fermentation of a given substrate with a given microbial population, thereby giving a reasonable simulation of what occurs in the rumen, and, as such, will be a useful tool for evaluation of ruminant feedstuffs.

One limitation to the use of the technique for forage evaluation is the lack of uniformity in methodology. This makes it difficult to compare results from different groups. An urgent task facing those who are using the technique is to remedy this situation. It should be possible to standardize the technique in terms of the kind of medium being used, the timing of inoculum collection and the diet of the donor animals. Steps have been made in this direction. Two ring tests were organized in 1994 and 1996 (Agricultural Development and Advisory Service (ADAS) – Jim Huntington and Caroline Rymer, personal communication). Preliminary results from the first test, in which all groups used their own equipment and methodology, were reported at an informal meeting held at the University of Reading in 1995, while results of the second test, in which the medium, inoculum collection and, to some extent, animal diet were standardized, have also been reported (Rymer, 1997).

The use of standard substrates, as pioneered for the Hohenheim gas test, may need to be considered in order to overcome the inevitable differences between inocula, not only between groups, but also between runs. Other important details which still need to be investigated include: medium (e.g. an ideal buffer concentration, presence of micronutrients and anaerobiosis) and treatment of inoculum to maximize cellulolytic organism activity; the use of blanks; sample preparation in terms of method and duration of drying and grinding; and, very importantly, the relationship between gas, VFA and microbial production within the *in vitro* system. Inevitably, compromises will have to be made between what would be ideal in terms of the metabolism and growth requirements of microorganisms and what is practical on a day-to-day basis. Some flexibility within the system will need to be maintained to allow for the requirements of different research questions, such as the potential ATP and/or biomass yield of forages.

Future Use and Avenues for Further Research

The need to evaluate the nutritive characteristics of ruminant feeds must increase in importance in coming years. However, the feed-evaluation systems which exist now are inadequate (Beever, 1993a) and are in a state of flux, as new methodologies are developed and new discoveries made concerning animal and microbial physiology. Determination of the fermentation kinetics of substrates for their routine evaluation is likely to be an important facet of evaluation systems of the future, particularly as the dynamics of nutrient utilization are taken into account.

Synchronization of energy and nitrogen supply to the rumen is considered to be an important approach to improve the efficiency of rumen fermentation (Rooke and Armstrong, 1989). To apply this principle in ruminant feed formulation, one needs sufficient data on rates of protein degradation and carbohydrate fermentation by rumen microbes. It is evident that energy supplements differ in their rate and extent of ruminal fermentation (Sutton, 1985; Krishnamoorthy *et al.*, 1991) and cumulative gas-production techniques could be used to generate such information *in vitro*.

Cumulative gas production is being used in combination with alkane analysis of forages as a measure of grazing intake in dairy cows (Sip van Wieren and Pablo Chilibroste, personal communication). This methodology could be extended further to investigate the value of feed consumed by wild animals, by analysing the alkanes present in their faeces and then carrying out a fermentation study of the plants that are then known to have been eaten.

In addition to routine screening and feed evaluation, the technique is also being used to study more fundamental aspects of gastrointestinal tract (GIT) fermentation, particularly in relation to plant cell walls and other fermentable carbohydrates. Valuable information is being obtained, particularly when used in combination with other techniques already available. For example, van Laar *et al.* (1999) have recently used cumulative gas production in conjunction with cell-wall sugar analysis at various time intervals to evaluate differences in the fermentation of different anatomical components of soybean cell walls (Fig. 10.9).

Given the flexibility of the technique in terms of medium chosen and inoculum used, it is also being adapted to investigate more fundamental questions relating the source of substrate to the amount of microbial biomass produced. For this kind of research, it is essential to remember that one is not only examining the test substrate, but also microbial behaviour in terms of that test substrate and the medium being used. For example, when investigating the relation between biomass and gas production, it must be realized that the microbes must have access to all possible micronutrients they may require, or the results from an *in vitro* test will bear little resemblance to what occurs *in vivo*.

Progress will continue to be made in terms of the technical details of the methodology. However, it is clear that once these details have been refined, the technique can be used to measure the dynamic interaction that exists between the substrate and microbes for fermentation in the rumen or, indeed, the rest of the gastrointestinal tract. Results of fermentation kinetics are likely to change our perceptions of both feed evaluation and the kinetics of microbial metabolism.

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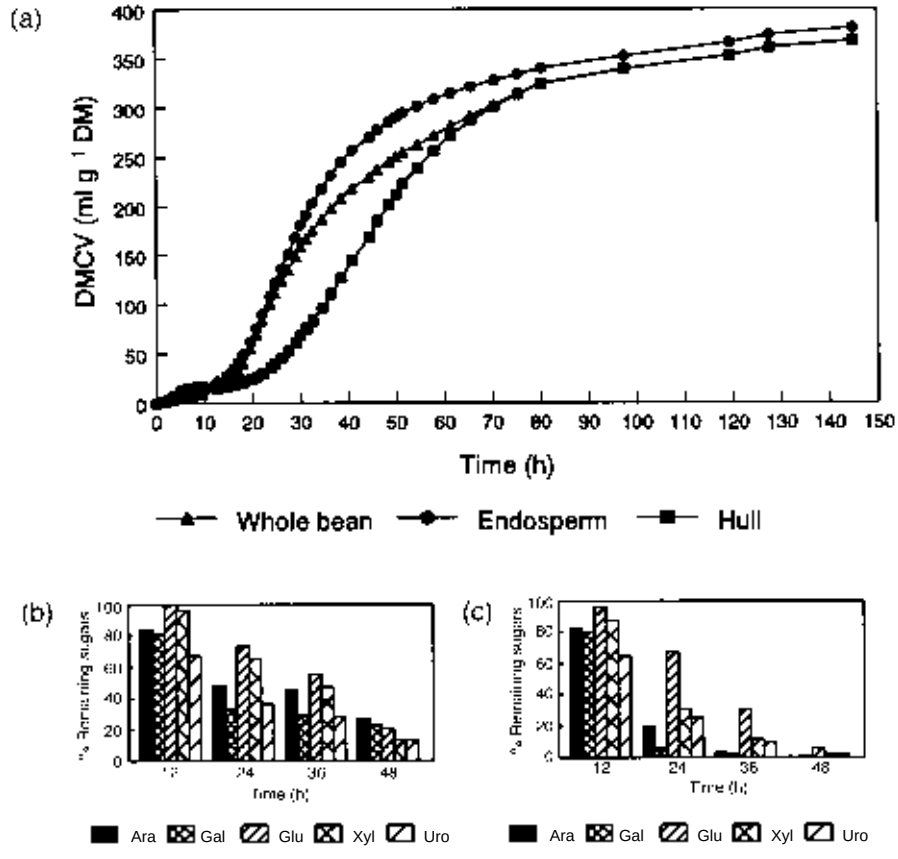

Fig. 10.9. Cumulative gas-production profiles of soybean hull and endosperm (a), and the progressive loss of cell-wall sugars: (b) hull, (c) endosperm (van Laar *et al.*, 1999). DMCV, dry matter cumulative volume; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Uro, uronic acid.

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11 Animal-based Techniques for the Estimation of Protein Value of Forages

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Introduction

Forages are important components of diets for ruminant farm animals. Not only do forages contribute to the energy supply of ruminants, but they also provide a significant amount of protein. In diets for dairy cows, good-quality grass can easily contribute 50% of the energy and up to 60% of their protein requirement. Proteins in feed are important sources of amino acids, the building blocks of many biologically active compounds in animals, of which proteins in muscles, connective tissues and enzymes are quantitatively the most important. The protein value of feeds for ruminants contains two important components: feed protein escaping microbial degradation and microbial protein. Protein degradation and microbial protein synthesis occur in the forestomachs and both sources of protein are subsequently hydrolysed in and absorbed from the small intestine. However, both processes are quite variable and their combined result determines the protein supply to the small intestine. This supply broadly depends on the combination of rate of degradation, rate of microbial growth and rate of outflow of feed particles and microbes. Therefore, a proper evaluation of the protein value of forages requires techniques that take these dynamics into account. Various *in vivo* techniques have been developed and this chapter gives a critical review of such techniques.

In Vivo Estimation of Protein Degradation and Microbial Protein Synthesis

The most widely applied way of measuring protein degradation and microbial protein synthesis *in vivo* is in animals equipped with a re-entrant or a T-piece cannula in the proximal duodenum. Total protein passing into the proximal duodenum is measured and sampled and the proportion of microbial protein is determined by using an appropriate marker.

 Forage Evaluation in Ruminant Nutrition (eds D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed)

Various surgical techniques for providing a ruminant with intestinal cannulae have been developed and discussed (McGilliard, 1982). The use of cannulated animals has a number of disadvantages. First of all, it is an invasive and very costly technique. Measuring intestinal digesta flow is also tedious (MacRae, 1975; Faichney, 1993; Reynolds *et al.*, 1994; Poncet *et al.*, 1995). Although digesta can be sampled reliably, flow rates may become affected after opening the cannulae for various reasons, such as changes in pressure (Sutton and Oldham, 1977; Faichney, 1993). Therefore, the use of markers is also recommended with re-entrant cannulae; another reason is because the measuring periods are usually too short to compensate for the often observed initial flow reduction after opening the cannulae. Use of markers, however, is a prerequisite when T-piece cannulae are used (Faichney, 1975, 1993).

Further limitations of measurements in cannulated animals are due to the difficulty in accurately measuring microbial protein (Hvelplund *et al.*, 1995). Various microbial markers have been developed. Examples were summarized and discussed (Harrison and McAllan, 1980; Tamminga, 1982; Broderick and Merchen, 1992), and these include nucleic acids (RNA, DNA), total purines, amino acids (2,6-diaminopimelic acid (DAPA), D-alanine) and radioactive as well as stable isotopes (sulphur-35 (^{35}S), phosphorus-32 (^{32}P), nitrogen-15 (^{15}N). Based on the assumption that feed protein or endogenous protein do not contain DAPA or RNA, the proportion of microbial N (MN) in the non-ammonia-N (NAN) entering the small intestine is estimated from:

MN/NAN = (DAPA/NAN in digesta)/(DAPA/N in microbes)
MN/NAN = (RNA/NAN in digesta)/(RNA/N in microbes)

As pointed out by Harrison and McAllan (1980) and Stern *et al.* (1994), the results obtained using different markers seldom agree. For reference material, bacteria isolated from strained rumen fluid by differential centrifugation are often used, but these may not represent the daily flow of bacteria out of the rumen and arriving at the proximal duodenum. The ratio between bacteria associated with the fluid (FAB) and those associated with feed particles (PAB) is subject to considerable variation. The proportion of bacteria associated with feed particles may vary between 50 and 80% (Owens and Goetsch, 1986; Oosting, 1993). Chemical composition differs between FAB and PAB (Merry and McAllan, 1983) and differences exist in outflow rate between rumen fluid and feed particles and, as a result, also between FAB and PAB. Besides, diurnal variation in composition of FAB and PAB is often not taken into account, a problem that can be overcome to some extent by taking samples more frequently and spread over a period of 24 h. As an alternative, bacteria have sometimes been isolated from duodenal digesta (Yang and Poncet, 1993). Estimating the contribution of protozoa is even more cumbersome (Harrison and McAllan, 1980; Schelling *et al.*, 1982). Currently, the two most commonly used microbial markers to differentiate between microbial and dietary protein are DAPA and purines. However, DAPA is absent in protozoa and some bacteria and is subject to extensive metabolism. In their extensive review on markers for quantifying microbial protein synthesis in the rumen, Broderick and Merchen (1992) concluded that a simultaneous determination of total purines and the use of the ^{15}N method has the best chance of getting reliable results. On the other hand, Stern *et al.* (1994) con-

cluded that 'because no absolute standard exists, it is impossible to state which marker method, microbial isolation procedure or site of microbial isolation provides the most accurate estimate of microbial N flow into the proximal duodenum'. The choice of method may also be determined by the availability of analytical equipment. Extremes are the analytical procedures for purines, which can easily be done in a spectrophotometer, and the use of ^{15}N, which depends on having access to quite costly isotope-ratio mass spectroscopy (IRMS) equipment.

Depending on the site of cannulation, the mix of undegraded feed protein and microbial protein may be diluted by a variable amount of protein originating from the animal itself and giving no net contribution to the postruminal protein supply. Methods for estimating endogenous contribution have been developed, but more frequently in non-ruminants than in ruminants (Tamminga *et al.*, 1995; Nyachoti *et al.*, 1997). A widely used method in pigs is that of the isotope dilution technique, often with ^{15}N leucine as precursor. In ruminants, ^{15}N-labelled feeds have been used (Van Bruchem *et al.*, 1997), but corrections for ^{15}N contamination of microbial protein passing along the small intestine are necessary.

Furthermore, when ruminal protein degradation is based on the appearance of protein in the small intestine, this measurement only gives the final outcome and it remains uncertain whether differences result from differences in rate of degradation or differences in rumen retention time or both. Yet another disadvantage of the use of cannulated animals is that, when mixed diets are fed, as is usually the case with dairy cows, no digestive information is obtained on the individual dietary ingredients. Again, the conclusion of Stern *et al.* (1994) was that:

> *in vivo* estimates of protein degradation are expensive, labour intensive, time consuming, and subject to error, associated with use of digesta flow rate markers, microbial markers and animals variation. Therefore, alternative procedures for measuring ruminal degradation of dietary protein are needed.

This has led to the development of alternative methods and in many countries the *in situ* method, in which nylon bags filled with the ingredient to be investigated are incubated in the rumen for various lengths of time, has been adopted as the reference method. This method also has its limitations, which will be discussed in the next chapter. An alternative method, which as yet has received little attention, is the rumen evacuation method (Robinson *et al.*, 1987). This method, when applied carefully at different times after feeding and combined with measuring the rate of passage out of the rumen (the reciprocal of rumen retention time), which is possible with the application of markers, can give useful information on the dynamics of rumen degradation and synthesis. It would make it possible to quantify PAB and FAB and, when appropriate outflow rates for fluid and particles are applied, give an estimate of microbial protein supply and its variation. An additional advantage would be that animals equipped with a rumen cannula only would suffice.

In Vivo Estimation of Postruminal Availability

Postruminal availability of protein can be measured *in vivo* as the difference between protein entering the proximal duodenum and protein passing out of the

terminal ileum. A complicating factor is that two sets of cannulae are needed, one in the proximal duodenum and one in the terminal ileum. Using re-entrant cannulae in dairy cattle certainly creates severe problems (Reynolds *et al.*, 1994). A summary of the apparent fractional absorption of NAN and amino acids is given in Table 11.1 (NRC, 1985).

As already discussed, a complicating factor is the presence of a variable amount of endogenous protein at both sites. Besides, what enters the duodenum is a mixture of undegraded feed protein and microbial protein, which can be differentiated in the proximal duodenum, but can no longer be differentiated at the terminal ileum. Solutions have been found in infusion studies, either on top of the normal passage (Schwarting and Kaufmann, 1979) or in animals kept on intragastric infusion (Hvelplund *et al.*, 1994). The techniques have been applied both with isolated rumen bacteria (Storm *et al.*, 1983) and with feedstuffs (Hvelplund, 1985). Both protein sources – feedstuffs in particular – contain non-protein dry matter (DM), which may induce the release of extra endogenous protein; this may explain why the results of infusion studies usually show a slightly lower intestinal digestion than the method based on mobile nylon bags. Using the technique of intragastric infusion offers a possibility to correct for endogenous contamination (Hvelplund *et al.*, 1994), with the restriction that the endogenous secretions of animals fed with intragastric infusion may not represent a practical situation.

An alternative approach is to insert small nylon bags, containing a small amount of the protein source to be investigated, into the proximal duodenum and to recover them either from the distal ileum or from the faeces. This mobile nylon-bag technique is now a widely applied method and further details on its suitability will be discussed in the next chapter.

Measuring the net appearance of absorbed amino acids in the portal blood has also been recommended as a means of estimating postruminal availability of protein. It should, however, be realized that the gut (wall) itself uses considerable and variable amounts of amino acids (MacRae *et al.*, 1996), with an apparent preference for non-essential amino acids.

Table 11.1. Summary of apparent fractional absorption of non-ammonia nitrogen and amino acids from the small intestine in ruminants (NRC, 1985).

	n	mean	SD	CV
Non-ammonia nitrogen				
Lactating cattle	12	0.65	0.04	0.07
Non-lactating cattle	17	0.66	0.04	0.06
Sheep	29	0.64	0.06	0.09
All	58	0.65	0.05	0.08
Amino acids				
Lactating cattle	21	0.69	0.05	0.08
Non-lactating cattle	11	0.62	0.06	0.10
Sheep	22	0.70	0.06	0.09
All	54	0.68	0.06	0.10

SD, standard deviation; CV, coefficient of variation.

Use of Flow Markers

As already mentioned, the use of intestinal cannulae requires the use of markers. Quite a variety of different markers have been applied. One category follows the passage of fluid, examples being polyethylene glycol (PEG) and various elements in a complex with ethylenediamino-tetra-acetic acid (EDTA), notably cobalt (Co)-EDTA and chromium (Cr)-EDTA. Examples of markers representing the passage of particles are chromic oxide (Cr_2O_3), Cr-mordanted neutral-detergent fibre (NDF) (Cr-NDF) and a wide variety of rare-earth elements, such as cerium (Ce), dysprosium (Dy), lanthanum (La), ruthenium (Ru), often applied as Ru-phenanthroline, and ytterbium (Yb). Rare-earth elements adsorb strongly to feed particles, and so are thought to represent the behaviour of feed particles. Migration between particles may cause problems, however (Faichney, 1993). Chromium oxide (Cr_2O_5) and titanium(IV) oxide (TiO_2) have also been widely used as markers, but, because of their high density, they may not represent either of the two fractions. A third category are the internal markers, such as lignin, lignin fractions (cellulose), indigestible acid-detergent fibre (IADF) and rumen-indegradable neutral-detergent fibre (RINDF) (Tamminga *et al.*, 1989a, b) and *n*-alkanes (Mayes *et al.*, 1995). A thus far under-explored type of internal marker is that based on carbon-13 (^{13}C), which seems applicable either based on the difference in natural abundance between C-3 and C-4 plant species (Südekum *et al.*, 1995) or based on artificially enriched plants (parts) grown in a carbon dioxide ($^{13}CO_2$)-rich environment (Svejcar *et al.*, 1993).

The most straightforward method is to infuse the marker continuously into the rumen at a constant rate. Intestinal flows can than be estimated by dividing the marker dose rate by the mean marker concentration in the digesta. When using T-piece cannulae, sampling of digesta may become problematic and non-representative. This seems particularly the case when the cannula is placed immediately behind the pylorus (P.H. Robinson and M.W. Bosch, personal communication). A solution may then be the double-marker method, a combination of a solute marker and a particle-associated marker, from which a reconstitution factor can be calculated (Faichny, 1993).

When a steady state has been achieved and is maintained by continuous infusion of a solute marker (*S*) and a particle-associated marker (*P*) and their concentrations are expressed as fractions of the daily dose per unit of digesta or its phases, the following relationship is valid:

$$R = (P_{DG} - Z \cdot S_{DG})/(Z \cdot S_{FP} - P_{FP})$$

Where *R* = the reconstitution factor, i.e. the number of units of fluid phase (FP) that must be added to (or removed from) one unit of digesta (DG) to obtain true digesta (TD), and *Z* = the marker concentration ratio, *P*/*S*, in TD. The concentration (C_{TD}) and digesta flow (F_{TD}) can than be calculated from:

$$C_{TD} = (C_{DG} + R \cdot C_{FP})/(1 + R)$$

and:

$$F_{TD} = 1/S_{TD}$$

However, this does not solve the problem of non-representative sampling within the particulate phase.

Markers can also be used to estimate the ruminal outflow of fluid or particles. In this case, a pulse dose of the marker is given into the rumen and fractional passage rates are calculated either from its dilution in rumen fluid or from its faecal excretion pattern with time, based on the assumption that fractional passage rates out of the rumen follow first-order kinetics (Grovum and Williams, 1973):

$$C_t = C_0 \cdot e^{-kt}$$

Where C_t and C_0 represent marker concentrations at times t and zero, and k is the fractional outflow rate.

A still unresolved question is whether external markers are superior to internal markers or vice versa. Till now, most researchers would argue it to be an advantage for a marker not to take part in the digestive process. However, inert markers may behave differently from feed particles, because they do not posses the right functional density and are not subjected to particle-size reduction or entrapment in the mat of fibrous material normally present in the rumen. Recent findings (Tamminga, 1993; Van Straalen, 1995) would suggest that passage out of the rumen of a feed particle to some extent depends on its degree of degradation. As a result, a relationship between degradation and passage cannot be ruled out. Under these circumstances, internal markers, particularly those which take part in the digestive process, could prove to be superior to external and inert markers. The use of ^{13}C-enriched feed components could possibly solve this problem.

Use of Purine-derivative Excretion as Index of Microbial Protein Supply

The *in vivo* methods discussed so far in this chapter all require cannulation of animals. With an increasing concern for animal welfare, there is a greater need to develop non-invasive or less invasive methods. The technique for the estimation of microbial protein supply using urinary excretion of purine derivatives (PD) as a parameter is a recent development in this line. Unlike the commonly used methods based on digesta flow measurement, this technique does not require any surgical preparation. Since only urine collection is needed, an estimation of microbial protein supply can be incorporated into N balance and digestibility trials without much additional labour input.

Urinary PD comprise allantoin, uric acid, xanthine and hypoxanthine collectively. All four components are found in urine of sheep, goats, red deer and llamas, but only allantoin and uric acid are found in urine of cattle and buffaloes. These derive mainly from the degradation of absorbed microbial purines (exogenous source) and a smaller fraction from the turnover of tissue nucleic acids (endogenous source). The endogenous PD excretion has been determined in several species, including sheep, cattle, goats, llamas and buffaloes. It has been repeatedly demonstrated in sheep and cattle that the daily PD excretion is directly related to the uptake of exogenous (i.e. intestinal) purines (Chen *et al.*, 1990b; Verbic *et al.*, 1990; Balcells *et al.*, 1991). Therefore, the excretion of PD can provide a quantitative estimate of the intestinal flow of microbial protein if the purine:protein ratio

in mixed rumen microbes is constant. A practical example of the application of this technique is given in Chen *et al.* (1992b). In a number of studies (Oosting *et al.*, 1995; Pérez *et al.*, 1996, 1997; Samaniego *et al.*, 1997), it was shown that estimates of microbial protein in sheep based on this technique were in close agreement with direct measurements using microbial markers (^{15}N, amino acid profile, DAPA and RNA). A numerical comparison is given in Table 11.2.

In using this technique, however, one should be aware of its limitations and uncertainties. The technique is based on two assumptions. The first assumption is that all the nucleic acid purines entering the small intestine are of microbial origin. Considering that residual dietary purines after microbial degradation may be negligible for most diets and that the quantity is difficult to determine in intact animals, at this stage this assumption may have to be accepted. However, special precaution needs to be taken when a large amount of fishmeal is fed. The second assumption is that the purine:protein ratio in mixed rumen microbes is a constant. Whether a dietary regime can drastically alter the value of this ratio is not known. There is not yet sufficient information to provide an answer, but earlier results (Schelling *et al.*, 1982) do not indicate a large variation.

Marked species differences in purine metabolism have been demonstrated in several species including sheep, cattle, buffaloes and llamas (Chen *et al.*, 1990a, 1996a; Bakker *et al.*, 1996; for a review, see Stangassinger *et al.*, 1995). It is important to note that different equations will need to be used with different animal species for the calculation of microbial N supply based on PD excretion. Equations have been established so far for European sheep (Chen *et al.*, 1990b) and cattle (Verbic *et al.*, 1990), the parameters of which have been confirmed by studies in separate laboratories (Balcells *et al.*, 1991; Beckers and Thewis, 1994).

Table 11.2. Duodenal-flow microbial N in sheep estimated with purine bases and other methods.

Duodenual flow	Bacterial marker	Flow of bacterial amino acid N (exp. 1) or total N (exp. 2)(g day^{-1})			
Experiment 1 (Oosting *et al.*, 1995) Diet:		UWS	AWS	AWSC	AWSP
MN1	DAPA	6.5	8.0	10.0	11.5
MN2	AA profile	6.8	8.4	11.7	11.0
MN3	Purine bases	6.7	10.5	11.3	12.0
MN2	Purine bases	4.8	7.2	12.0	11.7

Diets: UWS, untreated wheat straw; AWS, ammonia-treated wheat straw; AWSC, ammonia-treated wheat straw supplemented with casein; AWSP, ammonia-treated wheat straw supplemented with potato protein.

Experiment 2 (Pérez *et al.*, 1996) Diet:		0	220	400	550
MN1	^{15}N	6.2	9.0	14.0	14.4
MN2	Purine bases	4.8	7.2	12.0	11.7

Diets: lucerne (550 g day^{-1}) supplemented with 0, 220, 400 and 550 g of barley.

An equation derived from the sheep experiment of Chen *et al.* (1990b) is:

$$Y = (0.150\ W^{0.75}\ e^{-0.25X}) + 0.84X$$

and one derived from the cattle experiment of Verbic *et al.* (1990) is:

$$Y = (0.385\ W^{0.75}) + 0.85X$$

where Y is urinary excretion of total PD (mmol day^{-1}), $W^{0.75}$ the metabolic body weight and X the absorbed exogenous purine (mmol day^{-1}). The equation for sheep may also be applied to goats. However, there are strong indications that the equations developed using European animals may not work with animals in the tropics. Animals in the tropics appeared to excrete a smaller proportion of the plasma PD in the urine than those in temperate areas (Liang *et al.*, 1994; Chen *et al.*, 1996a). Recent data suggest that this proportion is rather consistent within each animal individual and is not affected by dietary regime, such as feed intake (P. Prasitkusol, unpublished). It is unlikely that one single equation will be applicable universally. In the future, specific equations will need to be established and validated for the target animal groups to which the method is applied. This may seem an insurmountable task, but methodologies are now being developed to make it easier to establish the equation (Chen *et al.*, 1997; IAEA, 1997). The accuracy of the results obtained based on the purine technique has to be assessed more extensively in the future. At this stage, one should not take the estimates of microbial N based on PD excretion as absolute values, but as values for comparative purposes.

Measurements of PD excretion have proved useful in many studies on microbial protein production from forage feeding. This technique has been used in studies to examine: (i) how the source and level of N in the diet affected microbial protein supply (Chen *et al.*, 1992a; Puchala and Kulaseck, 1992; Balcells *et al.*, 1993a; Giraldez *et al.*, 1993; Susmel *et al.*, 1994a, b); (ii) the efficacy of supplementing straw or silage with carbohydrates or concentrates (Chen *et al.*, 1992a; Balcells *et al.*, 1993b; Chamberlain *et al.*, 1993; Dewhurst *et al.*, 1994; Gomes *et al.*, 1994; Moorby *et al.*, 1994); (iii) the effect of phosphorus supply on microbial protein synthesis (Gunn and Ternouth, 1994; Scott *et al.*, 1994); and (iv) the effect of rumen outflow rate on microbial protein flow (Chen *et al.*, 1992b; Dewhurst and Webster, 1992).

Research has also been carried out to simplify the technique further for application in the field, where total urine collection is impractical. The results indicate that it is possible to use the averaged 'PD-to-creatinine' ratio in several spot urine samples collected within a day as an index of microbial protein supply (Chen *et al.*, 1995, 1996b), but the sensitivity is, of course, lower than that based on total urine collection. Spot testing may be of value only to indicate large differences. Therefore, where facilities allow, total urine collection is recommended. Recent studies indicated that the ratio of purine N to total N (called the 'purine nitrogen index') in spot urine samples could potentially be used to identify situations where rumen-degradable N (RDN) is inefficiently used for the synthesis of microbial protein (Chen and Jayasuriya, 1997). Similarly, it has been suggested that milk allantoin could be used as an indicator of microbial protein production in dairy cows. Its potential use may be limited, because concentrations in milk are much lower than in urine and milk allantoin as a proportion of the total excretion appears to vary with milk production (Stangassinger *et al.*, 1995).

Some technical details of the method are given in the laboratory manual prepared by Chen and Gomez (1992) and the International Atomic Energy Agency (IAEA) (1997). The chemical analysis of purine derivatives can be carried out with any of the following instruments: high-performance liquid chromatographs (HPLC), autoanalysers and spectrophotometers.

Application to Forages

Figure 11.1 illustrates the procedures that are currently used or available for the *in vivo* evaluation of the protein values of forages. The need to do so may arise, for example, in order to evaluate the nutritive value of new varieties of pasture or to compare the different methods of preservation or different ways of feeding the forage. Typically, the forages in question need to be fed as the sole or main component of the diet. Feeding trials should be conducted to measure the voluntary feed intake and the flow of dietary and microbial protein to the small intestine kg^{-1} digestible organic matter (OM) intake. There are two options, depending on the extent of surgical intervention to be imposed on the animals. One is to use animals fitted with a rumen cannula and a duodenal catheter to measure the flow of dietary and microbial protein into the small intestine. Microbial and flow markers need to be used. Protein availability can be measured if a cannula is also fitted at the ter-minal ileum. This approach involves painstaking labour input and few animals can be used at one time. Most earlier work for measuring the protein value of forages was done in this way (see review by Thomson and Beever, 1980).

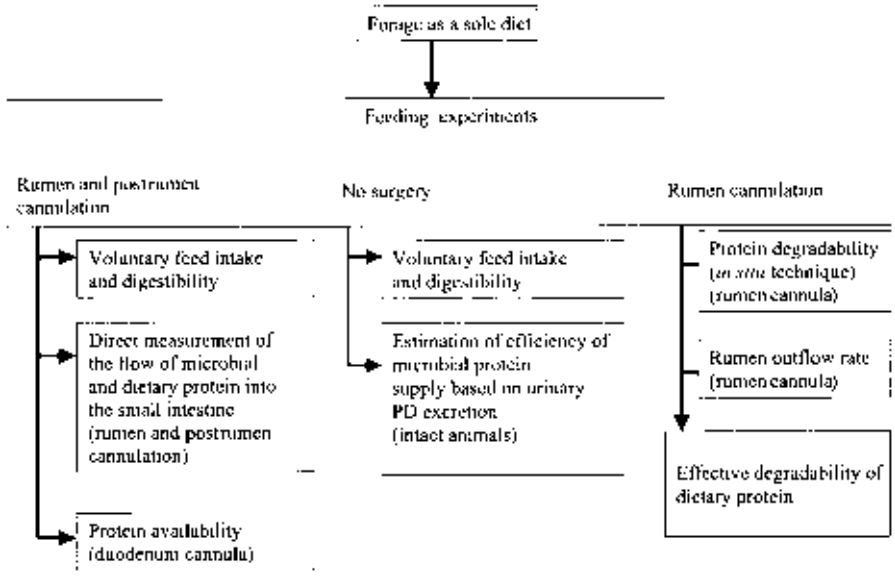

Fig. 11.1. Commonly used procedures for the estimation of the protein value of forages.

The alternative approach is to use an indirect estimation of microbial protein flow based on urinary PD excretion, which can be carried out on intact animals. The undegradable dietary protein contribution can be estimated based on protein degradability, using the *in situ* technique, and the rumen digesta outflow rate can be estimated in separate animals fitted with rumen cannula. As was discussed before, the latter approach does not give an absolute value for the flow of microbial protein and dietary protein and is thus more suitable for comparison. The simplicity of this approach outweighs its limitation when the object of the study is to compare two or more forages. There have been an increasing number of studies reported in which this approach is used. For instance, Verbic (1997) compared different methods of grass forage preservation (fresh-cut silage, wilted silage, highly wilted silage, formic acid-treated silage and hay). He reported that the hay and highly wilted silage had higher contents of rumen-escape protein kg^{-1} DM and a greater amount of microbial protein supply (estimated based on PD excretion) kg^{-1} digestible OM intake. Formic acid-treated silage also had a higher protein value than untreated silage. Verbic *et al.* (1997) used the same approach to compare maize silages made from dent- or flint-type hybrids. Silage made from the flint-type hybrid supported higher microbial protein production than that from the dent-type hybrid. O'Kiely *et al.* (1994) showed that addition of dry beet pulp to the ensiling of whole-crop fodder beet (*Beta vulgaris*) not only acted as an effluent absorbent but also improved the microbial protein supply from the silage. The intake of tropical grasses is often limited by their low N content and supplementation with *Leucaena leucocephala* improves intake and animal productivity. In coastal Kenya, where *Leucaena* production was affected by a psyllid pest, Abdulrazak *et al.* (1996, 1997) showed that *Gliricidia sepium* could be used as an alternative to *Leucaena* as a protein supplement to Napier grass or *Zea mays* stover.

The combined use of different methods often has added value in terms of extra information. From the *in situ* measurements, Verbic (1996) found that the rates of degradation of OM and protein were more synchronized in hay than in silage, and this may in part explain why highly wilted silage and hay have a greater efficiency of microbial protein production than fresh-cut silage. In using the *in situ* degradation measurements, most people would only carry out the measurements for DM or protein alone, but, with forages fed as the sole or main component of the diet, it pays to determine the degradation of both. The ratio of the content of RDN to that of degradable organic matter (DOMR) – ideally 32 g RDN kg^{-1} DOMR, according to the Agricultural Research Council (ARC, 1984) – should provide an indication of whether the RDN content of the forage is deficient or sufficient. Moreover, the amounts of RDN or OM released at different times after feeding can be calculated from the derivative of the degradation curves, and the variation in the amount of RDN released with time relative to that of OM provides an index of the synchronization of the N and energy supply. The term 'synchrony index' (Sinclair *et al.*, 1993) was proposed in this context. Although its practical significance has yet to be explored (Chamberlain and Choung, 1995), a measurement of the synchronization index may shed light on why forages vary in their protein value (i.e. in the efficiency of microbial protein production).

Further Research Needed

The aims of methods developed to measure the protein value of forages are accuracy, simplicity, cost-effectiveness and minimum surgical intervention (particularly to avoid postrumen cannulation).

In direct measurement of intestinal flow of microbial protein and dietary protein, chemical analysis is not a problem. The key element that affects the accuracy of the measurement is the determination of digesta flow. Although a lot of work has been done in this area and an increasing number of liquid and solid markers are being used, there is still a lack of an ideal marker that can represent all particle sizes/densities. The development of a simple and accurate method for digesta flow measurement is still required.

Direct measurement as an approach will probably not be developed much further, due to its need for surgical intervention. Its application will remain, however, for some time to come, particularly when absolute protein value measurements are needed.

It is to be expected that future developments will be in non-invasive methods (e.g. using PD for estimation of microbial protein supply). Further work on the technique based on PD excretion in urine includes the following areas: (i) the results need to be validated by simultaneous comparison with two other methods; and (ii) due to species differences in purine metabolism, it is most likely that specific 'calibration curves' will need to be constructed for each target animal.

Cost Implications

Table 11.3 summarizes the cost elements of the techniques covered in this chapter. These include invasiveness, labour and chemical analysis. Most of the techniques require the use of surgically modified animals. Not only is such surgery (including facilities, skills and staff involved) costly, but the care and maintenance of such animals, particularly animals with postrumen cannulation, is also costly. Animals may also lose their carcass value. In addition, measuring periods can be very labour-intensive. Besides, the invasiveness of surgical techniques meets increasing resistance from animal-welfare groups and the general public.

The major consideration for the cost of chemical analysis is the requirement for capital equipment. Where flow measurement using metal-based markers is needed, the basic requirement is often an atomic-absorption spectrometer. Determination of microbial markers in digesta samples requires an amino acid analyser (DAPA), ultraviolet (UV) spectrophotometer (RNA), liquid scintillation counter (^{35}S or ^{32}P) or mass spectrometer (^{15}N), depending on the choice of marker. RNA is commonly used, because its analysis does not need expensive equipment. Apart from the requirement for facilities to collect total urine, this technique for the indirect estimation of microbial protein supply based on urinary PD excretion appears to be the simplest and least expensive. The basic equipment required for the chemical analysis is a UV spectrophotometer, although chromatographic equipment can also be used. Apart from tracers, the reagents used for chemical analysis in most techniques are relatively inexpensive on a 'per sample' basis.

Table 11.3. Cost elements of various *in vivo* techniques for the estimation of protein value of forages.

	Cannulae	Flow measurement	Microbial marker	Others	Main cost elements	Equipment required for chemical analysis
Microbial protein						
Direct determination	Rumen, duodenum	Yes	RNA, DAPA, ^{15}N ^{32}P, ^{35}S		Surgery, labour, chemical analysis, loss of carcass value	DAPA: amino acid analyser/HPLC RNA: spectrophotometer ^{15}N: mass spectrometer ^{32}P and ^{35}S: liquid scintillation counter Flow markers: atomic absorption spectrometer
Indirect estimation (urinary marker)		No		Complete urine collection	Facility to collect total urine, chemical analysis	Spectrophotometer or HPLC
Undegradable protein						
Flow of undegradable protein	Duodenum	Yes			Surgery, chemical analysis	Flow markers: atomic absorption spectrometer Kjeldahl N
In situ (nylon bag)	Rumen	No			Surgery, labour	Kjeldahl N
Rumen-emptying	Rumen	Yes			Surgery, labour	Kjeldahl N
Postruminal availability						
Difference between duodenum and ileum	Proximal duodenum, terminal ileum	Yes			Invasiveness, labour, chemical analysis	Flow markers: atomic absorption spectrometer Kjeldahl N
Infusion into abomasum	Rumen, Abomasum,			Intragastric infusion	Surgery, labour	
Mobile nylon bag	Duodenum	No			Surgery, labour	Kjeldahl N

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12 *In Situ* Techniques for the Estimation of Protein Degradability and Postrumen Availability

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Introduction

Obtaining the protein value of a feed for ruminants, expressed as amino acids truly absorbed from the small intestine, which is now common according to the new protein evaluation systems, involves measurements of several characteristics of the feed (Verité *et al.*, 1987; AFRC, 1992; Tamminga *et al.*, 1994; Madsen *et al.*, 1995). These characteristics are related to the fate of the protein in the feed during its passage through the digestive tract and to the energy-yielding processes in the rumen (mainly fermentation of carbohydrates) which drive microbial protein synthesis in the rumen. Factors related to the feed protein are degradability of the feed protein in the rumen, content of amino acids in the undegraded crude protein and, finally, small-intestinal digestibility of the amino acids in the undegraded dietary protein. Microbial protein synthesis in the rumen is related to the energy made available during fermentation in the rumen, but also of importance is the content of amino acids in the microbial protein produced in the rumen and the digestibility of microbial amino acids in the small intestine.

To determine the contribution of amino acids truly absorbed due to consumption of a forage, it would be necessary to quantify the factors mentioned above and develop easy and reliable methods for estimating values for those factors, which are variable among feeds and also within feeds. This chapter is restricted to *in situ* methods used to estimate protein degradability in the rumen and intestinal digestibility of undegraded dietary protein.

Rumen Protein Degradation

Feed protein is, to a large extent, degraded in the rumen. Rumen protein degradability is therefore one of the most important qualitative factors determining the

 Forage Evaluation in Ruminant Nutrition (eds D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed)

protein value of a feed, as the protein degradability determines the supply to the rumen microbes of both ammonia and branched-chain fatty acids, as well as the supply to the small intestine of rumen-undegraded feed protein as a potential source for amino acid absorption.

Rumen protein degradation follows the scheme: protein → oligopeptides → dipeptides → amino acids → ammonia (Wallace, 1995; Cotta and Russell, 1996). The degradation of amino acids to ammonia is an intracellular process, but the degradation of protein to amino acids is an extracellular, but cell-associated, process (Cotta and Russell, 1996). According to Wallace (1995), the rate-limiting step in protein degradation is the degradation of oligopeptides to dipeptides from the N terminal.

It is well known from many studies that protein degradability can vary substantially both between and within feed groups. Therefore, estimation of protein degradability is of major importance in feed evaluation. As *in vivo* estimation of rumen protein degradability is problematic (see Chapter 11), other methods have been applied. Since the introduction of the new protein evaluation systems, the *in situ* (nylon bag, *in sacco*) method has been the most widely used method, and has commonly been used also as reference method for validation of other laboratory methods.

The technique to estimate rumen fermentation by incubation of small feed samples in fibre bags in the rumen was first used by Quin *et al.* (1938), who used silk bags to study degradation in the rumen of sheep. However, it was the introduction by Ørskov and McDonald (1979) of mathematical tools to weight the obtained data with a fractional rumen outflow rate and thereby transform the degradability values to the so-called effective protein degradability (EPD) which led to today's extensive use of the *in situ* method. Beside protein degradability, the method has been used to examine rumen degradability of dry matter (DM), organic matter, neutral-detergent fibre (NDF) (Stensig *et al.*, 1997) and starch (Tamminga *et al.*, 1990). Recently, the *in situ* method has been thoroughly reviewed by Huntington and Givens (1995); therefore, this chapter will mainly deal with those aspects that have major importance for forages.

Description of the in situ *method*

The principle in the *in situ* method is incubation of small feed samples in the rumen in fibre bags. The bags have pores small enough to retain the feed sample, but large enough to allow bacteria to enter the bags. Due to the small amounts of feed sample incubated, the feed under examination will not affect the rumen fermentation, and it is assumed that the conditions within the bag are similar to the conditions in the surrounding rumen content.

It has been attempted to standardize the *in situ* method, and the following method has been agreed upon for concentrate feeds by a European Economic Community (EEC)–European Association of Animal Production (EAAP) seminar (Oldham, 1987), as described in detail by Madsen and Hvelplund (1994). However, the standard is also commonly used for forages.

Bag specification
Bags should be made of polyester (Dacron) or nylon, with a pore size of 30–50 μm. Sample size should be 10–15 mg of material cm^{-2} of bag surface, and the ratio of width to length of the bags should be between 1:1 to 1:2.5.

Sample preparation
Samples for the incubation in the bags should normally be air-dry. For some materials that would otherwise be difficult to process, low temperature (less than 60°C) or freeze-drying is acceptable before milling through a 1.5–2.5 mm screen. After milling, there should be no fine sieving/screening to remove fine particles.

Animals and feeding
Animals should be cattle, sheep or goats, fed according to maintenance level or slightly above, with feeding twice per day and with a minimum interval between meals of 8 h. The diet should contain grass or legume hay plus concentrates, in the ratio two parts forage and one part concentrate on a DM basis, with a minimum of 13% crude protein in total ration DM. The concentrates used should contain as wide a mix of ingredients as possible, with a minimum of three sources of protein, and ideally the feedstuff under test should be included and high-starch concentrates should be avoided.

Replications and procedure
A minimum of three animals are to be used in each estimation, with incubation time 0, 2, 4, 8, 16, 24 and 48 h. Bags are to be inserted at either feeding time.

Washing procedure
For washing, cold water should be used and automatic machine washing is preferable for standardization, with a washing time of 10–15 min.

Compared with concentrates, it may be necessary to modify sample pretreatment, incubation times and treatment of the residues when forages are examined. Most forages need drying before milling. Here, it is very important to avoid heat damage of the protein, and therefore freeze-drying is recommended (López *et al.*, 1995). Compared with the original fresh forages, drying and milling can be argued to result in changes, which may affect the measured degradability. Therefore freezing and chopping or homogenizing the frozen material is used as an alternative (Huntington and Givens, 1997). Compared with dried and ground samples, the frozen and chopped samples will probably reflect the original fresh samples better. However, handling and weighing out of representative samples are easier with dried and ground samples.

With low protein and high fibre content, microbial colonization of the bag residues means that a significant proportion of the nitrogen (N) in the residues may be of microbial origin. This and other problems that are of special interest when evaluating forages will be discussed below.

Data presentation

The most detailed data presentation is to give the degradabilities measured at each incubation time, which can be used to draw the degradation profile (see Fig. 12.2).

For many feeds, first-order kinetics (equation 1) can describe the degradation profile satisfactorily (Ørskov and McDonald, 1979):

$$\text{Deg}(t) = a + b(1 - e^{-ct}) \quad (1)$$

where:

Deg(t) = fraction of protein degraded at time t of incubation
a = fraction of immediately degradable (soluble) protein
b = fraction of not soluble, but degradable protein
c = the fractional rate of degradation of fraction b (h^{-1})

The degradation profile describes the degradation of protein retained in the rumen. However, some protein will escape rumen degradation. Traditionally, the escape of protein from the rumen has been assumed to follow first-order mass action kinetics, according to equation (2) (Ørskov and McDonald, 1979):

$$R(t) = e^{-kt} \quad (2)$$

where:

$R(t)$ = fraction of protein retained in the rumen at time t of incubation
k = fractional outflow rate from the rumen (h^{-1})

The EPD can then be calculated according to equation (3) (Ørskov and McDonald, 1979):

$$\text{EPD} = a + b[c/(c + k)] \quad (3)$$

The EPD, which is the predicted *in vivo* rumen protein degradability, can also be calculated by the summation method, proposed by Kristensen *et al.* (1982), as shown in equation (4):

$$EPD = N_0 + \sum_{i=0}^{n}\left[\left(N_{t_{i+1}} - N_{t_i}\right) * \frac{e^{-kt_i} + e^{-kt_{i+1}}}{2}\right] \quad (4)$$

where:

N_0 = fraction of soluble protein determined by washing
N_{t_i} = fraction of degraded protein at incubation time t_i
i = incubation number, where $n + 1$ indicates the total number of the considered intervals and k and t are explained above

Equation (4) is a method that needs no assumptions regarding the degradation profile to follow a special model. Furthermore, this method requires only a pocket calculator to calculate EPD. However, with very few incubation times, the method may result in an underestimation of EPD, and also, if the potential degradability is not reached within the longest incubation time and this is combined with a low fractional outflow rate, it can result in an underestimation of EPD.

The assumption that the particle outflow from the rumen follows first-order mass action kinetics is questionable, especially for forages. Huhtanen *et al.* (1993) showed a selective retention (two-compartment model with an escapable and non-escapable pool) of particles from barley, barley fibre, rape-seed meal and soybean meal, which heavily affected calculated EPD. As the passage from the first to the second (escapable) pool is slower for forage particles than for concentrates (Huhtanen and Kukkonen, 1995; Stensig *et al.*, 1997), the assumption about simple first-order passage kinetics is probably more erroneous for forages than for concentrates. If this is true and two-compartment passage models are applied for calculation of EPD, this may result in a decrease in the protein value of forages compared with concentrates.

In vivo *validation*

In vivo estimation of rumen protein degradability requires a separation of the duodenal protein flow into undegraded feed protein (including peptides and amino acids of feed origin) and the rest, which consists of microbial and endogenous protein. Microbial protein can be estimated using different markers, but endogenous protein flow is difficult to estimate; therefore, *in vivo* estimation of the flow of undegraded feed protein is problematic. Furthermore, most concentrates and also many forages must be fed in combination with other feeds, and therefore only the flow of undegraded feed protein from the whole diet can be estimated, and not undegraded feed protein from the individual feeds. As the rumen protein degradation is a dynamic process, the effective protein degradation is highly dependent on the passage rate, as discussed above, and will therefore depend on, for example, feeding level. When validating nylon-bag data, it is therefore important also to judge the passage rate, used for calculation of the effective degradability, based on nylon-bag data in relation to the expected passage rate at the feeding level in the *in vivo* experiments used for validation.

Due to these difficulties only a few *in vivo* validations of the nylon-bag method have been performed. Madsen and Hvelplund (1985) validated the nylon-bag method on *in vivo* data, where microbial synthesis was estimated using diaminopimelic acid (DAPA) as a marker and endogenous non-ammonia nitrogen (NAN) was estimated as 0.27% of duodenal DM flow. Values for individual feeds were obtained using the difference approach and correcting for extramicrobial and endogenous protein when additional feeds (protein-rich concentrates, beet pulp and grass pellets) were added to a basal ration. The results are shown in Fig. 12.1. Figure 12.1 shows a reasonable general relationship between *in vivo* and nylon-bag data, but also reveals that there are severe differences between the two methods for some of the samples.

Vanzant *et al.* (1996) compared *in situ* with *in vivo* degradability for lucerne (alfalfa) and prairie hay, as shown in Table 12.1. Table 12.1 shows a reasonable agreement between *in vivo* and *in situ* protein degradability. However, with three different assumptions for endogenous N supply to the duodenal N flow, Vanzant *et al.* (1996) calculated *in vivo* lucerne protein degradabilities from 79 to 90% and prairie-hay protein degradabilities from 41 to 72%. These large variabilities in *in*

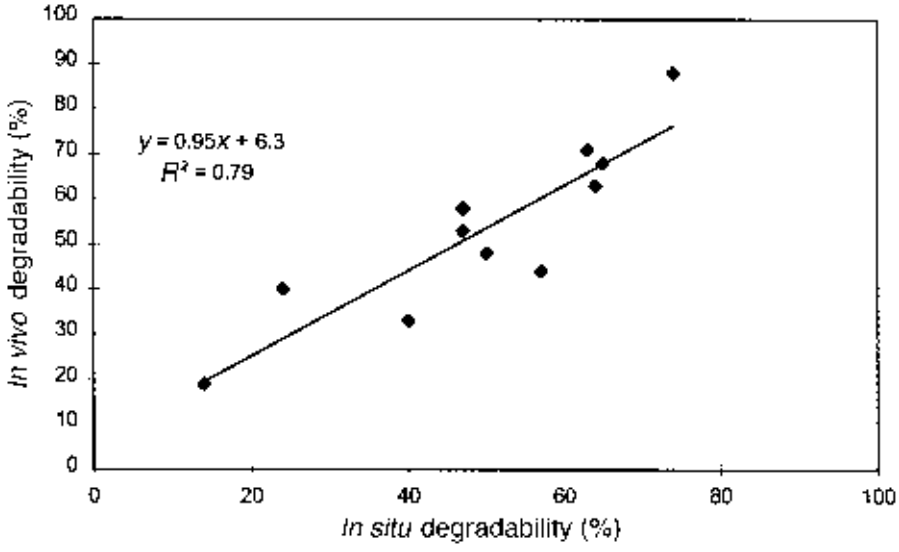

Fig. 12.1. The relation between *in vivo* and *in situ* protein degradability in protein-rich concentrates, beet pulp and grass pellets. *In situ* degradability is calculated using a fractional outflow rate of 8% h^{-1} (redrawn from Madsen and Hvelplund, 1985).

vivo estimates depending on endogenous flow estimates clearly show the problems faced in estimating *in vivo* degradability, and these problems will increase in severity with decreasing proportion of undegraded feed protein in the duodenal flow.

Compared with the extensive use of nylon-bag data, the validation on *in vivo* values has been very scarce and dubious, due to the lack of proper estimates of duodenal flow of endogenous N.

Loss of small particles from the bags

As a result of milling the samples, small particles are produced that can escape through the pores in the bags without any further degradation. According to equations (1), (3) and (4), this loss will be regarded as soluble and immediately degraded, which is probably not true.

Experience from our laboratory with concentrate feeds showed that effective protein degradabilities measured with the *in situ* method were not additive. Compounded feedstuffs often gave higher EPD values than expected from the EPD values obtained with the ingredients. The lack of additivity could be explained by a higher particle loss from the bags for the compounded feeds compared with the ingredients. This is probably due to extra milling and particle-size reduction during the mixing and pelleting process.

The extent of small-particle loss can be estimated as the difference between the loss from the nylon bags when they are only washed and the solubility measured on filter-paper. The method used to measure water solubility in our laboratory

Table 12.1. Comparison of *in vivo* with *in situ* effective protein degradability (%) (from Vanzant *et al.,* 1996).

	Lucerne	Prairie hay
In vivo	83.4	55.5
*In situ**	91.5	58.3
In situ†	87.2	57.2

* Incubated in the same cows as used for *in vivo* data.
† Incubated in steers fed brome hay *ad libitum*.

is as follows: 0.5 g of sample (same milling as in *in situ* study) is weighed out in a beaker, 40 ml tap water is added and the sample is soaked for 1 h at room temperature (≈ 20°C). Then the material is transferred to an N-free filter-paper with retention value 2 and washed four times with 40 ml of water. The residual N is determined and the water-soluble N is calculated as the difference between the original amount of N and the N in the residue.

Assuming the particle loss is degraded similarly to the particles remaining in the bag, corrections can be made for the loss. Individual degradability values, *a*, *b* and *c* values and values for EPD can be corrected.

Individual degradability values (Weisbjerg *et al.*, 1990):

$$DEG_{cor}(t_i) = DEG(t_i) - P[1 - ((DEG(t_i) - (P + SOL))/(1 - (P + SOL)))] \quad (5)$$

a, *b* and *c* values (Weisbjerg *et al.*, 1990):

$$a_{cor} = a - P \quad (6)$$

$$b_{cor} = b + P[b/(1 - (P + SOL))] \quad (7)$$

$$c_{cor} = c \text{ (as this is the assumption)} \quad (8)$$

EPD values (Madsen *et al.*, 1995):

$$EPD_{cor} = SOL + [((1 - SOL)/(1 - (P + SOL))) \times (EPD - (P + SOL))] \quad (9)$$

where:

$DEG_{cor}(t_i)$ = corrected degradability at incubation time t_i
$DEG(t_i)$ = measured degradability at incubation time t_i
P = loss of small particles
SOL = water solubility
EPD_{cor} = corrected effective protein degradation
EPD = measured effective protein degradation

The effect of correction for particle loss on the degradation profile is shown in Fig. 12.2. The *a* value (intercept) will be reduced by a value that corresponds to the estimated particle loss and, with increasing incubation time, the corrected curve will approach the measured curve, as shown in Fig. 12.2. The particle loss can be large, as shown in Table 12.2. The assumption, used in equations (5)–(9), that the

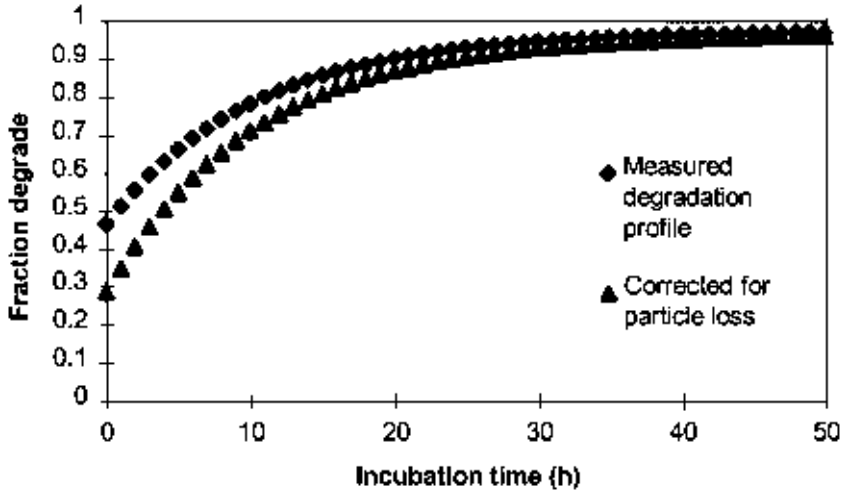

Fig. 12.2. Degradation profile for protein in ryegrass (values from Table 12.4).

particle loss would be degraded similarly to the fraction remaining in the bag after washing is certainly not true for some feeds, e.g. oat grain, where the particle loss is very high (from 42 to 67% of total N in our laboratory) and the remaining protein is, to a large extent, hull-associated protein. In such situations, correction for particle loss will result in severe underestimation of EPD, as the assumption used for correction will not be true.

As shown in Table 12.4, whole crop also gives large particle losses and, as whole crop is also a feed with a heterogeneous composition (straw and grain), similar problems to those with oat grain can be expected. In our laboratory, we examined how correction for particle loss affected whole crop of barley, field beans, peas and two samples of wheat in an experiment, where water solubility and *in situ* degradability were determined both on the whole crop and on samples that had been split up into the straw part and grain part. The results are shown in Table 12.2. It is obvious from Table 12.2 that the particle loss from whole crop is higher for the grain fraction than for the straw fraction. However, except for barley whole crop, the corrected EPD (EPD II) values obtained for the whole crop correspond reasonably well to the corrected EPD calculated from the grain and straw fraction. Therefore, based on these results, corrections for particle loss seem reasonable also for whole-crop cereals.

Reproducibility

One of the major problems with the *in situ* method is the poor reproducibility. In a ring test with participation from 23 laboratories representing 17 different countries, very poor reproducibility was found (Madsen and Hvelplund, 1994). For soybean meal, cottonseed meal, coconut meal, fish-meal and barley, effective degradabilities

Table 12.2. The effect of correction for particle loss on whole-crop cereals and parts of whole-crop cereals.

	Proportion of N in grain and straw	% N in DM	EPD I*	EPD II†	N lost with particles‡
Barley					
Grain	0.380	1.44	86.5	75.4	42.1
Straw	0.620	1.18	68.6	59.5	19.6
Whole crop		1.18	73.3	58.5	32.0
Calculated§		1.28	75.4	65.5	28.2
Wheat sample 1					
Grain	0.314	1.72	87.1	78.6	40.4
Straw	0.686	1.08	62.2	45.6	25.7
Whole crop		1.30	71.8	55.6	29.0
Calculated§		1.28	70.0	56.0	30.3
Wheat sample 2					
Grain	0.584	1.55	92.9	83.9	45.1
Straw	0.416	0.71	69.7	51.5	24.2
Whole crop		1.04	83.5	68.0	31.5
Calculated§		1.20	83.2	70.4	36.4
Field bean					
Grain	0.123	3.56	80.1	62.9	40.5
Straw	0.877	2.72	79.5	69.0	21.6
Whole crop		2.76	80.1	69.5	23.7
Calculated§		2.82	79.6	68.2	23.9
Peas					
Grain	0.288	4.06	91.3	85.7	38.1
Straw	0.712	3.15	74.0	69.0	17.2
Whole crop		3.41	76.3	69.5	22.1
Calculated§		3.41	79.0	73.8	23.2

* Effective protein degradability calculated at 8% passage rate without correction for particle loss.
† Effective protein degradability calculated at 5% passage rate with correction for particle loss.
‡ % of total N.
§ Values for grain and straw are weighted according to the proportion they make up of whole-crop N.

(mean ± standard deviation) of 63% ± 11; 51% ± 9; 47% ± 8; 23% ± 6 and 72% ± 8, respectively, were found. Standard deviations of this magnitude means that the obtained values cannot be considered as absolute values and that one should be very careful when values from different laboratories are mixed. However, the ranking of the feeds within the laboratory was reasonably consistent.

In a ring test between the Nordic countries (unpublished), where standardization of the *in situ* method has been attempted for many years, the reproducibility was better for barley, rape-seed meal and artificial dried grass (mean ± standard deviation) – 68% ± 7; 64% ± 4 and 42% ± 4, respectively – but these differences between laboratories were still too large if values were to be adopted from other laboratories.

A major part of the difference between laboratories seems to be due to differences in the washing procedure (Madsen and Hvelplund, 1994), and the difference between laboratories can be extremely high even with a high degree of standardization, as shown in a Nordic ring test. Some of the poor reproducibility can be explained by the repeatability. Data from our laboratory showed the following means and standard deviations for EPD when measurements were repeated five times for barley, rape-seed meal and artificial dried grass: 65% ± 3, 64% ± 3 and 45% ± 2, respectively. The variation within the laboratory is mainly due to variation between periods and, to a smaller degree, variation between cows and between bags within cow and period (van der Koelen *et al.*, 1992). Especially the N disappearance after just washing showed a substantial variation between periods, supporting the theory that variation in disappearance after washing (0 h incubation) is one of the major sources for variation in EPD within and between laboratories.

Microbial contamination

The principle in the *in situ* method is that microbes should enter the bag and degrade the feed in a similar way to that in the rumen. Therefore, the samples in the bag will be colonized by microbes. The normal washing procedure after incubation removes degraded feed and a large part of the microbes, but some microbes will be so attached to the residues that they will not be removed. This contamination of the residues with microbes will have only a minor influence on DM degradation, but, due to the high protein content in microbes, protein degradation can be underestimated, especially in high-fibre and low-protein feeds (Varvikko and Lindberg, 1985). As many forages are high in fibre and some are low in protein, microbial contamination of the residues can severely affect the EPD estimation of forages.

Different methods have been used to overcome this problem. Chilling, sonication and stomaching have all been used for removal of bacteria, and stomaching has been shown to be the most effective method (Michalet-Doreau and Ould-Bah, 1992). T. Hvelplund and J. Lindberg (unpublished) compared protein degradability estimated with four different methods using ^{15}N-labelled feeds. The four estimates were true degradability obtained as ^{15}N degradability, degradability based on residual N after machine washing, degradability based on residual N after the residues had been treated in a stomacher and degradability based on residual N after NDF boiling of the residues, as shown in Table 12.3.

It can be seen from Table 12.3 that machine washing as the only treatment resulted in an underestimation of the protein degradability, and the underestimation became more pronounced with increasing maturity of the crop. The use of the stomacher resulted in an overestimation of rumen protein degradability for feeds like whole-crop cereals and underestimation for feeds like straw and grass. These results indicate that the stomaching process does not remove all microbes, but increases the particle loss in feeds like whole crops. Therefore, the stomaching method is not perfect, but is clearly superior to no treatment.

In our laboratory, we have used the stomacher (individual residues are treated in the stomacher for 5 min) for all forage samples for the last 10 years. However,

Table 12.3. Effective protein degradability in different crops estimated with the *in situ* technique using four different methods (N = normal procedure, S = treatment of the residue in a stomacher, ^{15}N = ^{15}N degradability, NDF-N = N residue in the bag estimated as NDF-N) and calculated according to Kristensen *et al.* (1982), using a passage rate of 8% h^{-1} (from T. Hvelplund and J. Lindberg, unpublished).

Feed	Phase of vegetation (days after germination)	Stage of maturity	N	S	^{15}N	NDF-N
Pea whole crop	56	Full blooming	0.79	0.80	0.78	0.93
Pea straw I	77	Start of filling	0.72	0.77	0.75	0.86
Pea straw II	90	Filled pea shell	0.61	0.72	0.68	0.83
Pea straw III	104	Matured	0.48	0.56	0.50	0.72
Barley whole crop	56	Start of heading	0.43	0.60	0.53	0.72
Barley straw I	77	Milky stage	0.20	0.40	0.47	0.66
Barley straw II	90	Yellow stage	−0.12	0.20	0.33	0.54
Barley straw III	104	Matured	−0.19	0.14	0.31	0.52
Mixture A*	104	Matured	0.66	0.75	0.66	0.90
Mixture B*	104	Matured	0.45	0.50	0.48	0.77
Barley straw IV		Matured	−0.01	0.13	0.48	0.63
Ryegrass hay	51	4–5 leaves	0.65	0.66	0.70	0.84

* Composition of mixture: barley straw + pea straw + pea seeds; A, original; B, NaOH-treated.
NaOH, sodium hydroxide.

use of the stomacher is a very time-consuming process. Therefore, Michalet-Doreau and Ould-Bah (1989) derived equations (equations 10 and 11) that make it possible to correct EPD calculated at 6% h^{-1} passage rate for the microbial contamination from information about the crude-protein content of the feed or, better, information about both crude-protein and NDF content:

$$\Delta EPD = 20.2 - 0.674(\%\ \text{crude protein in DM}) \quad (10)$$

$$\Delta EPD = 6.4 - 0.353(\%\ \text{crude protein in DM}) + 0.170(\%\ \text{NDF in DM}) \quad (11)$$

where ΔEPD is the value which should be added to the measured (uncorrected) EPD.

Escape of soluble protein

According to equations (3) and (4), soluble protein is assumed to be immediately degraded in the rumen. For forages like silage, with a protein solubility as high as 84%, as shown for wheat whole-crop silage in Table 12.4, the fraction is close to the estimated EPD, even if corrections are made for particle loss. The fractional outflow rate of fluid from the rumen is high (≈ 12–15% h^{-1}), compared with particle outflow, which means that the degradation rate of the soluble protein should be very high if no soluble proteins are to escape, and this indicates that the present method results in an overestimation of rumen protein degradability for feeds high in soluble protein. Aaes and Kristensen (1997) measured fluid passage rate and the

Table 12.4. Degradation characteristics for different forages, measured *in situ*.

		% N in DM		*a*		*b*		*c*			% N lost with particles‡	
Feed	OBS*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	EPD†	Mean	SD
Beets												
Fodder beets	10	1.14	0.12	0.759	0.023	0.198	0.037	0.080	0.035	0.88	11.5	2.6
Grass												
Clover grass	78	3.38	0.79	0.428	0.069	0.543	0.073	0.115	0.022	0.81	18.2	4.0
Italian ryegrass	1	2.83	–	0.466	–	0.497	–	0.129	–	0.82	24.0	–
Cocksfoot	21	2.80	0.84	0.446	0.116	0.539	0.144	0.095	0.034	0.80	15.9	4.5
Ryegrass	19	3.37	0.92	0.467	0.088	0.509	0.090	0.098	0.036	0.80	18.0	4.3
Legumes												
Lucerne	22	3.27	0.46	0.577	0.064	0.362	0.058	0.103	0.028	0.82	–	–
Galega	4	2.83	0.13	0.355	0.029	0.577	0.047	0.074	0.019	0.70	16.4	2.8
White clover	4	4.20	0.81	0.582	0.094	0.408	0.589	0.066	0.025	0.81	–	–
Red clover	11	3.54	0.73	0.272	0.150	0.692	0.149	0.099	0.018	0.73	13.7	3.4
Field bean	3	2.68	0.28	0.653	0.107	0.263	0.118	0.082	0.015	0.82	28.1	4.5
Vetches	1	3.40	–	0.610	–	0.322	–	0.168	–	0.86	15.1	–
Peas	18	3.11	0.33	0.671	0.080	0.248	0.058	0.070	0.028	0.82	28.3	14.2
Whole crop												
Barley	11	1.34	0.11	0.564	0.121	0.369	0.235	0.074	0.073	0.78	24.6	6.6
Oat	1	1.16	–	0.691	–	0.173	–	0.195	–	0.83	47.4	–
Triticale	12	1.53	0.59	0.555	0.080	0.310	0.110	0.145	0.093	0.79	16.5	5.7
Wheat	49	1.32	0.30	0.629	0.083	0.257	0.085	0.079	0.103	0.79	26.3	10.1
Rye	18	1.71	0.69	0.637	0.077	0.256	0.098	0.138	0.076	0.82	26.4	5.3
Grass silage												
Clover grass	38	2.61	0.65	0.758	0.057	0.191	0.053	0.097	0.025	0.88	9.8	3.6
Cocksfoot	3	2.87	0.72	0.648	0.051	0.293	0.024	0.100	0.016	0.84	12.8	1.8
Ryegrass	2	2.12	0.06	0.749	0.010	0.200	0.016	0.095	0.008	0.88	19.8	1.6
Legume silage												
Field beans	2	2.78	0.11	0.633	0.019	0.298	0.012	0.082	0.013	0.82	29.9	2.8
Vetches	4	3.05	0.60	0.723	0.042	0.189	0.058	0.093	0.014	0.85	–	–
Peas	5	2.85	0.35	0.795	0.120	0.157	0.105	0.107	0.032	0.90	16.9	4.1
Whole crop silage												
Barley	10	1.72	0.15	0.823	0.066	0.127	0.061	0.025	0.015	0.87	19.7	2.4
Wheat	28	1.39	0.25	0.839	0.057	0.075	0.040	0.081	0.120	0.89	19.8	6.0
Maize	5	1.71	0.13	0.798	0.020	0.147	0.042	0.019	0.006	0.84	28.3	5.2
Hay												
Clover grass	5	2.32	0.37	0.400	0.097	0.554	0.098	0.059	0.016	0.70	10.1	0.4

* Number of observations.
† EPD calculated from the mean *a*, *b* and *c* values, using a passage rate of 5% h^{-1}.
‡ % of total N. Number of observations for particle loss are in most cases less than the number of observations used for the calculation of degradation characteristics. Samples were milled through a 1.5 mm screen.
SD, standard deviation.

content of soluble amino acids in rumen fluid of cows fed either fresh clover, fresh ryegrass or grass silage and found, especially for grass silage, a significant contribution of rumen-soluble amino acid N to the duodenal amino acid N flow. As the content of soluble amino acid N was highest for silage and the DAPA content in

the soluble amino acid N fraction was very low, this indicates that most of the soluble amino acid N was originating from the feed and not from endogenous or microbial origin. This is in agreement with several studies that show that the degradation rate of soluble proteins and amino acids in the rumen is far from infinite (Mangan, 1972; Volden *et al.*, 1998). This implies that proper estimation of rumen protein degradation, at least for feeds with a high proportion of the N in soluble amino acid N, as in silage, will necessitate that the soluble *a* fraction is weighted, with the ratio between rate of degradation of soluble protein ($k_{d-fluid}$) and the rate of passage of fluid ($k_{p-fluid}$) as shown in equation (12):

$$EPD = [a(k_{d-fluid}/(k_{d-fluid} + k_{p-fluid})] + [b(c/(c + k))] \quad (12)$$

where EPD, *a*, *b*, *c* and *k* are as explained under equation (3).

Fluid passage rate has been determined in many experiments and it would be easy to find appropriate values for calculation of EPD. Rate of degradation of soluble proteins has rarely been determined, as it is difficult to trace the soluble proteins, as some end up in ammonia and some in microbial protein. Mangan (1972) estimated the half-life of soluble proteins. For casein it was 20 min and for ovalbumin the half-life was 180 min. Assuming first-order kinetics, this results in rates of degradation of 210% h^{-1} and 23% h^{-1} for casein and ovalbumin, respectively, and assuming a fluid passage rate of 13% h^{-1} this will result in rumen degradabilities of 94 and 64%, respectively. Peltekova and Broderick (1996) estimated *in vivo* degradation rates for casein (48.6% h^{-1}) and for soluble proteins from lucerne hay and lucerne silage (27.3 and 28.5% h^{-1}, respectively), but the proportions of soluble true protein were small in both lucerne hay and silage. However, inclusion of the soluble fraction in the potentially escapable pool also raises the question as to how far the protein should be degraded to be regarded as degraded. From a nutritional point of view, it is the amino acid supply of feed origin to the intestine that is interesting, and amino acids will be lost from the feed amino acid pool when they are either degraded to ammonia or used for microbial amino acid synthesis.

Values for forage protein degradation

In Table 12.4, values for protein degradation obtained in our laboratory for forages are shown. Large differences have been observed between feeds, but the most striking difference is the difference between fresh and ensiled feeds. The ensiling process results in a large increase in the water-soluble fraction of the protein, and thereby in the *a* value. The *b* value is reduced similarly to the increase in *a*, but the changes result in increased EPD values. The values in Table 12.4 show EPD values for silage of clover plus grass, ryegrass, pea whole crop, barley whole crop, and wheat whole crop, which are 7 to 10 percentage-units higher than for fresh feeds.

Crude-protein versus amino acid degradation

The measured EPD values are normally used directly for prediction of amino acid protein degradability, although they are based on crude protein (total N × 6.25)

degradability. In forages, non-amino acid N generally makes up a bigger part of total N than in concentrates, which can make it more problematic to use total N values as predictors for amino acid N. In a study with 14 different concentrate ingredients, Weisbjerg *et al.* (1996) found, as a mean, no differences in crude protein and amino acid protein effective degradability, although small differences were found for some of the feeds. However, water solubility was considerably higher for crude protein than for amino acid protein. In a study with eight fresh and ensiled forages, Skiba *et al.* (1996) found a higher solubility/degradability at short incubation times for crude protein compared with amino acid protein, a difference which was so pronounced that EPD values were also considerably higher for crude protein compared with amino acid protein, as shown in Table 12.5.

The lower degradability of amino acids compared with crude protein is in accordance with results obtained by Susmel *et al.* (1989) for ensiled lucerne. These results indicate that, for forages, the use of crude-protein degradabilities results in an overestimation of amino acid protein degradation. However, in most protein evaluation systems, this is probably more than counteracted in an overestimated assumption with respect to the amino acid N proportion of total N in forages, especially in silages.

Intestinal Digestibility of Undegraded Feed Protein

Estimates of the true digestibility of protein and amino acids in the small intestine, from experiments in which undegraded protein was infused into the intestine of ruminants, are scarce. Applying regression techniques, Tas *et al.* (1981) reported a value of 0.82 for the true digestibility of undegraded amino acids passing to the duodenum of sheep fed a diet of grass and concentrates. Hvelplund (1985)

Table 12.5. Effective rumen degradability of crude protein (N) and total amino acids (AA) from different roughages determined *in situ* (from Skiba *et al.,* 1996).

	Effective degradability*		Effective degradability,* corrected for particle loss	
	N	AA	N	AA
Clover grass silage	0.885	0.858	0.818	0.792
Clover grass I	0.769	0.739	0.597	0.581
Ryegrass	0.762	0.722	0.648	0.606
Clover grass II	0.763	0.722	0.631	0.614
Winter wheat whole-crop silage	0.899	0.858	0.795	0.712
Pea whole-crop silage	0.857	0.838	0.702	0.635
Artificial dried grass	0.597	0.502	0.506	0.414
Galega	0.700	0.663	0.568	0.523

* 5% passage rate.

measured the increments in protein and amino acids passing the terminal ileum when different proteins, which had previously been exposed to some degradation in the rumen, were infused into the abomasum of sheep. This experiment showed that the protein and amino acids in different undegraded proteins were digested to a variable degree in the small intestine. Thus, protein and amino acid digestibility of undegraded feed protein varies, especially if the protein sources are treated to protect the protein against degradation. Protection may influence digestibility negatively if the protection is overdone, as shown by Kaufmann and Lüpping (1982) and Hvelplund (1985). Because of the variable digestibility of undegraded feed protein, there is a need to assess the digestibility of undegraded dietary protein for all types of feeds. However, *in vivo* experiments and the use of infusion techniques for the estimation of postruminal digestion of undegraded dietary protein can only be applied to a limited extent, as they are costly and time-consuming. Therefore, alternative methods have been evaluated.

The mobile nylon-bag method

The mobile-bag method for estimation of intestinal protein digestibility was introduced for pigs by Sauer *et al.* (1983). Since then, the method has also been used to study intestinal digestibility in ruminants and has been introduced in many different laboratories (Hvelplund, 1985; Rae and Smithard, 1985; Rooke, 1985; Voigt *et al.*, 1985; DeBoer *et al.*, 1987). Intestinal digestibility, using the mobile-bag technique, is obtained as the disappearance of protein from the bags during passage through the intestine. Many factors may influence the disappearance using this procedure to predict digestibility, including bag-cloth characteristics, pepsin–hydrochloric acid (HCl) pretreatment of the sample and place of recovery.

Pore size of the bag cloth

An appropriate pore size of bag cloth should allow free inflow of digestive enzymes into the bag and free outflow from the bags of nutrients originating from digested feed, without losing feed particles that have not been degraded. Pore sizes of bag material ranging from 9 to 53 μm have been reported (Vanhatalo, 1995) and comparative data from different sources indicate that the difference in the disappearance obtained due to bag pore size tends to be rather small and thus of minor importance. However, the pore size of the bags is a compromise between an efficient flushing of the bags with intestinal contents during the passage through the intestine and avoiding losses of undegraded feed particles from the bags. Based on this, a bag pore size of approximately 10–15 μm can be recommended and seems to be a good compromise with respect to the points mentioned above, provided, however, that the free surface area of the bag cloth is large enough (> 5%), as pointed out by Vanhatalo (1995).

Pepsin–HCl treatment

Exposure of feed residues to pepsin–HCl treatment is a normal process during passage through the abomasum. In the mobile-bag procedure, however, pretreatment with pepsin-HCl is not always included, as the necessity for this is doubted, mainly because there are acidic conditions in the proximal part of the ruminant small intestine. This extends the activity of pepsin also to the section where the mobile bags are inserted. In some situations, the influence of pretreatment is very pronounced, as observed for formaldehyde-treated soybean meal, where the pH in the abomasal content used for the pretreatment varied from 2.21 to 3.22, which resulted in an intestinal disappearance varying from 0.75 to 0.54 (Hvelplund, 1985). As a consequence, pretreatment with pepsin–HCl at a pH of 2.4 is recommended, as this pH value was found to represent the average conditions in the abomasum of cattle, based on measurements of abomasal content from a donor cow and values obtained from the literature (Hvelplund, 1985).

Place of recovery: ileum vs. faeces

In the modern protein evaluation systems, the small-intestinal digestibility is an important parameter. This implies that the digestibility between duodenum and ileum should be measured. However, recovery of bags from the ileum cannulae is difficult, compared with recovery from the faeces. Place of recovery may influence the estimated disappearance of protein from the mobile bag due to the activity in the large intestine. An increase in disappearance from ileum to faeces is obtained if further breakdown of protein takes place in the hind-gut. If available substrate for microbial digestion is present in the bag residue, the possibility for microbial growth also exists and, if the microbes are strongly adhering to the residues, this will lead to an increase in protein content and, thereby, decreased protein disappearance during passage through the hind-gut. Comparative studies, reviewed by Vanhatalo (1995), have shown that collecting bags from faeces instead of from the ileum usually only causes a slight increase in protein disappearance from the bags with plant-based concentrates, nearly no impact on protein feeds of animal origin and a variable response on different forages, which is reflected in the amount of substrate left in the bags for microbial fermentation.

In a study including different forages labelled with ^{15}N, the influence of place of recovery on both true (^{15}N) and apparent (Kjeldahl-N) disappearance was estimated (Jarosz *et al.*, 1994). The results from this study (Table 12.6) showed that the disappearance of Kjeldahl N underestimated true N disappearance at both recovery sites. Kjeldahl-N disappearance measured using bags recovered from the faeces generally agreed with estimates of ^{15}N disappearance measured using bags recovered from the ileum, except for very mature high-fibre feeds. This leads to the conclusion that bags recovered from the faeces can be used to predict true small-intestinal disappearance.

Table 12.6. Disappearance of Kjeldahl nitrogen (N_K) and ^{15}N from mobile bags recovered at the terminal ileum (TI) or from the faeces (F), and statistical significance of place of recovery on disappearance, as well as significance between apparent (N_K) and true (^{15}N) nitrogen digestibility. Each value is the mean of three observations (from Jarosz *et al.*, 1994).

	Disappearance				Test probability (*P*)			
	N_K		^{15}N		TI vs. F		N_K vs. ^{15}N	
Feedstuffs*	TI (g kg^{-1})	F (g kg^{-1})	TI (g kg^{-1})	F (g kg^{-1})	N_K	^{15}N	TI	F
Pea whole crop	946	970	955	978	0.06	0.008	0.2	0.4
Pea straw I	911	936	913	950	0.003	0.001	0.7	0.02
Pea straw II	833	870	858	895	0.02	0.5	0.02	0.02
Pea straw III	636	641	804	846	0.8	0.03	0.0003	0.0001
Barley whole crop	885	896	930	949	0.2	0.06	0.004	0.001
Barley straw II	669	626	735	765	0.3	0.2	0.01	0.02
Barley straw III	565	470	646	677	0.003	0.4	0.04	0.0003
Mixture A†	730	775	809	859	0.009	0.02	0.003	0.001
Mixture B†	907	917	941	957	0.3	0.1	0.03	0.002
Ryegrass hay	852	873	908	922	0.2	0.06	0.04	0.002

* For stage of maturity of the crop, see Table 12.3.
† Composition of mixture: barley straw + pea straw + pea seeds; A, original; B, NaOH-treated.
NaOH, sodium hydroxide.

Description of the mobile-bag procedure

No attempts have been made to standardize the mobile-bag procedure between laboratories and, to our knowledge, no ring test has been performed. The procedure mentioned below is the one that is used at Research Centre Foulum to estimate total digestibility of protein, from which the digestibility of rumen-undegraded protein can be calculated.

Bag specification and sample preparation

Nylon material with a pore size of 11 μm and with a 6% open bag area is used for preparation of bags measuring 6 cm × 6 cm. The feed is milled through a 1.5 mm screen before weighing. Sample size is *c.* 1 g of material per bag. If the feed is bulky (e.g. some roughages), the sample size is reduced to 0.8 g. The bags are closed by heat-sealing.

Incubation procedure

For each feed, six bags are prepared. The bags are incubated in the rumen for 16 h, with two bags in each of three cows. Incubation for 16 h is used for all feeds at present, as this incubation time is believed to reflect the influence of rumen metabolism on the feed before passage to the intestine, but this needs further clarification. After incubation, the bags are washed in a washing-machine, using cold water (2 × 22 l). After this, the bags are soaked for 1 h in a 0.004 mol l^{-1} HCl solution at pH 2.4. Then, the bags are incubated for 2 h in a pepsin–HCl solution at a pH of 2.4 (100 mg pepsin 1:10,000 l^{-1} of 0.004 mol l^{-1} HCl solution) in a shaking waterbath at 40°C. The bags are then inserted into the small intestine through the duodenal cannula in three cows, with two bags per feed in each cow. A maximum of 12 bags are introduced into the duodenum per cow per day. After passage through the intestine, the bags are recovered from the faeces and washed in a washing-machine, using cold water (2 × 22 l), and, finally, the residues are quantitatively transferred to N-free filter-paper and analysed for nitrogen.

In vivo *validation: relation between disappearance from bags and true digestibility*

Disappearance of protein from the mobile bag during passage through the intestine is assumed to be related to the true digestibility of protein in the intestine, and this assumption has been tested in a limited number of experiments, where the true digestibility was estimated in infusion experiments or with the ^{15}N dilution method (Hvelplund, 1985; Varvikko and Vanhatalo, 1990; Todorov and Girginov, 1991; Hvelplund *et al.*, 1994; Vanhatalo and Varvikko, 1995). Good agreement was obtained in the experiment by Todorov and Girginov (1991) and, as illustrated in Fig. 12.3, in the experiment by Hvelplund *et al.* (1994), whereas the agreement was less satisfactory for the other experiments. The reason for this may be ascribed to different factors with respect to the bag method used and the method used for obtaining *in vivo* values as discussed by Vanhatalo (1995). Further research is needed to clarify the relationship between disappearance from the bags and true intestinal digestibility, but, until this has been tested further on a wide range of

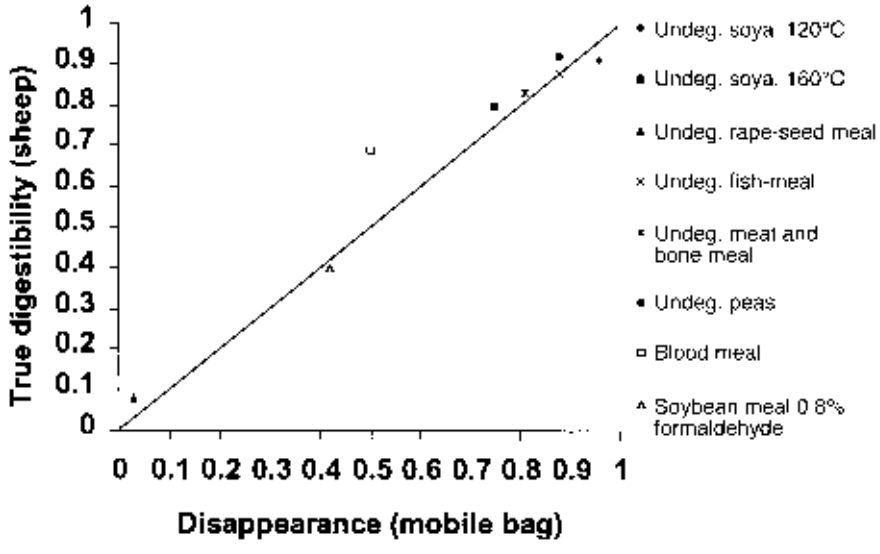

Fig. 12.3. The relationship between the disappearance of protein from the nylon bags and the true digestibility of protein in the small intestine of sheep. —, $x = y$ (from Hvelplund *et al.,* 1994).

feeds, it is reasonable to treat the results from the mobile bag as direct estimates of the true intestinal digestibility.

Practical use of mobile-bag results

The value needed in the protein evaluation systems is the digestibility of rumen-undegraded feed protein at the actual rumen degradability. This digestibility can be predicted from total digestibility of the original feed protein and information on the actual rumen degradability according to equation (13) (Hvelplund *et al.*, 1992), as illustrated in Fig. 12.4:

$$TD = (UDN - TU)/UDN \quad (13)$$

where:

TD = true digestibility in the small intestine of undegraded feed protein
UDN = fraction of undegraded dietary protein
TU = fraction of true indigestible protein in the feed

The equation assumes that the fraction of true digestible protein in the feed, which is estimated from experiments with the mobile bag, is equivalent to maximum rumen degradability. The equation has been found valid for a variety of different concentrates and roughages, where total digestibility has been estimated as the disappearance from mobile bags passing through the intestine. The practical implication

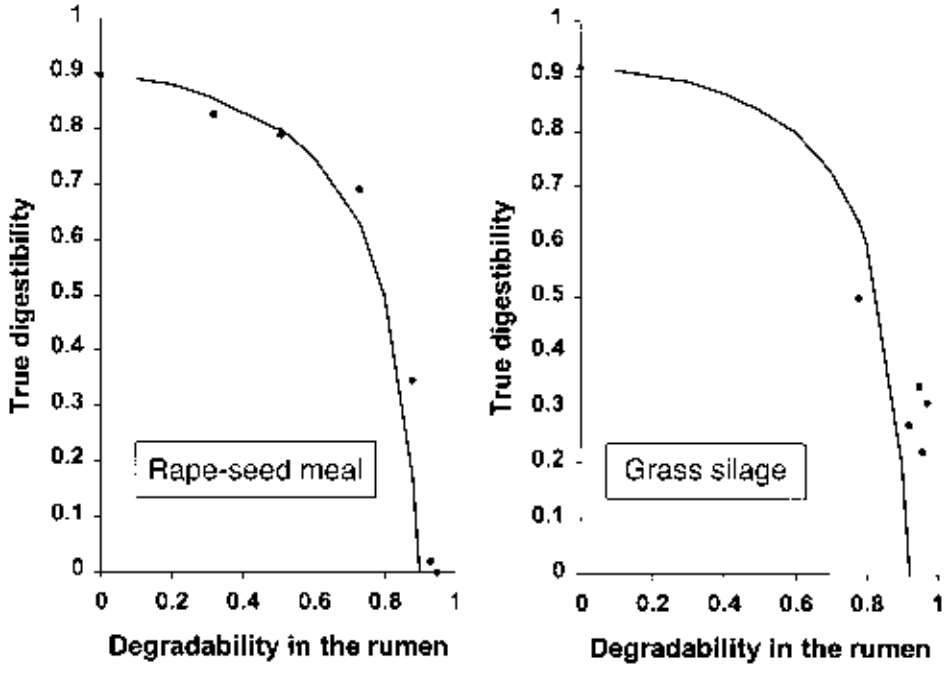

Fig. 12.4. The relationship between intestinal digestibility of undegraded feed protein and rumen protein degradability on two different feeds. ▲ True digestibility of intact feed protein used to estimate the digestibility (curve) from equation 13. ● True digestibility observed on samples of undegraded dietary protein (from Hvelplund *et al.,* 1992).

of this is that the intestinal digestibility of undegraded dietary protein cannot be considered as a constant value for a particular feed, but will vary according to the actual degradability in the rumen. If rumen degradability is assumed to vary according to feeding level, as recommended in the British protein evaluation system (AFRC, 1992), this also means that the same feed will have different intestinal digestibility of the undegraded dietary protein. This is illustrated for different roughages in Table 12.7, where the intestinal digestibility of undegraded dietary protein is calculated at two different rumen degradabilities, based on a passage rate of 2% h^{-1} or 5% h^{-1}.

The assumption that total digestibility can be estimated as small-intestinal disappearance from the mobile bags can be questioned. Mgheni *et al.* (1994) showed that, for tropical roughages, the potential degradability in the rumen was substantially higher than the digestibility of original protein in the small intestine measured with the mobile-bag technique. This is probably due to the content of tannins and to a high proportion of cell-wall-bound nitrogen in tropical roughages, which cannot be solubilized by intestinal enzymes but may be digested in the rumen, due to the combined action of proteolytic and cellulolytic microbial enzymes. This is also true for temperate roughages at a late stage of maturity, as

Table 12.7. Total digestibility of original protein (TDIG), degradability calculated at a passage rate of 2 or 5% h^{-1} (DEG) and the digestibility of the undegraded dietary protein at these degradabilities (DIG).

		Passage rate (2% h^{-1})		Passage rate (5% h^{-1})	
	TDIG	DEG	DIG	DEG	DIG
Clover grass silage	0.937	0.922	0.192	0.885	0.452
Clover grass I	0.937	0.865	0.533	0.769	0.727
Ryegrass	0.952	0.869	0.664	0.762	0.798
Clover grass II	0.963	0.875	0.704	0.763	0.844
Winter wheat whole-crop silage	0.906	0.906	0.000	0.899	0.069
Pea whole-crop silage	0.951	0.907	0.473	0.857	0.657
Artificial dried grass	0.797	0.722	0.269	0.597	0.496
Galega	0.830	0.782	0.220	0.700	0.433

shown by Vanhatalo *et al.* (1996). Similar problems may occur with other feeds, such as grains, in which protein is protected by starch, which limits the access of intestinal enzymes (Volden and Harstad, 1995), or with some protein-rich feeds, such as rape-seed meal, with a relatively high fibre content (Vanhatalo and Varvikko, 1995). Therefore, for estimation of total digestibility, it is recommended to include preincubation of the mobile bags in the rumen and, for temperate feeds, 16 h seems reasonable.

Crude-protein versus amino acid digestibility

The mobile bag is usually used to measure crude-protein digestibility, but the values are also assumed to represent amino acid digestibilities, as amino acid digestibility is the value required in the modern protein evaluation systems. In a recent study by Weisbjerg *et al.* (1996), which included 15 different concentrates, it was shown that total amino acid digestibilities were similar to crude-protein digestibilities. Large differences in amino acid digestibilities were observed among different concentrates, with the highest for protamyl (0.98) and the lowest for cottonseed cake (0.84). With respect to individual amino acids, glutamic acid and arginine had higher and threonine and glycine lower digestibilities, compared with total amino acids, whereas the remainder of the individual amino acids were similar to total amino acid digestibility.

The mobile nylon-bag method has also been used to evaluate the digestibility of protein and amino acids in different roughages. In one study, it was found that digestibility of amino acids was higher than for protein (Skorko-Sajko *et al.*, 1994), whereas no differences were observed in the study by Skiba *et al.* (1996), as shown in Table 12.8. Both studies, however, showed that big differences exist in the digestibility of both protein and amino acids among the different roughages.

Table 12.8. Total digestibility of original protein (N), total amino acids (AA), lysine (LYS) and methionine (MET) in different forages (from Skiba *et al.*, 1996).

	N	AA	LYS	MET
Clover grass silage	0.937	0.939	0.942	0.932
Clover grass I	0.937	0.939	0.951	0.946
Ryegrass	0.952	0.944	0.961	0.939
Clover grass II	0.963	0.959	0.970	0.961
Winter wheat whole-crop silage	0.906	0.890	0.857	0.882
Pea whole-crop silage	0.951	0.946	0.953	0.927
Artificial dried grass	0.797	0.798	0.806	0.749
Galega	0.830	0.832	0.827	0.863

Values for total digestibility of original protein in forages

Table 12.9 shows values obtained in our laboratory for total digestibility of original protein in different forages. The mean digestibility varies between 0.86 and 0.94, except for straw, where the digestibility is 0.65. Within each feed, there is a considerable difference between maximum and minimum values, which is mainly a reflection of stage of maturity of the crop (mowing time), but there is also some influence of cut (first or later cuts).

Table 12.9. Total digestibility of original protein in different forages.

Feed	Number of samples	Mean digestibility	Maximum digestibility	Minimum digestibility	SD
Alfalfa	27	0.922	0.954	0.866	0.027
Clover grass	20	0.897	0.911	0.715	0.068
Ryegrass	32	0.889	0.958	0.751	0.053
Cocksfoot	20	0.884	0.941	0.753	0.044
White clover	6	0.918	0.932	0.885	0.019
Red clover	13	0.939	0.973	0.886	0.028
Lupin whole crop	15	0.934	0.967	0.908	0.014
Lupin whole-crop silage	7	0.925	0.960	0.900	0.025
Horsebean whole crop	4	0.864	0.897	0.839	0.026
Triticale whole crop	12	0.915	0.967	0.879	0.030
Barley whole crop	14	0.863	0.931	0.769	0.056
Wheat whole crop	59	0.897	0.956	0.834	0.031
Rye whole crop	32	0.931	0.966	0.884	0.027
Pea whole crop	16	0.906	0.945	0.857	0.025
Grass silage	9	0.919	0.933	0.897	0.011
Wheat straw	2	0.652	0.669	0.634	0.026
Barley straw	1	0.642	–	–	–

SD, standard deviation.

Future Developments

In situ techniques for the estimation of protein degradability in the rumen and intestinal digestibility of rumen undegraded dietary protein require access to cannulated animals. *In situ* methods are therefore not acceptable as routine methods in feed evaluation, and laboratory methods need to be developed. A lot of effort has been devoted to this field in recent years, but universal methods to predict degradability, which can be used on a broad spectrum of feeds, have not yet been developed. The reason for this can probably be ascribed to a number of factors. An *in vitro* method may give satisfactory results against the *in situ* method in one laboratory on a limited number of samples, but fail in another laboratory. The reason for this could be the low reproducibility of the *in situ* method, as shown in the European ring test (Madsen and Hvelplund, 1994). To proceed in developing, testing and implementing laboratory methods, including the *in situ* method, it is necessary to have feed samples available where 'true' *in vivo* degradability has been estimated at a standardized feeding level, as degradability is a dynamic process that is dependent on a balance between the rate of degradation and the rate of outflow from the rumen. However, it is difficult to estimate 'true' *in vivo* degradabilities, as this requires a separation of duodenal protein flow in undegraded feed protein, microbial protein and endogenous protein. Microbial protein can be estimated with reasonable precision, using different microbial markers, but further research is needed to obtain more trustworthy estimates of endogenous protein flow, if undegraded feed protein is to be estimated by difference.

The values used in protein evaluation systems are effective protein degradabilities and, to obtain these values, the nylon-bag results have to be weighted with a passage rate. The traditional approach, using first-order kinetics, seems to be insufficient to describe the passage behaviour of feed particles from the rumen, especially for forages. As the assumptions about passage are of major importance both for the actual values obtained for effective degradabilities and for the ranking of feeds, further research in passage kinetics is necessary.

Intestinal digestibility of rumen-undegraded protein has only been investigated in a few *in vivo* experiments, and the mobile bag has commonly been accepted as giving values that can be regarded as equal to estimates of *in vivo* digestibility. However, at present, the number of comparisons between *in vivo* estimates and the mobile-bag method are scarce and have not been fully conclusive, and further comparisons are necessary to accept the mobile-bag method as a general reference method.

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13 Enzymatic and Microbial-cell Preparation Techniques for Predicting Rumen Degradation and Postruminal Availability of Protein

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Need for an Alternative Technique to *In Situ*

Current feeding systems recognize the need to select feeds based on the supply of ruminal degradable and undegradable protein (Madsen, 1985; INRA, 1989; NRC, 1989; ARC, 1984). Several systems have incorporated dynamic aspects of nutrient degradation in the rumen (Sniffen *et al.*, 1992), estimates of amino acid supply (Madsen, 1985; INRA, 1989; O'Connor *et al.*, 1993) and intestinal protein digestion (Madsen, 1985; INRA, 1989; Sniffen *et al.*, 1992) into their models. Most systems have used *in vivo* and *in situ* data to develop feed tables. Determination of protein degradation in the rumen using these techniques is expensive, labour-intensive and time-consuming for industry and field application. The advantages and disadvantages of these techniques have been discussed in previous chapters. Although the *in situ* procedure has often been used as a reference method to compare degradation values, ring-test studies have shown that there is substantial variation among and within laboratories on estimates of protein degradation obtained with this technique (Madsen and Hvelplund, 1994; Wilkerson *et al.*, 1995). Most of this variation depends on diet and individual animals, and it is difficult to control. Two additional problems are encountered when using the *in situ* technique for measuring protein degradation in forages: (i) forages may have a high proportion of water-soluble materials, which the technique will erroneously consider degradable; and (ii) the effect of microbial contamination may be more important in forages, because of high fibre and low protein content. To take full practical advantage of the new feeding systems, it is necessary to develop techniques that allow fast, affordable, reliable and accurate measurements of ruminal degradation and intestinal digestion of proteins. These techniques need to be validated with true

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estimates of protein degradability and digestibility, but these measurements are difficult to obtain *in vivo* or *in situ* (Stern *et al.*, 1997). In the absence of true estimates with which to validate *in vitro* techniques, the use of values obtained with the *in situ* technique provides the best available data. However, the limitations of this technique should be carefully evaluated when using these values to validate other techniques.

Enzymes and Activities Needed

Proteins are linear chains of amino acids, linked together by peptide bonds. Protein degradation and utilization in the rumen require the action of different proteases, peptidases and deaminases. The rate and extent to which protein degradation occurs will depend on the type and concentration of enzymes and on the number of susceptible peptide bonds and their accessibility. The secondary and tertiary structure of proteins will influence the accessibility of proteases to specific peptide bonds, thereby affecting the rate and extent of ruminal protein degradation (Wallace and Kopecny, 1983). Because the number of different bonds within a single protein is large, the synergistic action of different proteases is necessary for complete protein degradation.

Several studies revealed that the predominant protease type produced by ruminal bacteria is a cysteine protease, while serine protease (0–41%), metalloprotease (9–30%) and aspartic protease (2–15%) are generally present at lower levels (Brock *et al.*, 1982; Kopecny and Wallace, 1982; Prins *et al.*, 1983). Several authors (Brock *et al.*, 1982; Prins *et al.*, 1983; Wallace and Kopecny, 1983) reported that trypsin-like activity was the predominant proteolytic activity in the rumen. Lower chymotrypsin-like activity has also been identified. Peptide breakdown is accomplished mainly by the activity of a dipeptidyl peptidase, while leucine and alanine aminopeptidase and carboxypeptidase have been found at lower concentrations (Wallace and McKain, 1991; McKain *et al.*, 1992; Depardon *et al.*, 1996). The complexity of the rumen and the difficulty of simulating its proteolytic activity suggest that the role of quantitatively unimportant microorganisms or enzymes may play a strategic role in protein degradation. For example, Wallace (1985) demonstrated that the combined activity of two ruminal bacteria resulted in a protein degradation larger than the one obtained from the sum of both activities individually (Fig. 13.1). Therefore, it appears that a combination of enzymes with the same activities and specificities as those of the rumen environment would be required to fully simulate ruminal degradation of proteins. However, the relative importance of each protease or peptidase changes with type of diet (Nuget and Mangan, 1981) and animal (Wallace and Cotta, 1988), creating further difficulties in understanding and modelling ruminal protein degradation.

In addition to the proteolytic activity needed for protein degradation, other non-proteolytic enzymes may be required. Most proteins interact with other nutrients within feeds. Assoumani *et al.* (1992) demonstrated that starch interferes with protein degradation, and the addition of amylase increased total protein degradation of cereal grains by between 6 and 20 percentage units. Debroas and Blanchart (1993) found that neutral-detergent fibre (NDF) protein was degraded by proteolytic

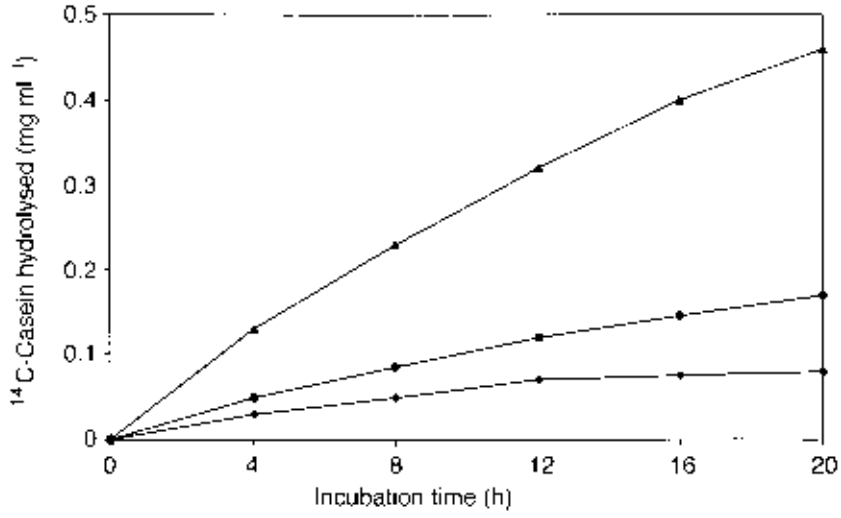

Fig. 13.1. Effect of pure cultures of *Selenomonas ruminantium* (♦), *Streptococcus bovis* (●) or a coculture of both (▲) on hydrolysis of carbon-14 (^{14}C)-casein (adapted from Wallace, 1985).

bacteria only after microbial depolymerization of cellulose began. Kohn and Allen (1995b) also reported an increase in protein degradation (from 42.4 to 53.1%) when cellulases were added to an *in vitro* proteolytic technique, and suggested that fibre interference may be particularly important for protein degradation during long-term incubation of forages. Therefore, it appears that protein degradation in the rumen requires the presence of several proteolytic and non-proteolytic enzymes. *In vitro* systems should provide these same activities to accurately simulate ruminal degradation of proteins.

Cell-free Enzymes versus Microbial-cell Preparations

Ruminal proteolysis is the result of the activity of an array of enzymes synthesized by bacteria, protozoa and fungi. Approximately 70–80% of ruminal microorganisms are attached to undigested feed particles in the rumen (Craig *et al.*, 1987), and 30–50% of these have proteolytic activity (Prins *et al.*, 1983). The role of protozoa in ruminal protein degradation is mostly related to the degradation of feed particles and bacterial proteins (Wallace and Cotta, 1988), but their overall effect on protein degradation is still controversial. The significance of the proteolytic activity of fungi has not been clearly established, but it is likely that they play an important role in the degradation of plant cell-wall proteins (Wallace and Munro, 1986). Development of an *in vitro* method should attempt to simulate the activities and interactions of these microorganisms. Several approaches have been used, from incubations with commercially available enzymes of non-ruminal origin to complex simulations of ruminal fermentation (Stern *et al.*, 1997).

There has been considerable interest in the development of techniques using commercial enzymes to predict protein degradation in the rumen (Table 13.1). These techniques should be cheaper, easy to standardize and completely independent from the animal. Krishnamoorthy *et al.* (1983) used a protease from *Streptomyces griseus*, because its endo- and exopeptidase activities were similar to those found on major proteolytic ruminal bacteria. Ruminal proteolysis *in vitro* was simulated by choosing an enzyme concentration (0.066 units ml^{-1}) that would provide a proteolytic activity similar to that of whole ruminal fluid. Feed samples were subjected to proteolysis for 18 or 48 h to resemble mean ruminal retention times for grain and roughages, respectively. Undegradable nitrogen estimated *in vitro* was moderately correlated (r^2 = 0.61) with values estimated *in vivo*, and suggested that the technique could be used for comparing ruminal protein degradation of feedstuffs on a relative basis. Other research (Aufrère and Cartailler, 1988; Terramoccia *et al.*, 1992; Susmel *et al.*, 1993) also reported good correlations between *S. griseus* protease and *in situ* estimates of protein degradation (Table 13.1). The most comprehensive validation study of the *S. griseus* protease *in vitro* technique was conducted by Aufrère *et al.* (1991). Ninety-seven concentrate feeds were incubated for 1 and 24 h with the *S. griseus* protease, and estimates of ruminal protein degradation were highly correlated to *in situ* results (r^2 = 0.89). In contrast, other authors reported moderate to poor correlations (from r^2 = 0.21 to r^2 = 0.67) between *S. griseus* protease and *in situ* estimates of protein degradation (Table 13.1), suggesting that this enzyme may not be specific for simulation of ruminal degradation across a wide variety of feeds. Fahmy *et al.* (1991) studied the pattern of protein degradation by *S. griseus* protease activity, using gel electrophoresis, and reported that the type of proteins degraded and resulting end-products were different *in situ* and *in vitro*, suggesting that the enzyme did not have the same specificity as that found in the rumen. Therefore, it appears that the relationship between *S. griseus* protease and *in situ* protein degradation is an empirical association, rather than a solid simulation of ruminal proteolytic activity. Few efforts have been made to validate this technique for forages. Aufrère *et al.* (1989) indicated that the use of the crude-protein content and estimates of *S. griseus* protease degradation could be used for predicting protein degradation of hay (r^2 = 0.79; n = 21).

Another commercial enzyme used for *in vitro* determination of protein degradation is a neutral protease extracted from *Bacillus subtilis*. Protein degradation by *B. subtilis* protease resulted in high correlations with ruminal degradation estimated *in situ* (Roe *et al.*, 1991, r^2 = 0.78; Assoumani *et al.*, 1992, r^2 = 0.92). However, these tests were conducted with few samples and the technique has not been tested with forages. A different neutral fungal protease extracted from *Aspergillus orizyae* (Poos-Floyd *et al.*, 1985) has also been tested in a small number of concentrate samples with positive results (Table 13.1).

Ficin is a plant protease with a wide spectrum of activity extracted from *Ficus glabatra*. Poos-Floyd *et al.* (1985) reported that the highest correlation between enzymatic and ruminal protein degradation *in vivo* was obtained after 1–4 hours digestion with ficin (r^2 = 0.76), but the relationship decreased as incubation time increased. Aufrère and Cartailler (1988) also observed a high correlation (r^2 = 0.94) between protein degradation obtained with 24 h of *in situ* versus *in vitro* ficin incubations, although variation was lower using the protease from *S. griseus*.

Table 13.1. Summary of validation studies conducted using commercially available enzymes.

Enzyme	Samples*	Comments	r^2 †	SE‡	Reference
S. griseus	12C/2F	pH = 8/18–48 h	0.61		Krishnamoorthy *et al.*, 1983
S. griseus	9C	1 h	0.67		Poos-Floyd *et al.*, 1985
S. griseus	12C	pH = 8/1–24 h	0.90	7.0	Aufrère and Cartailler, 1988
S. griseus	21F	pH = 8/1–24 h	0.79	3.9	Aufrère *et al.*, 1989
S. griseus	3C2F	pH = 8	0.07	21.0	Kopecny *et al.*, 1989
S. griseus	96C	pH = 8/1–24 h	0.89	5.8	Aufrère *et al.*, 1991
S. griseus	6C	12 h	0.21	88.0	Roe *et al.*, 1991
S. griseus	8C	pH = 6.5	0.91	8.1	Assoumani *et al.*, 1992
S. griseus	8C		0.77	9.6	Terramoccia *et al.*, 1992
S. griseus	10C	pH = 6.5	0.72		Susmel *et al.*, 1993
S. griseus	13FC	pH = 8	0.39	9.8	Tománková and Kopecny, 1995
Ficin	9C	4 h	0.76		Poos-Floyd *et al.*, 1985
Ficin	42 FC	4 h	0.04		Lindquist *et al.*, 1989
Ficin	4F	4 h	0.20		Lindquist *et al.*, 1989
Ficin	6C	12–24 h	0.01	4.2	Roe *et al.*, 1991
Ficin	23C/38F	pH = 7/2–3 h	0.92		Kosmala *et al.*, 1996
Bromelain	9C	1 h	0.72		Poos-Floyd *et al.*, 1985
Bromelain	13FC	pH = 7.2	0.73	6.5	Tománková and Kopecny, 1995
Bromelain	40C	pH = 7.2	0.70	7.1	Tománková and Kopecny, 1995
Bromelain	28C	pH = 7.2	0.19	10.2	Tománková and Kopecny, 1995
Bromelain	41F	pH = 7.2	0.53	5.8	Tománková and Kopecny, 1995
Bromelain	68F	pH = 7.2	0.55	7.3	Tománková and Kopecny, 1995
Bromelain	9F	pH = 7.2	0.77	4.5	Tománková and Kopecny, 1995
Papain	9C	1–4 h	0.72		Poos-Floyd *et al.*, 1985
Papain	3C2F	pH = 6	0.81	4.5	Kopecny *et al.*, 1989
Papain	13FC	pH = 6	0.59	8.1	Tománková and Kopecny, 1995
B. subtilis	6C	12h	0.78	30	Roe *et al.*, 1991
B. subtilis	8C	pH = 6.5	0.92	7.6	Assoumani *et al.*, 1992
A. orizyae	9C	4–8–24 h	0.90		Poos-Floyd *et al.*, 1985
Alkalase	3C2F	pH = 9	0.35	13.6	Kopecny *et al.*, 1989
Fromase	3C2F	pH = 6	0.14	6.5	Kopecny *et al.*, 1989
Chymosin	3C2F		0.00	21.0	Kopecny *et al.*, 1989
Trypsin	3C2F		0.00	14.6	Kopecny *et al.*, 1989
Trypsin and fromase	3C2F		0.09	8.2	Kopecny *et al.*, 1989

* Type of feed: C = concentrate; F = forage; the value indicates the number of samples used in each study.
† Coefficient of determination between *in vitro* and *in vivo* or *in situ.*
‡ Standard error of estimate.
S. griseus, Streptomyces griseus; *B. subtilis, Bacillus subtilis*; *A. orizyae, Aspergillus orizyae.*

Kosmala *et al.* (1996) showed that the incubation of feed samples with 9 or 18 units of ficin 100 mg^{-1} of sample crude protein from concentrates and forages, respectively, resulted in an overall $r^2 = 0.92$ ($n = 61$) compared with *in situ*. When the regression was performed only with forages ($n = 38$), the coefficient of determination was slightly lower ($r^2 = 0.85$), probably due to the potential interaction with fibre components of forages. These results disagree with observations by Lindquist *et al.* (1989) and Roe *et al.* (1991), as shown in Table 13.1.

Other enzymes tested, including fromase, alkalase, chymosin, or trypsin (Kopecny *et al.*, 1989), pepsin, pancreatin and protease type XIV (Siddons *et al.*, 1985), have not yielded positive results (Table 13.1). Luchini *et al.* (1996a) used a mixture of enzymes (*S. griseus* protease, chymotrypsin and proteinase K; or trypsin, carboxypeptidase B, chymotrypsin and carboxypeptidase A) in an attempt to provide similar proteolytic activity and specificity to those found in the rumen. However, these mixtures failed to simulate the proteolytic activity of strained ruminal fluid.

Two other plant proteases, bromelain and papain, have also been tested to estimate protein degradation in the rumen. Bromelain yielded moderate correlations (Table 13.1), ranging from $r^2 = 0.55$ for forages (Tománková and Kopecny, 1995) to $r^2 = 0.72$ for concentrates (Poos-Floyd *et al.*, 1985). Higher correlations were obtained by Kopecny *et al.* (1989) using papain in five different feeds ($r^2 = 0.81$). Poos-Floyd *et al.* (1985) also obtained moderate correlations ($r^2 = 0.72$) between *in vivo* estimates of protein degradation and papain, although ficin and neutral fungal protease had slightly better correlations. However, these results are in conflict with those reported by Tománková and Kopecny (1995).

Other factors, in addition to proteolytic enzymes, may affect protein degradation of forages in the rumen. For example, Tománková and Kopecny (1995) showed that the addition of α-amylase to a bromelain solution improved the coefficient of determination between *in situ* and bromelain digestion from 0.26 to 0.81. The positive effect of amylases on protein degradation has also been reported by other authors (Aufrère and Cartailler, 1988; Assoumani *et al.*, 1992). It appears that the use of α-amylase was necessary when studying protein degradation of feeds with a starch content of 230 g kg^{-1} or greater (Assoumani *et al.*, 1992). Although it has not been tested in forages, this observation may be of particular interest when measuring protein degradation of maize silage, where starch may account for as much as 400 g kg^{-1} of the dry matter (DM). Furthermore, the addition of cellulolytic enzymes resulted in an increase in protein degradation from 424 to 512 g kg^{-1} after 16 h incubation with ruminal enzymes (Kohn and Allen, 1995b). Similar results were obtained by Abdelgadir *et al.* (1996) when forages were pretreated with 8000 units of cellulase before undergoing an *in vitro* digestion with *S. griseus* protease. On the other hand, factors related to the incubation medium may also play an important role in *in vitro* protein degradation. Protein conformation and solubility, and therefore degradability, depend on the pH of the medium. Assoumani *et al.* (1992) indicated that, as pH was reduced from 8 to 6.5, correlation between *S. griseus* protease and *in situ* protein degradation increased from $r^2 = 0.68$ to $r^2 = 0.91$. The presence of reducing factors also affects *in vitro* protein degradation (Kohn and Allen, 1995b). These authors reported that, as the environment was more reduced, more protein was degraded (Fig. 13.2).

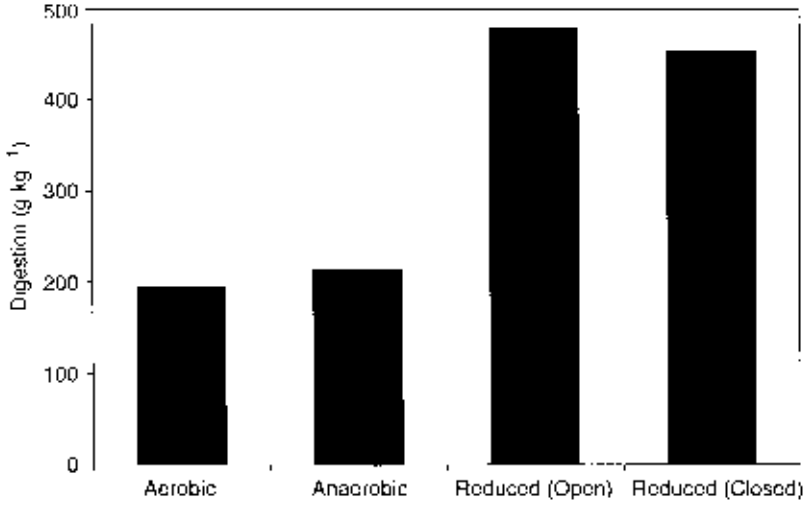

Fig. 13.2. Effect of the addition of a reducing agent to an *in vitro* incubation medium on protein degradation of soybean meal after 16 h digestion with enzymes extracted from the rumen (adapted from Kohn and Allen, 1995b).

It is apparent from data available on *in vitro* protein degradation that the use of non-ruminal enzymes and incubation conditions different from those of the rumen are unlikely to successfully mimic the complex interactions occurring during protein degradation by ruminal microbes. At best, these methods provide an empirical relationship with estimates of ruminal degradation of proteins. Because of these limitations, caution is required when using results obtained with these techniques (Mahadevan *et al.*, 1987; Roe *et al.*, 1991; Luchini *et al.*, 1996a).

A more physiological approach would be to use proteolytic enzymes isolated from the ruminal contents. Most protease and peptidase activities in ruminal contents are associated with the outer side of bacterial cell walls, and can be liberated by treatment with detergents or gentle shaking (Brock *et al.*, 1982; Kopecny and Wallace, 1982; Prins *et al.*, 1983). Susmel *et al.* (1993) used cell-free strained rumen fluid as inoculum and reported a moderate correlation (r^2 = 0.69) between *in situ* and *in vitro* estimates of protein degradation. Mahadevan *et al.* (1987) proposed an extraction method based on release of enzymes after acetone, butanol and chilled-water rinses. These extractions allowed the recovery of approximately 30–35% of the proteolytic activity of strained ruminal fluid. Enzymes could be stored at −20°C for at least a year without losing proteolytic activity. Kohn and Allen (1995a) slightly modified this method and increased recovery of proteolytic activity in ruminal fluid up to 62%. The proteolytic activity did not decrease with time of incubation, and the addition of new enzyme after 8 h of digestion did not increase estimates of protein degradation at 16 h (Kohn and Allen, 1995b). These observations suggest that enzymatic activity was not limiting, and that long-term incubation periods are possible. When this enzyme preparation was evaluated on feed samples (Kohn and Allen, 1995b), protein degradation was lower compared

with values from the National Research Council (NRC, 1989). It was suggested that some enzymes may be selectively eliminated or inactivated by the extraction method, or that some enzymes may function differently when associated with the microbial membrane. However, the technique successfully showed relative differences between forages, stage of maturity and methods of forage preservation (Kohn and Allen, 1995c). The use of enzymes extracted from the rumen may provide the basis for further research on a technique that may be low in cost, fast and reliable. However, it is necessary to carefully observe methods of enzyme isolation, determine optimal ratio of substrate protein to proteolytic activity and provide all necessary conditions of the incubation medium to simulate protein degradation.

It is likely that the complex interactions involved in ruminal degradation of protein will be best simulated by live ruminal microorganisms. Initial studies on protein degradation used *in vitro* incubations of strained ruminal fluid (Little *et al.*, 1963). Feeds were incubated with the inoculum under anaerobic conditions and protein degradation was calculated from the ammonia nitrogen that was released. However, the final ammonia nitrogen concentration was the result of both protein degradation and ammonia nitrogen (NH_3-N) utilization by ruminal microbes, with the latter being greatly influenced by the amount and nature of carbohydrates fermented. For example, Broderick (1982) showed that readily fermentable carbohydrates, such as maize and sorghum grains, resulted in negative NH_3-N release because microbial uptake was greater than NH_3-N released.

To prevent nitrogen utilization by ruminal microorganisms, Broderick (1987) developed an *in vitro* inhibitor procedure (IIV) for estimating ruminal protein degradation by using chloramphenicol and hydrazine sulphate to inhibit microbial protein synthesis and amino acid and ammonia nitrogen utilization by ruminal microbes. Because neither of these inhibitors depresses proteolytic activity, the IIV method might be expected to yield useful estimates of protein degradation kinetics. A limited substrate incubation was used to guarantee the first-order kinetic reaction required for calculations of rates of degradation. Results demonstrated that soluble proteins have different rates of degradation and are not completely degraded. Using the IIV technique, Broderick *et al.* (1988) reported that rates of protein degradation of 18 protein supplements ranged from 0.103 to 0.813 h^{-1}, yielding estimates of protein degradation ranging from 730 to 940 g kg^{-1}. Rates of degradation estimated with the IIV technique were slightly higher, but were highly correlated with *in situ* estimates (r^2 = 0.92, n = 7). Broderick *et al.* (1988) suggested that the lower rates of degradation observed *in situ* could be partially explained by: (i) microbial growth inside the bags, which may reduce apparent rates of degradation; (ii) the microbial population inside the bag may be different compared with the rumen environment; and (iii) the efflux of material from the bag may leave a less degradable material inside the bag, but the soluble fraction may not be all degradable. As a result, the *in situ* method may overestimate degradation of the soluble fraction and underestimate rate of degradation. In contrast, the relatively short incubation time in the IIV (a maximum of 4 h) may overestimate rates of degradation of feeds containing a large pool of rapidly degraded proteins. The IIV technique has been successful in identifying differences in protein degradation of concentrates (Broderick *et al.*, 1988) and forages (Broderick and Buxton, 1991; Broderick and Albrecht, 1997). Recent data by Broderick and Albrecht (1997)

provided information on rates of degradation of forage protein obtained *in vitro* that agree with those reported *in vivo* (Table 13.2).

However, the IIV technique has some limitations: (i) the accumulation of ammonia and amino acids may result in end-product inhibition of enzymes; (ii) microbial activity decreases with time due to the difficulty of maintaining anaerobic conditions and pH; (iii) growth inhibitors are expected to eventually interfere with enzyme synthesis and microbial growth, affecting protein degradation; (iv) degradation rates determined for feeds with high levels of ammonia and free amino acids (as in forage silages) are less accurate because breakdown of more slowly degraded residual proteins must be computed from the appearance of additional ammonia and amino acids in the presence of high background nitrogen; and (v) quantification of degradation rates from the slope of slowly degraded proteins (rates less than 0.01 h^{-1}) is less accurate.

Broderick and Clayton (1992) used alternative mathematical models based on the Michaelis–Menten approach to overcome some of the problems associated with limited substrate degradation. Results using this new approach were obtained more rapidly (2 h) and were more consistent with *in vivo* estimates of ruminal protein degradation. Dialysed ruminal fluid has also been used to reduce background nitrogen contamination and increase the accuracy of estimated rates of degradation (Broderick and Albrecht, 1997).

Neutze *et al.* (1993) modified the inhibitor *in vitro* method by estimating the degradable protein fraction as trichloracetic acid (TCA)-soluble nitrogen. Estimates of the soluble and insoluble potentially degradable fractions and rates of degradation were different from those obtained *in situ*, but estimated protein degradation was strongly correlated to *in situ* values ($r^2 = 0.75$). The coefficients of

Table 13.2. Estimates of the rate and extent of degradation of the potentially degradable pool of protein at two rates of passage (adapted from Broderick and Albrecht, 1997).

			Rate of degradation†	Escape protein‡	
	n	B_0*	(h^{-1})	0.06 h^{-1}	0.03 h^{-1}
Alfalfa	2	94.3	0.219	20.3	11.4
White clover	1	94.6	0.269	17.3	9.5
Red clover	2	95.1	0.140	28.5	16.8
Kura clover	1	96.3	0.220	20.6	11.6
Cicer milkvetch	2	94.4	0.213	20.8	11.7
Crownvetch	1	93.2	0.219	20.0	11.2
Canada milkvetch	1	94.7	0.252	18.2	10.1
Birdsfoot trefoil	10	93.9	0.197	21.9	12.4
Grassland Maku	2	95.4	0.037	59.0	42.7
Sainfoin	6	95.2	0.027	65.9	50.4
Lespedeza	12	96.2	0.009	83.2	73.2

* Potentially degradable protein fractions.
† Estimates using the inhibitory *in vitro* technique (Broderick, 1987).
‡ Estimated as $B_0 \times (k_p/(k_p + k_d)$, at passage rates ($k_p$) of 0.06 and 0.03 h^{-1}.

variation of the degradation parameters were smaller *in vitro* than *in situ*, which indicates that the *in vitro* system was more accurate.

To avoid the use of inhibitors of nitrogen utilization by microbial cells, Hristov and Broderick (1994) developed a system using $^{15}NH_3$ to quantify microbial nitrogen utilization. The advantage of this method is that it allows for normal microbial growth and reduces the potential for end-product inhibition. Degradation rates were computed as appearance of NH_3-N plus net microbial protein synthesis. Results showed similar trends to the limited-substrate IIV method, but average degradation was about 28% greater in the uninhibited system, suggesting that the inhibition of microbial growth in the IIV technique results in a reduction of proteolysis. Estimates of protein degradation of seven protein supplements were consistent with previously reported values. This method may be of particular interest in the analysis of samples with high non-protein nitrogen content as percentage of total protein, such as legume and grass silages, although the technique still needs to be tested on these types of samples. Peltekova and Broderick (1996) used this modified procedure to estimate the rate and extent of protein degradation in lucerne (alfalfa) hay and silage, using a two-compartment model. Estimates of ruminal protein degradation were comparable with NRC (1989) values. An *in vitro* method based on use of $^{15}NH_3$ to estimate microbial nitrogen utilization may serve as a more theoretically sound procedure with which to compare other *in vitro* tests that estimate protein degradability.

Raab *et al.* (1983) used a different approach to determine nitrogen utilization by bacteria. Their method consisted of adding graded amounts of fermentable carbohydrate to an *in vitro* fermentation system with the protein source to be tested. Gas production and NH_3-N concentrations were measured up to 24 h. Extrapolation of NH_3-N release to zero gas production was assumed to correct for NH_3-N and amino acid utilization for microbial growth, because zero gas production represents no incorporation of nitrogen into microbial protein. The technique assumes that gas production is a reflection of microbial growth and that this relationship is linear over the conditions of the experiment. Estimates of protein degradation were similar to those obtained *in vivo*. However, a total of 15 incubation tubes were required per sample tested, which may impose a limit on its routine application.

Nuget and Mangan (1981) used an *in vitro* fermentation system to test if rates of proteolysis could be measured *in vitro*. Rates of degradation of fraction I leaf protein compared well with previously reported estimates obtained *in vivo* and *in vitro*. Results using this *in vitro* system showed an initial rate of proteolysis that was approximately 82% of that *in vivo*, and only slowed significantly after 2 h incubations. When the fraction I leaf protein was labelled with carbon-14 (^{14}C), the system allowed for a precise study of the kinetics of protein degradation.

All batch-culture systems require the use of a microbial inoculum and incubation conditions similar to those of the ruminal environment. Because about 75% of the proteolytic activity was recovered in the particulate fraction of the rumen contents (Brock *et al.*, 1982), it is necessary to obtain ruminal samples with solid-phase-associated bacteria. Chilling and blending of whole rumen contents will suffice to recover a large amount of its proteolytic activity (Craig *et al.*, 1984). Estimates of protein degradation may be affected by the donor animal, time of

ruminal fluid collection and type of diet (Wallace and Cotta, 1988). However, Furchtenicht and Broderick (1987) indicated that changes in proteolytic activity of the inoculum were largely due to its microbial numbers and that the variation in proteolytic activity among inocula obtained from cows fed different diets could be reduced by standardizing the inoculum to a constant microbial weight. Luchini *et al.* (1996b) tested the possibility of using preserved ruminal microorganisms as inoculum. The use of frozen microbes in the IIV technique of Broderick (1987) resulted in lower proteolytic activity, but feeds were ranked in the same order as with fresh ruminal fluid. This approach may be promising, but needs further validation. Other factors, such as pH and anaerobic and reducing conditions, also affect microbial protein degradation and should be carefully controlled to allow for optimal microbial fermentation (Grant and Mertens, 1992). Available information suggests that the refinement of *in vitro* ruminal-fluid incubations may provide an appropriate method for measuring ruminal protein degradation. However, the need for ruminally cannulated animals still creates a problem for routinely using *in vitro* ruminal-fluid incubations by industry.

There have been very few attempts to use a combination of pure microbial cultures to simulate ruminal fermentation. If *in vitro* rumen microbial fermentation is able to provide reasonable estimates of protein degradation, it is possible that a combination of major proteolytic, peptidolytic, amylolytic and cellulolytic bacteria may provide the basis for a standardized *in vitro* technique based on ruminal microbial activities. At the present time, only the research conducted by Laycock *et al.* (1985) has attempted this approach. A mixture of *Butyribivrio* sp. and *Streptococcus bovis* was used to predict protein degradation of soybean meal. However, after 24 h incubation, estimates of protein degradation were about half of those obtained *in situ*. It is possible that a combination of other microorganisms may improve estimates of protein degradation, but this hypothesis needs to be examined further.

The most physiological approach to determining protein degradation *in vitro* would be to design a system that carefully simulates ruminal fermentation. Various continuous-culture fermentation systems have been designed to simulate the ruminal environment, enabling the study of ruminal microbial ecology and digestion of nutrients (Hoover *et al.*, 1976; Czerkawski and Breckenridge, 1977; Teather and Sauer, 1988; Fuchigami *et al.*, 1989; Miettinen and Setälä, 1989). Advantages of these systems compared with *in vivo* measurements include reduction in cost, time and variation among experimental units. Also, there are no complications from endogenous sources, and digesta flow markers are not required, because passage rates are regulated and measured directly. However, similar to *in vivo* measurements, reliable techniques are required for isolation of microbial cells and for differentiation of effluent digesta into microbial and dietary N fractions. The most commonly used continuous-culture systems are the single-flow, Rusitec (Czerkawski and Breckenridge, 1977) and the dual-flow continuous culture (Hoover *et al.*, 1976). The Rusitec system has a single outflow, and residence time in the rumen is simulated by placing feedstuffs into nylon bags inside the reaction vessel for 48 h. Recently, Prevot *et al.* (1994) evaluated the Rusitec and concluded that the system cannot reproduce the *in vivo* fermentation because ciliated protozoal populations, and probably many bacterial species, are eliminated from the fermenter. The dual-flow continuous-culture system developed by Hoover *et al.* (1976) has been successful

in maintaining ruminal fermentation parameters and microbial populations similar to those found in the rumen. Hannah *et al.* (1986) and Mansfield *et al.* (1995) compared ruminal fermentation patterns and nutrient flow between *in vivo* and the dual-flow continuous-culture fermenters. The continuous-culture system was able to obtain digestibility values for organic matter, total non-structural carbohydrate, fibre and protein within physiological ranges, although some digestibility measurements were significantly different compared with *in vivo* data. Some of the differences in fermentation parameters observed between the dual-flow continuous-culture fermenters and the rumen can be attributed to lack of absorptive capacity and defaunation *in vitro*. However, it should be noted that interpretation of results *in vitro* and *in vivo* were similar for 80% of the individual parameters evaluated, supporting the dual-flow continuous-culture system as an excellent model for studying ruminal microbial fermentation (Mansfield *et al.*, 1995). Although the protozoal population was much lower in fermenters, Mansfield *et al.* (1995) found small differences between *in vivo* and *in vitro* systems in total and cellulolytic counts and no differences in amylolytic, proteolytic and fungal counts. High protozoal numbers have been accomplished by intermittent mixing and compartmentalization (Teather and Sauer, 1988; Fuchigami *et al.*, 1989). These systems are excellent tools for research and modelling of ruminal fermentation, but are elaborate and expensive, require inoculation with ruminal digesta and may not be suitable for routine analysis of microbial digestion for individual feed ingredients.

Can Enzymes Estimate Rate of Degradation?

Rates of energy and protein degradation affect energy and protein utilization by ruminal microorganisms and the animal. Feeds are composed of a mixture of proteins that may have different degradation patterns. Pitchard and Van Soest (1977) proposed that there are different pools of proteins with different rates of degradation within each feed. Overall degradation in the rumen is a function of pool sizes and rates of degradation and passage of the potentially degradable pools.

Determination of rates of degradation requires that the digestion process follows first-order kinetics. Therefore, *in vitro* procedures should be designed to contain an excess of enzyme (Krishnamoorthy *et al.*, 1983). Krishnamoorthy *et al.* (1983) and Aufrère and Cartailler (1988) observed that rates of degradation with the *S. griseus* protease were higher than those obtained by the *in situ* method. Similarly, Kosmala *et al.* (1996) and Coblentz *et al.* (1997) observed that degradation rates of feed proteins were faster with ficin than those estimated *in situ*. Rates of degradation estimated with ficin were inconsistent with those obtained *in situ*, suggesting that ficin was not suitable for measuring dynamics of protein degradation in the rumen (Kosmala *et al.*, 1996). Roe *et al.* (1991) compared the dynamics of protein degradation *in situ* and with *S. griseus*, ficin and neutral proteases. However, none of these *in vitro* tests resulted in degradation curves that had consistent relationships with those generated by the *in situ* technique. Inconsistencies in rates of degradation were also found when untreated and chemically treated soybean-meal protein fractions were digested *in vitro* by bacterial proteases, pepsin or trypsin (Lynch *et al.*, 1988). Several authors suggested that adjusting the protease concen-

tration to provide a similar proteolytic activity to that found in the rumen could provide better estimates of rates of protein degradation (Krishnamoorthy *et al.*, 1983; Kosmala *et al.*, 1996; Coblentz *et al.*, 1997). Mahadevan *et al.* (1987) tested rates of degradation of insoluble feed protein, using enzymes extracted from the rumen and an *S. griseus* protease. Results revealed considerable differences in degradation rates between the two techniques. However, the ruminal enzymes were more consistent with reported values. It was suggested that the use of non-ruminal enzymes may not be adequate for the determination of rates of protein degradation. Kohn and Allen (1995b) also obtained more reasonable estimates of rates of degradation of soybean meal and lucerne hay proteins (0.15 and 0.06 h^{-1}, respectively) using 2 h incubations with ruminal enzyme extract. At the present time, it appears that the IIV technique of Broderick (1987), with recent modifications (Hristov and Broderick, 1994; Broderick and Albrecht, 1997), may be the most adequate *in vitro* method for determining rates of protein degradation. The use of enzymes extracted from the ruminal fluid may also provide reasonable estimates of rates of degradation, but the technique needs further testing.

The question that needs to be answered is how accurate should the measurements of rates of protein degradation be. From a scientific standpoint, accuracy should always be an objective. For field application, accuracy will depend on its impact on estimates of effective ruminal degradation. Figure 13.3 demonstrates the effect of different rates of passage and degradation on changes in estimates of effective ruminal degradation. A feed protein with 20% soluble protein, 60% potentially degradable fraction and 20% undegradable fraction was used for the model. Degradation was calculated using the model of Ørskov and McDonald (1979).

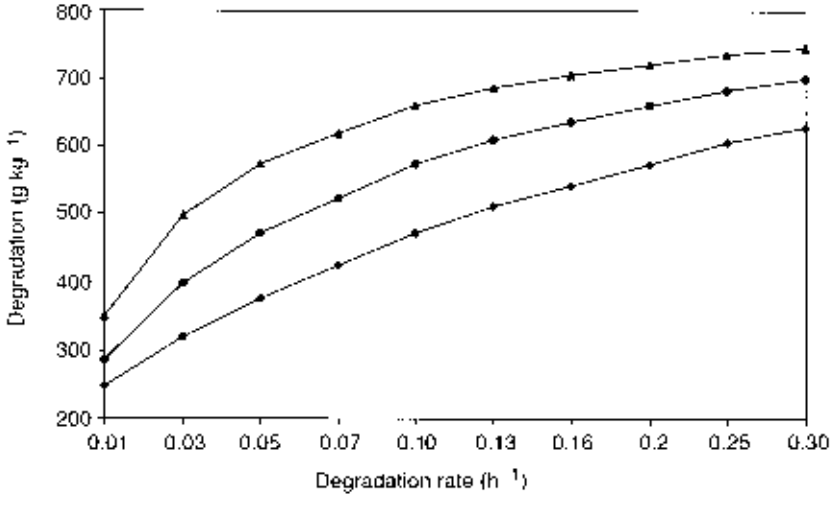

Fig. 13.3. Effect of rate of degradation on estimated effective degradation at different passage rates: forage-particles pool (k_p = 0.03 h^{-1}, ▲); concentrate-particles pool (k_p = 0.06 h^{-1}, ●); and liquid pool (k_p = 0.12 h^{-1}, ◆).

From Fig. 13.3, it can be concluded that: (i) for all rates of passage, the importance of an accurate estimate of degradation rate is greater at low degradation rates; and (ii) rate of degradation is more important than rate of passage at low degradation rates, but the opposite occurs at high degradation rates. Therefore, small errors in the estimation of rates of degradation in the insoluble fraction of forages (low passage rates) may have a great impact on estimates of degradability within a range of rates of degradation between 0 and 0.15 h^{-1}. For example, a change from 0.03 to 0.05 h^{-1} in degradation rate of an insoluble forage protein with a rate of passage of 0.03 h^{-1} results in an increase in degradation from 500 to 575 g kg^{-1} (an increase of 75 g kg^{-1}). In contrast, the same increment from 0.20 to 0.23 h^{-1} only affects estimates of degradation from 722 to 731 g kg^{-1} (an increase of 0.9 percentage units). In contrast, soluble protein flows with the liquid fraction and rates of passage are higher (i.e. 0.12 h^{-1}). Under these conditions, an error in the estimation of rates of degradation will have a smaller effect on estimated degradability, although this effect will influence estimates of degradation over a wider range of degradation rates (Fig. 13.3). Unfortunately, *in vitro* techniques are less accurate as degradation rate decreases. It is important to keep these observations in mind when attempting to improve *in vitro* techniques for industry application.

Intestinal Digestion

Protein supply from forages represents a high proportion of total protein intake. In preserved or heat-damaged forages, the amount of undegradable protein reaching the small intestine may be considerable. The large differences in intestinal digestion of undegraded protein from forages (from 554 g kg^{-1} to 860 g kg^{-1}; Frydrych, 1992) justify the need to incorporate these estimates into new feeding systems. *In vivo* and *in situ* techniques to measure intestinal digestion of proteins and their advantages and disadvantages have been discussed elsewhere (Stern *et al.*, 1997).

The Association of Official Analytical Chemists (AOAC, 1984) approved a standardized pepsin digestion procedure for estimating unavailable nitrogen. Pepsin-insoluble nitrogen (PIN) was strongly correlated to total-tract unavailable nitrogen in forages (Goering *et al.*, 1972; Shelford *et al.*, 1980). In contrast, Britton *et al.* (1986) and Zinn and Owens (1982) indicated that PIN was a poor predictor of total-tract crude protein (CP) digestibility. Zinn and Owens (1982) reported that about 200 g kg^{-1} of the nitrogen leaving the ileum was soluble in acid–pepsin solution, suggesting that PIN may underestimate unavailable protein of feeds. In addition, total-tract digestion does not provide information on site of protein digestion in the gastrointestinal tract.

Because of the limited spectrum of specificity of any single enzyme, it is unlikely that single enzymatic techniques will be able to predict protein digestion in the small intestine. The most commonly used enzymatic technique was developed by Akeson and Stahmann (1964). The system was designed to simulate abomasal (pepsin) and intestinal (pancreatin) digestion. Results were highly correlated (n = 12; r^2 = 0.98) with rat growth studies. Antoniewicz *et al.* (1992) compared results from the mobile-bag technique with pepsin–pancreatin digestion in four protein supplements treated with five different doses of formaldehyde. Results obtained

with the two techniques were strongly correlated (r^2 = 0.90). Van Straalen *et al.* (1993) also reported a strong correlation (n = 28; r^2 = 0.83) between the mobile-bag technique and a pepsin–pancreatin digestion test.

More recently, Calsamiglia and Stern (1995) developed a three-step procedure, modified from that of Akeson and Stahmann (1964), to estimate intestinal digestion of proteins in ruminants. After 16 h of ruminal preincubation, samples were incubated for 1 h in a hydrochloric acid (HCl)–pepsin solution, followed by a 24 h phosphate buffer–pancreatin digestion. Results of the pancreatin digestion step were highly correlated (r^2 = 0.83) with 34 duodenal samples from which small-intestinal protein digestion was determined *in vivo* (Fig. 13.4). The technique has been succesfully applied to numerous feeds (Yoon *et al.*, 1994; Howie *et al.*, 1996; Calsamiglia and Stern, 1997; Calsamiglia *et al.*, 1997).

Further Research Needed

All *in vitro* techniques currently available for protein quality evaluation have limitations (Stern *et al.*, 1997). Efforts should continue in the search of a scientifically sound technique that allows for further understanding of protein degradation and utilization by ruminal microbes and the ruminant animal. Aspects of protein digestion that should be studied in the near future include the following.

1. The search for a fast, inexpensive and reliable *in vitro* technique should continue. It is likely that current techniques may be sufficient for specific purposes (e.g. industry quality control). However, few techniques have been tested and validated

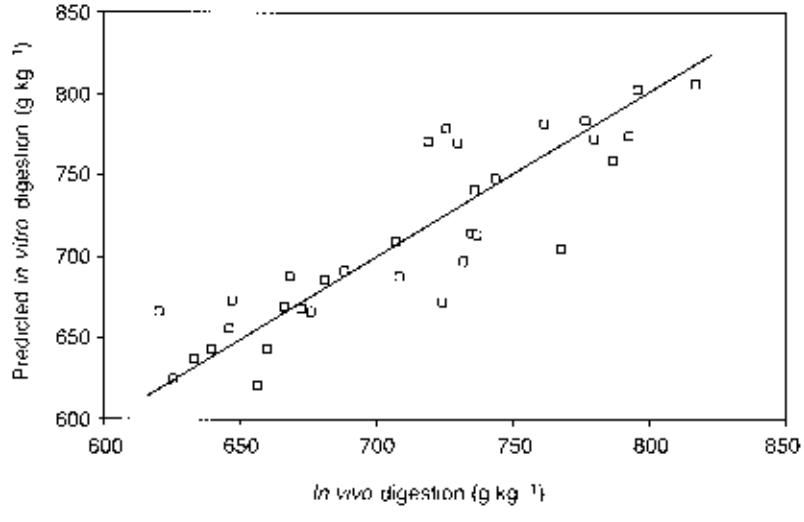

Fig. 13.4. Observed *in vivo* intestinal digestion of protein versus predicted value, using the three-step *in vitro* procedure (n = 34; r^2 = 0.83) (from Calsamiglia and Stern, 1995).

for forages. Recent research suggests that enzymatic techniques should carefully consider the use of combinations of proteolytic, peptidolytic and non-proteolytic enzymes (amylases and cellulases) in a medium where conditions (pH, redox potential, etc.) are maintained similar to those of the ruminal environment.

2. New techniques should be developed to determine pool sizes and their rates of degradation and ruminal passage. The application of pool sizes and their respective rates of degradation and passage may allow for modelling of the dynamics of protein availability in the rumen and true protein passage to the small intestine.

3. Feeding systems are incorporating daily requirements of amino acids. None of the *in vitro* techniques currently available has addressed the issue of amino acid profile of ruminally undegraded and intestinally digested protein. Therefore, efforts should also concentrate on the development of *in vitro* techniques capable of determining the amino acid profile of the undegraded protein in the rumen and of the digestible protein in the small intestine.

Cost Implications

One of the major advantages of *in vitro* techniques is the reduction in cost and labour compared with *in vivo* and *in situ* procedures. Cost of commercially available enzymes and reagents per analysis is generally very low and many samples may be processed at the same time, making these techniques suitable for industry application. The need for ruminal fluid in several of the techniques discussed is still a problem for their routine application in the field. However, when compared with other *in vivo* or *in situ* techniques, these *in vitro* techniques are still generally faster and cheaper. The cost of determining rates of degradation is higher than measuring extent of degradation, because more measurements are required. Some techniques (e.g. those of Raab *et al.*, 1983; Hristov and Broderick, 1994) are more complex and expensive, but may have the potential of yielding results of rates of degradation and effective degradability more in accordance with the physiological mechanisms found *in vivo*.

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14 Characterization of Forages by Chemical Analysis

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What is Worth Measuring?

The best evaluator of forage quality is animal performance. Intake, digestibility and efficiency of utilization are characteristics of forages that determine animal performance, with variation in intake accounting for 60–90% of the variation in digestible energy (Mertens, 1994). It would therefore be desirable to measure those forage characteristics that relate most closely to intake and digestibility. Chemical fractions that have been associated with intake and digestibility include fibre, lignin and protein (Cherney and Mertens, 1998). A routine system of chemical characterization of forages for ruminants, then, must include determination of fibre, lignin and protein and critical to these is an accurate dry matter (DM) determination. Some characterizations, such as the determination of water-soluble carbohydrates (WSC), may be of particular importance in silage research. Others, such as tannin assays, starch and soluble fibres, may be of particular importance for some types of forages and some feeding situations. The worth of any particular assay, other than for fibre, lignin, and protein, is thus dependent on the researcher's objectives and the likely economic impact of not performing the assay. If an anti-quality factor limits production, it may become the most important factor to analyse for.

What can it Tell us about Forages?

Chemical analyses can provide valuable information about the actual chemical constituents influencing digestion, unlike *in vitro* methods (Van Soest, 1994). Component chemical analyses, often expensive, can provide important biochemical information, leading to a better understanding of the factors that may limit animal performance. Chemical characterization methods cannot give a direct estimate of nutritive value, but rather rely on statistical association to measure digestibility

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and intake. Using these statistical associations, characterization of forage fibre, lignin, protein and other chemical components are used increasingly to predict animal performance.

Chemical analyses, along with the use of models, are increasingly used to predict performance. Forage chemical analyses can be used to identify factors in forages that may be limiting animal performance (Minson, 1981).

Chemical Characterization Methods

Dry-matter concentration

While not considered a chemical characterization *per se* by many, DM determination is included here because accurate determination of forage DM is essential for successful characterization of any other chemical constituent. Analytical results must be reported on a total DM basis for meaningful comparisons among feeds. Additionally, the accurate determination of DM is critical for balancing the diets of ruminants. Clancy *et al.* (1977) demonstrated that small errors in DM determination were amplified in the calculation of quality parameters, such that significant differences in quality resulted solely from the method of DM determination. Fresh forage or samples preserved as hay have several options for DM determination, while samples collected after ensiling must account for volatile compounds in ensiled forages. Options for determination of DM directly or by chemical means will be briefly discussed here (Table 14.1).

Oven-drying is commonly used for DM determination. At a drying temperature of 100°C, mechanically trapped water is evaporated, leaving chemically bound water (Brusewitz *et al.*, 1993). The amount of water removed from the sample is directly related to drying temperature, although a small amount of chemically bound water remains in samples dried at temperatures as high as 135°C. Officially approved oven-drying methods, according to the Association of Official Analytical Chemists (AOAC), include drying at 100°C under a vacuum of 1.3×10^4 Pa for 5 h (AOAC-934.01, 1990), drying samples in a forced-air oven at 135°C for 2 h (AOAC-930.15, 1990), or drying at 105°C for 16 h (AOAC-967.03, 1990). An officially approved method in the UK involves drying in a forced-air oven at 100°C for 18 h (MAFF-1, 1986). Further analyses should not be attempted on the dried sample, as drying at 100°C will alter some forage compounds making them less available (Van Soest, 1994).

A two-step procedure is generally used in the USA. Wet samples are dried in a forced-air oven at 55 or 60°C overnight to determine a partial DM value. Samples are then ground and can be used for the second step in total DM determination or for other analyses. The second step of the DM procedure usually involves one of the AOAC procedures.

Fermented feeds contain volatile components, which may be lost during the drying process. Direct laboratory measurement of water content of an as-received silage sample can reduce or eliminate DM determination errors due to loss of volatile components. Toluene distillation is one method for the direct measurement of water in a forage sample. Its use is warranted only for fermented samples,

Table 14.1. Comparison of laboratory methods for determining forage moisture contents (adapted from Brusewitz *et al.*, 1993).

Method	Operating principle	Moisture range	Accuracy*
Hot-air oven	Drying	0–1000	Good for dried forage, poor for silage
Hot-air oven	Drying with correction for volatiles	0–1000	Good
Toluene distillation	Boiling	0–900	Good
Toluene distillation	Boiling with correction	0–900	Very good for silages
Saponification	Chemical binding of water	0–1000	Very good
Karl Fischer	Titration	0–1000	Excellent
Gas chromatography	Physical separation of water from other extracted chemical	0–1000	Excellent
Near-infrared Reflectance Spectroscopy	Electromagnetic properties	0–400	Very good

* Precision for all listed procedures is ±10 g kg^{-1}.

due to the hazards associated with toluene (NFTA-2.3.1, 1993). The small amount of water not removed from the sample has been assumed to offset the volume of volatile components in the distilled-water phase (NFTA-2.3.1, 1993). A more accurate method is to measure the volatile components in the water phase in order to adjust the volume of water for these components (Dewar and McDonald, 1961; AOAC-925.04, 1990). Most studies have concluded that uncorrected toluene distillation results in higher DM content than oven-drying at a range of temperatures (Larsen and Jones, 1973).

The Karl Fischer titration method (Fischer, 1935) is based on the reaction of water with iodine in the presence of pyridine and methanol. This method also involves hazardous chemicals, and the relatively small sample size analysed will increase potential sampling error. Karl Fischer titration for DM determination has been considered too time-consuming for routine analyses (Hood *et al.*, 1971; Robertson and Windham, 1983), although others considered it a simple, rapid procedure for silage samples (Galletti and Piccaglia, 1988; Kaiser *et al.*, 1995).

Gas-chromatography procedures have been developed to measure the water content of forage (Fenton *et al.*, 1981; Burdick and McHan, 1982). Water and alcohol are separated using a gas chromatograph and the ratio of water to alcohol in unknown samples compared with the standards allows determination of sample water content. Accuracy and repeatability are excellent.

Because they can contain significant quantities of volatile compounds, fermented feeds present special problems. Several methods of correcting silage DM

content for loss of volatile components are used. Corrections to oven-dried silage DM have been made by analysing samples for fermentation products (Fatianoff and Gouet, 1969; Dulphy *et al.*, 1975). Galletti and Piccaglia (1988) did not find significant differences between Karl Fischer titration values and oven-dried values, after oven-dried silage values were corrected for fermentation products. Equations have been proposed for estimating a toluene DM determination from oven-dried grass-silage samples (Haigh and Hopkins, 1977; Barber *et al.*, 1984; Haigh 1995a, b, c). Such equations will work only if the exact oven-drying procedure is used. Loss of DM during drying is a function of the type and concentration of volatiles present, as well as oven temperature (Minson and Lancaster, 1963).

Kaiser *et al.* (1995) compared eight DM determination methods for silages. In general, silage × method interaction was highly significant, due to greater differences among methods at low silage DM compared with high silage DM. Kaiser *et al.* (1995) concluded that Karl Fischer DM values can be estimated in feed testing laboratories with sufficient accuracy from oven DM values and prediction equations.

Fibre determination

The proximate analysis system (Weende method) has been used for almost 150 years. Components of proximate analysis include crude protein, ether extract, crude fibre, ash and, by difference, nitrogen (N)-free extract (Fisher *et al.*, 1995). While this method is simple, repeatable and relatively inexpensive, there are several problems associated with the method that caution against its use. Ether removes waxes and some other compounds not considered digestible. The crude-protein determination assumes that all N is contained in protein with an N content of 16%, which is incorrect. Crude fibre does not provide an accurate representation of the least digestible fibrous part of the feed in many cases (Sollenberger and Cherney, 1995). Inversely, nitrogen-free extracts are not always a reasonable estimate of the highly digestible carbohydrate fraction. Crude-fibre digestibility exceeds nitrogen-free extract digestibility in about 30% of feedstuffs (Van Soest, 1994), with the largest error occurring in tropical grasses and straws. Perhaps the most serious error is the assumption that crude fibre contains all dietary cellulose, hemicellulose and lignin (Fig. 14.1). For these reasons, crude fibre and nitrogen-free extract are not and should not be routinely used. Crude protein has long been the standard for evaluating the protein value of forages and is still acceptable for many conditions (Vérité, 1980).

The primary standard for fibre evaluation of forages in the USA is the Van Soest system of fibre analysis (Marten, 1981). The Van Soest system separates carbohydrates into fractions based on nutritional availability (Sollenberger and Cherney, 1995). This separation results in a more reasonable estimate of the structural carbohydrates than does crude fibre (Fig. 14.1) and allows for prediction of other indices of forage quality, such as digestibility (Van Soest, 1994). The Van Soest system does not fractionate feeds into chemically pure fractions. It must be remembered, however, that what is important is the nutritional uniformity of the fraction in question.

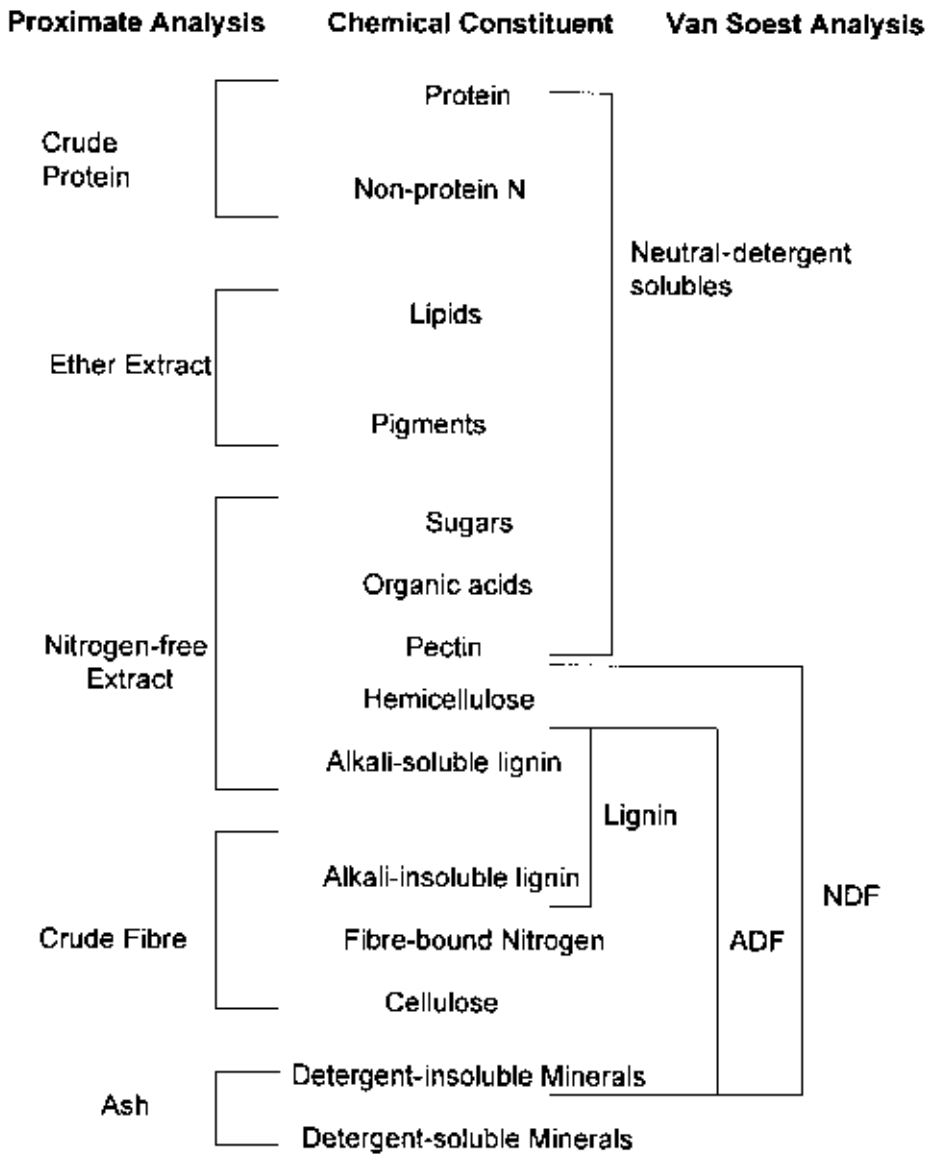

Fig. 14.1. Contrast of Weende system and Van Soest system of carbohydrate analysis (adapted from Fisher *et al.*, 1995). ADF, acid-detergent fibre; NDF, neutral-detergent fibre.

The Van Soest system of fibre analysis has remained relatively unchanged over the years, although a number of modifications have been proposed for special samples (Mascarenhas-Ferreira *et al.*, 1983). Current recommended procedures for neutral-detergent fibre (NDF) (Van Soest *et al.*, 1991) recommend elimination of sodium sulphite, discussed further below, and decalin from the original procedure

(Goering and Van Soest, 1970) and replacement of 2-ethoxyethanol with triethylene glycol. Other research has suggested that 2-ethoxyethanol is not necessary for the determination of NDF in forages and should be eliminated rather than replaced (Cherney *et al.*, 1989). Triethylene glycol may be used where excessive foaming is a problem during filtration. The use of amylase to aid in the removal of starch is also recommended (Van Soest *et al.*, 1991).

Starch contamination of NDF, particularly in those forages containing grain, can lead to overestimates of the NDF value (Robertson and Van Soest, 1977). Insufficient removal of starch from a sample often leads to difficulties with filtration, resulting in elevated NDF values (Cherney *et al.*, 1989). Generally, the use of heat-stable α-amylase is recommended (Van Soest *et al.*, 1991). Pretreatment with urea to remove starch may be necessary in forages with large concentrations of starch (Van Soest *et al.*, 1991).

Sodium sulphite, originally used to reduce nitrogenous contamination in fibre analyses, was removed from the system because it was thought to solubilize some lignin (Van Soest *et al.*, 1991). Recently, the inclusion of sodium sulphite in the NDF solution of high-protein forage samples was re-evaluated (Hintz *et al.*, 1996). Addition of 0.5 g sodium sulphite decreased nitrogen concentration in fibre and lignin values and reduced within-sample variance. Use of sodium sulphite in high-protein forages is therefore recommended.

Recently, a new modification, using the ANKOM fibre analyzer (ANKOM Technology, Fairport, New York), has been developed, which uses preweighed fibre filter bags (Komarek *et al.*, 1994). Fibre values obtained using this method compared to conventional systems were similar (Komarek *et al.*, 1994). This system may be advantageous for silage samples or samples with high soil contamination, which are routinely difficult to filter, as the ANKOM system does not require filtration. This system also offers advantages of reduced initial cost and reduced labour over the conventional system.

Another modification of the NDF system worthy of mention is the micro-NDF method (Pell and Schofield, 1993). There are numerous instances when the amount of sample available may be too small for conventional analysis. In the micro-NDF method, samples in the range of 10–50 mg are placed in 50 ml serum bottles with neutral-detergent solution and autoclaved for 60 min at 105°C. Results compare favourably with standard Van Soest fibre methodology (Van Soest *et al.*, 1991; Pell and Schofield, 1993).

Of the fibre fractions, NDF relates best to feed intake, representing the total insoluble fibre matrix (Van Soest, 1994). This makes NDF the most important fibre fraction to consider. The intended use of acid-detergent fibre (ADF) is as a preparative residue for the determination of cellulose, lignin, Maillard products, silica, acid-insoluble ash and acid-detergent-insoluble N (ADIN). Despite the statistical association in some cases, there is no valid theoretical basis to link ADF to digestibility (Van Soest *et al.*, 1991).

Modified ADF, slightly higher in acid concentration (0.5 mol l^{-1}) and longer in boiling time (MAFF-30, 1986) than Van Soest ADF, is common for fibre analysis in the European Union (EU). The original study for the method (Clancy and Wilson, 1966) reported that prolonged boiling with acid of higher concentration reduced bound nitrogen and improved the relationship between fibre and

digestibility. The use of modified ADF as a means of assaying for heat damage and unavailable protein is precluded, because this system includes oven-drying at 95°C as a preliminary step (Van Soest, 1994).

Lignin

Lignin and associated phenolics are most often identified as the chemical constituents in cell walls most limiting digestion (Van Soest, 1994; Buxton *et al.*, 1996). There are a number of procedures used to determine lignin in forage samples (Table 14.2). Insolubility of lignin in 72% sulphuric acid is the basis of most procedures (Barnes, 1973). Measurement of lignin in forage species is complicated by the presence of proteins, chemically bonded cinnamic acids (Iiyama and Wallis, 1990), Maillard products and other non-lignin substances insoluble in 72% sulphuric acid (Van Soest and Robertson, 1985). Klason lignin (72% sulphuric acid method), suitable for most woody species, leads to an overestimation of lignin, because protein coprecipitates with lignin (Norman and Jenkins, 1934). Most methods to determine lignin in forages, therefore, involve preliminary removal of protein, either by enzymes (Crampton and Maynard, 1938) or by pretreatment with acid-detergent solution (Van Soest, 1973).

Barnes (1973) suggested that problems associated with the use of lignin concentration and digestibility could be summarized as: (i) complex and unknown structure of lignin; (ii) lack of a standardized lignin procedure; and (iii) the inherent variation in lignin content among forages. Some 25 years later, the definition or concept of what constitutes lignin is still subject to debate (Van Soest, 1993) and there is, as yet, no universally accepted lignin standard. As to the third point, Van Soest (1993) suggested that a very large part of the diversity between species in the relationship between lignin content and DM digestibility is due to interaction with NDF content. If lignin is expressed on a fibre basis, lignin's relationship to digestibility appears to be reasonably uniform across legumes and grasses (Van Soest, 1993). It would thus appear logical to report lignin on a fibre basis, or to include fibre values when reporting lignin.

Giger (1985) performed a thorough review of current lignin methodology and concluded that a pure analytical lignin fraction, without any lignin loss or contamination by proteins, carbohydrates, cutins or tannins, is not possible with current methods. Methodological insufficiencies may be due to the complexity and diversity of the 'lignin fraction' (Barnes, 1973; Giger, 1985; Van Soest, 1994). Van Soest (1994) suggested that lignin assays be evaluated with the following criteria: (i) lignin recovery in digestion balances; (ii) degree of correlation between lignin and digestibility; (iii) low nitrogen content of lignin preparation; and (iv) recovery of phenolic matter. While this may not be a 'true definition' of lignin (Van Soest, 1994), it is certainly an operational definition. Lignin's value is in its relationship to digestibility or indigestibility. None of the methods listed in Table 14.2 or the more extensive list of Giger (1985) is clearly better based on the four criteria listed above. Some of the newer procedures, such as pyrolysis mass spectrometry, may be beneficial in determining the true structure of lignin. At this time, however, the equipment may be too expensive for use in a routine evaluation scheme. For any

Table 14.2. Methods for the determination of lignin.

Method	Comment	Reference
Quantitative		
Gravimetric		
Klason lignin	Most severe limitation is protein contamination	Giger, 1985
Acid-detergent lignin (ADL)	May contain cutin and other non-lignin carbohydrates	Van Soest, 1973
Difference after lignin removal		
Permanganate lignin	Yields higher values than ADL; cutin excluded, but may contain polyphenolic substances	Van Soest and Wine, 1968
Chlorite	Values may be higher than permanganate	Collings *et al.*, 1978
Triethylene glycol–HCl	Yields values similar to permanganate lignin; lignin preserved	Edwards, 1973
Absorbance	Most of these methods require calibration with gravimetric method	
Acetyl bromide	Protein and non-lignin fractions may contribute to absorbance	Morrison, 1972
Modified acetyl bromide	Non-lignin fractions cause little interference	Iiyama and Wallis, 1990
Bjorkman's ball-milling	Low lignin yields, carbohydrate contamination; used for structural studies	Lam *et al.*, 1990
Saponification	Only applicable to monocotyledonous plants containing ester bridges	Lau and Van Soest, 1981
Calorimetric	Heats of combustion vary due to lignin structure, cannot be used across genera	Schramm and Bergner, 1969
Pyrolysis mass spectrometry	Equipment expensive, must have definition of lignin; can distinguish differences between different lignin chemical methods	Morrison and Mulder, 1995
Non-quantitative	Used for anatomical studies	
Phloroglucinol–HCl		Akin *et al.*, 1990
Chlorine–sulphite		Akin *et al.*, 1990
Coupling with diazonium compounds		Akin *et al.*, 1990
UV fluorescence		Hartley *et al.*, 1990

HCl, hydrochloric acid; UV, ultraviolet.

new lignin method to be routinely used in evaluation, it would have to improve on the relationship with indigestibility, as well as be competitive with respect to time and effort *vis-à-vis* currently used methods (Van Soest, 1994).

Starch

In situations where forages containing high starch levels are fed, specific analysis for starch may be useful. Assays for the reliable extraction and hydrolysis of starch have been developed (Faichney and White, 1983). Accuracy of starch analysis, however, is dependent on the specificity of enzymes used in the analysis and on complete hydrolysis of starch to glucose (Hall, 1997). Starch digestibility is probably as critical as starch quantity in determining its value in a forage sample, and can have a large impact on animal performance (Allen *et al.*, 1997). Methods that evaluate starch digestibility quickly and reliably in the laboratory have not yet been developed, so starch digestibility is not currently routinely determined (Allen *et al.*, 1997).

Water-soluble carbohydrates

WSC concentration in pre-ensiled forage allows assessment of the available supply of energy for lactic acid production during ensiling, and therefore is an important measurement for studies involving these forages. Insufficient WSC will inhibit fermentation, while excess WSC provides substrate for undesirable organisms during storage and feed-out. Forage monosaccharides, glucose and fructose, the oligosaccharides, sucrose, melibiose, raffinose and stachyose, and fructosans can be extracted with cold water (McDonald and Henderson, 1964). Cold-water extractions of legumes and subtropical and tropical grasses do not yield starch, because amylose is partially soluble in hot water, while amylopectin is not soluble in water (Smith, 1981). Procedures that solubilize starch by enzyme or dilute-acid methods, as well as the sugars, are referred to as total non-structural carbohydrate methods (Smith, 1981).

If not inactivated, forage enzyme systems will quickly modify WSC concentration. Rapid freezing, followed by freeze-drying, can minimize modifications to WSC concentration, although freeze-drying does not inactivate enzymes. Rapid oven-drying will also minimize changes in WSC. Enzymes have been inactivated with hot water or ethanol (Kerepesi *et al.*, 1996), but cold-water extraction is recommended. Hot water may solubilize fibre components (McDonald and Henderson, 1964). Protein solubility and other interferences can be minimized by extracting sugars with alcohol solutions (Wiseman *et al.*, 1960).

Spectrophotometric methods used in the UK, involving the use of anthrone, are described elsewhere (Yemm and Willis, 1954; Thomas, 1977; MAFF-14, 1986). Total WSC concentration may also be determined using a spectrophotometric method based on the reaction of phenol–sulphuric acid with carbohydrate (Dubois *et al.*, 1956).

Other carbohydrates

Analysis for sugar components may yield valuable basic information. Methods used will depend on the carbohydrates present and the degree of fractionation required (Moore and Hatfield, 1994). Traditionally, schemes for fractionating carbohydrates were long and laborious (Van Soest, 1994). The development of automated high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC) equipment has eliminated this problem, but other problems remain. General schemes cannot be applied without knowing the peculiarities of the forage involved (Van Soest, 1994).

Soluble carbohydrates are approximately 98% digestible (Van Soest, 1994), but the diversity of carbohydrate fractions included prevents their treatment as a uniform nutritional entity. One fraction, soluble fibre, which includes non-strarch polysaccharides, such as pectic polysaccharides, is of particular interest. Unlike sugars and starches, with which they may be coextracted, soluble fibre is not digested by mammalian enzymes. Compared with starch and sugars, which tend to ferment to lactic acid and result in decreases in ruminal pH, soluble-fibre fermentation tends not to produce lactic acid (Hall *et al.*, 1997). The practical implications of this for ruminant nutrition, particularly high-producing dairy cows, justifies the separation of soluble carbohydrate into a fibre and non-fibre fraction. Colorimetric or chromatographic methods to extract fibre have problems with incomplete extractions, partial degradation of carbohydrates or interference by other substrates (Hall, 1997). Recently, a gravimetric method, using the neutral-detergent solution of the Van Soest method, has been proposed to estimate the neutral-detergent-soluble fibre fraction in forages. This method is relatively simple and inexpensive, suggesting its possible use in routine evaluation schemes for forages containing significant quantities of pectic substances.

Nitrogen fractions

Total N content is usually determined by macro- or micro-Kjeldahl methods. Samples are digested in sulphuric acid, with a copper sulphate or copper–selenium (MAFF-48, 1986; AOAC-984.13, 1990) catalyst. Nitrogen is converted to ammonia, distilled and titrated. A variety of other macro- and micro-Kjeldahl methods are available, based on similar principles. Total N content can also be determined effectively by combustion in elemental analyser instruments (Emteryd *et al.*, 1990). These instruments have become more affordable, and there are fewer environmental concerns related to chemical use and disposal than with traditional Kjeldahl methods. Because the N content of forage crude protein is not always 16%, it is suggested that N content be used with the correction factor for crude protein.

Total N may be only 60–80% true protein in fresh and ensiled forages, with the remainder being non-protein N and unavailable N (Van Soest, 1994). Simple crude-protein concentration determination is inadequate to describe protein quality, particularly in high-protein fresh or ensiled forages. Ideally, any method of assessing protein quality should be able to describe the degree to which a protein contributes to bacterial crude protein and undegraded intake protein (Broderick,

1994). Models such as the Cornell net carbohydrate and protein system (CNCPS) model (Fox *et al.*, 1992) have developed the need for even more comprehensive fractionation of forage proteins (Licitra *et al.*, 1996).

Protein solubility has long been used as a technique for determining degradation characteristics of proteins (Broderick, 1994). An ideal method for determining soluble protein would be a single laboratory analysis, and there has been much research into this methodology. Solubility and extraction methods for protein fractions are subject to many of the limitations of carbohydrate solubility methods (Van Soest, 1994). Unlike carbohydrate fractionation, however, in which the Van Soest method is standard in the USA for evaluating carbohydrate fractions in forages, there is no such standardization with protein fractions. This sometimes leads to difficulties in interpreting reported values for nitrogen fractions.

Sniffen *et al.* (1992) recently proposed the use of a series of chemical fractionations to identify five N components in crude protein. It provides a rational basis for the fractionation of protein, based on nutritional availability, much like the carbohydrate fractionation system of Van Soest (1994). A suggested methodology for these N components is reported by Licitra *et al.* (1996). This suggested methodology is outlined in Table 14.3.

Non-protein nitrogen is traditionally the protein passing into the filtrate after precipitation with a protein-specific reagent (Licitra *et al.*, 1996). Trichloroacetic acid (Krishnamoorthy *et al.*, 1982) and tungstic acid (Pichard and Van Soest, 1977) are two commonly referenced methods. These methods differ in the size of the amino acids included in the precipitate. Other methods are also available (Licitra *et al.*, 1996). The choice of the method will depend on the objective of the experiment.

Table 14.3. Suggested partitioning of nitrogen and protein fractions in forages (adapted from Licitra *et al.,* 1996).

Fraction	Estimation or definition	Enzymatic degradation	Classification*
Non-protein N	Not precipitable	Not applicable	A
True protein	Precipitate with tungstic acid		
True soluble protein	Buffer soluble but precipitable (TP-IP)	Fast	B_1
Insoluble protein	Buffer insoluble	Variable	B_2
Neutral-detergent soluble protein	IP-NDIP		
ND-insoluble protein, but soluble in AD	Protein insoluble in ND, but soluble in AD	Variable to slow	B_3
Insoluble in acid detergent	Includes heat-damaged protein and N associated with lignin	Indigestible	C

* From Pichard and Van Soest, 1977; Van Soest, 1994.
TP, true protein; IP, insoluble protein; ND, neutral detergent; AD, acid detergent; NDIP, neutral-detergent-insoluble protein.

A borate phosphate buffer (Krishnamoorthy *et al.*, 1982), which ensures pH stabilization, is recommended to estimate soluble true protein as the difference between nitrogen and non-protein nitrogen (Licitra *et al.*, 1996). Other fractions (Table 14.3) are based on Kjeldahl analysis of acid-detergent and neutral-detergent residues. The ADIN fraction is assumed to be indigestible (Licitra *et al.*, 1996). This fraction is also an indication of heat damage in silages (Van Soest, 1994). The neutral-detergent-insoluble protein fraction is that protein fraction which is not soluble in neutral-detergent protein (Licitra *et al.*, 1996).

Tannins

Tannins may modify the digestibility of dietary protein and structural carbohydrates in forages used for ruminants (Mueller-Harvey and McAllan, 1992; see also Chapter 20, this volume). This may be considered to be an anti-quality component or a desirable attribute, depending on the type of tannin and its concentration in forage (Broderick, 1995; Reed, 1995). High concentrations (50–100 g kg^{-1} DM) are generally considered toxic to ruminal microorganisms, while lower concentrations reduce the soluble non-protein nitrogen content of silage and improve efficiency of protein utilization (Albrecht and Muck, 1991).

Numerous tannin extraction and quantification procedures have been developed (Table 14.4). Most procedures are specific for condensed tannins. The majority have some difficulty associated with their use, either because the procedure is not specific or because the procedure is long and laborious. Additionally, toxic effects of tannins are strongly influenced by an interaction between animal species, animal adaptation and type of tannin, so that it may be difficult to predict (Robbins *et al.*, 1991). Because of these factors, no one procedure has emerged as the prevalent method in tannin analysis. An improved method, based on precipitation with trivalent ytterbium (Giner-Chavez *et al.*, 1997), exhibits promise for more routine quantification of tannins in forages.

Tannins are not prevalent in grasses or most temperate legumes. Routine evaluation for tannins for these forages is not necessary. Tannins are prevalent, however, among dicotyledonous forbs, shrubs and tree leaves (Haslam, 1979). For animals consuming diets with these types of forages, performance may be adversely affected. In these situations, energy or nitrogen content may not limit performance, but rather this anti-quality aspect of tannins. In these cases, it becomes important to routinely analyse for tannins. Other anti-quality forage components should be handled similarly (see Chapter 20).

Vitamins and minerals

Minson (1981) stated that a complete analysis of the elements would be his first priority where animal production was low. Forages clearly provide an important source of vitamin A and E and the minerals essential for growth (Fisher *et al.*, 1995). There are no simple procedures that can be routinely used for vitamin analysis in forages (Fisher *et al.*, 1995). Forages can be prepared for mineral analysis

Table 14.4. Methods for isolating and quantifying tannins.

Method	Comment	Reference
Extraction		
Absolute methanol		Terrill *et al.*, 1990
Aqueous methanol		Terrill *et al.*, 1990
Aqueous acetone		Terrill *et al.*, 1990
Aqueous acetone with ascorbic acid and diethyl ether	Ascorbic acid minimizes oxidation of tannins, but can interfere with subsequent analysis Some high molecular weight condensed tannins not soluble	Terrill *et al.*, 1992; Reed, 1995; Giner-Chavez *et al.*, 1997
Hot methanol		Reed, 1995
Isolation methods		
Sephadex LH-20	Commonly used method	Makkar and Becker, 1994
Trivalent ytterbium	Newer method yields results similar to Sephadex LH-20	Reed *et al.*, 1985; Giner-Chavez *et al.*, 1997
Binding procedures	Non-specific isolation	
Protein		Makkar *et al.*, 1988
Polyvinylpyrrolidine (PVP)		Makkar *et al.*, 1993
Polyethylene glycol (PEG)		Silanikove *et al.*, 1996
Quantification methods		
Gravimetric	Crude estimate of tannins	
Trivalent ytterbium	Does not work well for low levels of tannins	Reed, 1995
PVP	Not specific for condensed tannins	Makkar *et al.*, 1993
Colorimetric	All require standards; internal standards required	
Redox	Subject to numerous interferences	Hagerman and Butler, 1989
Vanillin–HCl	Commonly used, condensed tannins and flavan-3-ols specific	Broadhurst and Jones, 1978; Reed, 1995
Acid butonol	Best assay for condensed tannins; very specific	Hagerman and Butler, 1989
Protein binding	Less precise than colorimetric, may relate to biological effects	
Radial diffusion	Depends on protein structure and reaction conditions	Hagerman and Butler, 1989
Radioactivity		
^{14}C PEG	Pre-extraction of tannins is not required	Silanikove *et al.*, 1996

HCl, hydrochloric acid; ^{14}C, carbon-14.

by dry ashing at 550°C or wet digestion with nitric acid (AOAC-968.08, 1990). Mineral solutions are then analysed by atomic absorption spectrophotometry or flame photometry for individual elements or by inductively coupled plasma emission spectrophotometry for multiple elements. These procedures are labour-intensive and, unless a mineral deficiency or toxicity is expected, would not warrant routine analysis except for the macrominerals.

Chemical Analyses versus *In Vitro* Techniques

Many of the chemical characterization assays are designed to estimate *in vivo* digestibility. Numerous studies have concluded, however, that chemical methods are usually less highly correlated with *in vivo* methods of measuring forage quality than microbial and enzymatic methods (Barnes, 1973; Marten, 1981; Van Soest, 1994). The inability of a single chemical analysis or solubility index to accurately predict an *in vivo* parameter is due to the complexity of biological systems (Barnes, 1973). Legumes and grasses may have the same *in vivo* digestibility but be considerably different in chemical composition. Conversely, forages may be similar in chemical composition (e.g. NDF) but considerably different in digestibility (Cherney *et al.*, 1990). It is likely, therefore, that *in vitro* and/or *in situ* assays will continue to be used (Van Soest, 1994).

In vitro assays are not without difficulty. Standardization of techniques can be difficult and many factors will affect results (Marten, 1981; Cherney *et al.*, 1993). Soluble protein is often determined by direct *in vivo* measurement, although results obtained using this methodology have not always been successful and are difficult for commercial application (Broderick, 1994). Chemical characterization of forages is easier, quicker and less expensive than conducting *in vitro*, enzymatic or *in situ* assays (Weiss, 1994). Intensifying environmental and welfare concerns will make maintaining animals for research or commercial analyses increasingly difficult.

Microbial methods are sensitive to most intrinsic factors that limit *in vivo* digestion of a forage and can yield information concerning rates of digestion (Van Soest, 1994). Microbial methods, however, provide little information regarding the actual chemical constituents influencing digestion. Information from both of these types of analyses is likely to continue to be needed.

Future

The use of modelling to predict animal performance will continue to increase. Many of these models will rely heavily on chemical characterization, because of the speed, repeatability and generally low cost of chemical characterization. Fibre, lignin and protein assays will continue to be important, due to their strong association with factors affecting animal performance. Other assays, such as soluble-fibre and starch analyses, will become increasingly important as our level of understanding of the factors affecting animal performance increases. Automation and refinement of equipment will make chemical characterization faster and more repeatable.

There are numerous methods available for fibre, lignin, protein and other

chemical components. It is likely that new methods will continue to be developed. As with lignin, it is imperative that any new method be judged on its ability to improve on the relationship with intake or indigestibility, as well as be competitive with respect to time and effort *vis-à-vis* currently used methods. In addition, because there may be numerous assays to determine the same component, such as lignin, it is the responsibility of the researcher to know the limitations of their methods and what they actually are analysing (e.g. permanganate lignin versus acid-detergent lignin). Researchers should also take special care to report the exact methodology that they are using. With the advent of new methodologies and a better understanding of factors limiting animal performance, the chemical characterization of forages will continue to be a worthwhile field of endeavour.

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15 Near-infrared (NIR) Spectroscopy: an Alternative Approach for the Estimation of Forage Quality and Voluntary Intake

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Introduction

The demand for objective estimates of forage quality has increased significantly in recent years for several reasons. The intensive feeding of housed animals in Europe and North America requires constant monitoring of diet composition to ensure consistent levels of milk or meat production. In Australia, where grazed pasture is the foundation of the major livestock industries, the growth and quality patterns of pastures vary widely. Hence real-time decisions have to be made by producers on whether or when to purchase and utilize feed supplements (Flinn and Downes, 1996). Selection or breeding for nutritive value is now considered an important factor by many forage-plant breeders, with the need often to test a very large number of lines.

The widely used but empirical techniques to measure fibre content of forages are labour-intensive and hence expensive, even with modern digestion equipment. The highly regarded *in vitro* (Tilley and Terry, 1963) and enzyme-based techniques for estimating digestibility of forages require approximately one week to complete.

The physical, non-destructive technique of near-infrared (NIR) spectroscopy has been described as 'the most practicable and exciting analytical technique to hit the agricultural and food industries since Johann Kjeldahl introduced the Kjeldahl test' (Williams and Norris, 1987). Since its introduction in the 1960s for measuring moisture in grains (Norris and Hart, 1965), the number of analytical applications of NIR in quality control has expanded dramatically. The technique is also 'clean and green', in that no chemicals are used and there are no health and safety problems.

NIR spectroscopy represents a radical departure from conventional analytical methods, in that the entire sample of a forage is characterized in terms of its absorption properties in the NIR region, rather than separate subsamples being treated

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with various chemicals to isolate specific components. This forces the analyst to abandon his/her traditional narrow focus on one sample and one analyte at a time and to take a broader view of the relationship between components within the sample and between the sample and the population from which it comes.

The main disadvantages of NIR spectroscopy are the high initial cost of the instrumentation and its reliance on chemometrics (Norris, 1989a), leading to its traditional but unfair reputation as a 'black box', due to a lack of understanding of the way in which NIR spectra relate to composition.

This chapter reviews the principles and practice of NIR spectroscopy in the assessment of forage quality, and outlines the major steps required for successful use of the technique. Other reviews (see Givens *et al.*, 1997) have also extended into the use of NIR spectroscopy for predicting the nutritive value of foods for animals and humans.

NIR Spectra: What are we Really Measuring?

Spectroscopy literally means looking at light and is based on the interaction of electromagnetic radiation with the matter to be analysed. The theory of NIR spectrosocopy is covered in depth in the books of Williams and Norris (1987) and Osborne *et al.* (1993).

The NIR region of the electromagnetic spectrum lies between the visible and infrared regions, and is usually defined by the wavelength range 700–3000 nanometres (nm) (Norris, 1989b). However, most analytical use of NIR is between 1100 and 2500 nm. NIR spectra are essentially plots of reciprocal $\log_{10}$ reflectance (log 1/*R*) versus wavelength. The spectra appear as smooth, rolling lines with few well-defined features (Murray, 1988), but they consist of many overlapped bands, since the reflectance spectrum of a forage sample is the summation of the spectra of its major chemical components. The challenge is to extract useful information on nutritive value of forages from the reflectance data.

Prominent absorptions in NIR spectra of forages (Fig. 15.1) include water, with two bands at 1940 nm (combination band) and 1450 nm (first overtone band); aliphatic carbon (C)-hydrogen (H) bands (lipids) at 2310, 1725, 1400 and 1210 nm; and oxygen (O)-H bands (carbohydrates) around 2100 and 1600 nm (Murray, 1983). The nitrogen (N)-H absorptions due to amide structures present in protein occur predominantly at 2180 and 2055 nm (Wetzel, 1983), but these are often masked by the broad O-H band at 2100 nm. It is the shape of the spectral line, or the rate of change in slope with wavelength, that conveys chemical information (Murray and Williams, 1987). Hence first- or second-derivative plots of log 1/*R* are useful, as they can resolve overlapping bands into component absorptions, which may appear as shoulders on larger peaks, as well as minimizing the effect of particle size (Shenk and Westerhaus, 1994). The second derivative is particularly useful, as it produces a negative peak that almost coincides with the wavelength position where the log 1/*R* peak occurs (although frequently not apparent from the log 1/*R* spectra) (Fig. 15.2).

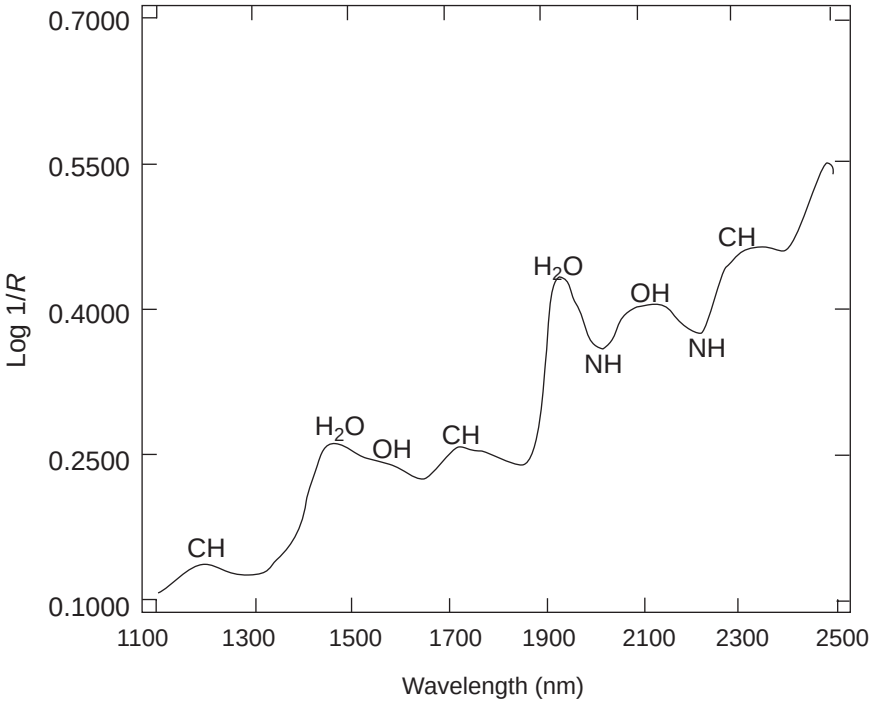

Fig. 15.1. NIR log 1/*R* spectrum of hay sample showing locations of absorptions due to major functional groups.

NIR Instruments and Sample Presentation Methods

There are many instrument types available for NIR analysis, and a detailed comparison is beyond the scope of this chapter (see Osborne *et al.*, 1993). However, the three major types of devices for wavelength selection are monochromators, filters and diode arrays. Monochromators scan the whole wavelength range by using a grating or prism and they provide maximum versatility. Tilting-filter instruments have three or six tilting filters, which, in effect, allow segments of spectra to be scanned. Fixed-filter instruments measure log 1/*R* at discrete wavelengths only, but are quite satisfactory for applications where the constituents are well defined and the appropriate wavelengths well known. Diode-array instruments use no moving parts for wavelength selection and have the advantage of being able to scan at high speed.

There are basically two alternative procedures for presenting a forage sample to an NIR instrument. Small sample cups are used for finely ground, uniform materials and large rectangular cells are used to pack unground and often high-moisture heterogeneous sample (Shenk and Westerhaus, 1994). Until recently, most NIR forage analysis has been done using dried, ground samples and the recommended procedures for drying (if required), grinding, mixing and packing are well established

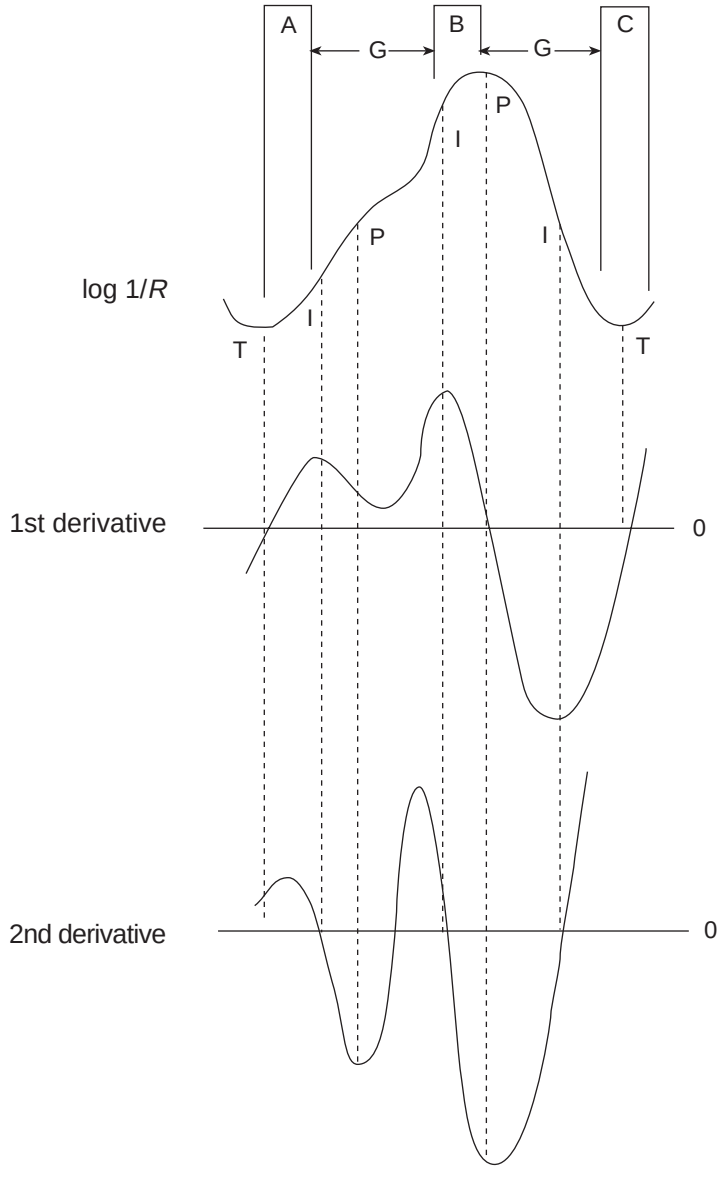

Fig. 15.2. The relationship between log 1/*R* and first- and second-derivative log 1/*R* spectra. The first derivative is computed from the difference (A − B), whilst the second derivative is computed from (A − B) − (B − C), or A − 2B − C (after Murray, 1988). A, B, C, segments; G, gap; T, trough; P, peak; I, point of maximum inflection.

(Shenk and Westerhaus, 1994). However, there are advantages in analysing forages in their natural (undried), unground state, due to reduced sample preparation time and, in the case of fermented forages, avoidance of analytical errors due to losses of volatile compounds by oven-drying.

NIR Data Processing and Calibration

Calibration is the mathematical process required to relate NIR optical measurements to the desired constituent or property used to define the nutritional quality of forages. Calibration is the key to successful use of the NIR technique, and many different approaches have been proposed. There are a number of major steps which are essential to obtain a satisfactory working calibration, i.e. sample selection, acquisition of spectra and reference data, pretreatment of spectral data, derivation of the regression model and validation of the model.

Sample population structuring

Selection of the appropriate samples is critical for a successful calibration. Dardenne (1996) confirmed that extended time spent on the development of appropriate calibration sample sets is rewarded by more stable and accurate calibrations. A decision must first be made on the intended use of the calibration, e.g. if the purpose is to analyse ryegrass samples, the calibration population should consist only of ryegrass. Conversely, if many different types of hay are to be analysed, all types must be represented in the population. The calibration population must include all sources of variation likely to be found in future unknown samples of similar material, such as chemical, physical and botanical characteristics (Windham *et al.*, 1989; Shenk and Westerhaus, 1994). The traditional method of sample selection involved either structured or random sampling from a population, using information already available on chemical or botanical composition (Abrams, 1989). A more convenient procedure is to choose representative samples from a large population on the basis of their spectral characteristics, thus reducing the number of samples requiring reference data. Software is available for this purpose, with one approach using algorithms known as CENTER and SELECT, where spectral boundaries for a product are first established using principal-component scores and then samples are selected from defined neighbourhoods from within the population (Shenk and Westerhaus, 1991).

There is no easy answer to the question of how many samples are needed for calibration. For agricultural products, calibrations based on fewer than 50 samples are seldom satisfactory. Windham *et al.* (1989) concluded that 50–100 samples were adequate for narrow-based or 'closed' populations, whereas 150 or more samples were necessary for broad-based or 'open' populations. With recent advances in software and computer power, it is now possible to establish calibration populations or 'libraries' containing hundreds or even thousands of samples, which are necessary for multi-product forage calibrations (Shenk and Westerhaus, 1993).

Quality of reference analyses

The quality of reference-method analysis has a crucial effect on the accuracy of NIR calibrations. Poor calibration accuracy has often been incorrectly blamed on NIR instruments, when, in most cases, the laboratory reference method and other

associated factors were at fault (Williams, 1987). Before assessing the accuracy of an NIR calibration, the error associated with the reference method should be known, but this is often ignored.

Pretreatment of spectral data

Because NIR spectra are affected by particle size, light scatter and path-length variation, pretreatment of the spectral data can often (but not always) improve calibration accuracy. Commonly used corrections include standard normal variate–detrending (SNV-D) (Barnes *et al.*, 1989), multiplicative scatter correction (MSC) (Martens and Naes, 1989) and derivatization. Detrending removes the linear and quadratic curvature of each spectrum, SNV scales each spectrum to have a standard deviation of 1.0 and MSC expands or contracts each spectrum and shifts it up or down to look most like the target spectrum (usually the mean of a file of spectra). First- or second-derivative mathematical treatments are most frequently employed.

Calibration methods

Multiple regression techniques relating NIR absorbance values (*X* variables) at selected wavelengths to reference values (*Y* variables) have the longest history of use in NIR calibration, and two common variations are step-up and stepwise regression. Step-up regression selects the *X* variable most highly correlated with *Y* and then progressively adds other *X* variables with the next highest partial correlations. This process is stopped when no additional improvement in correlation is found, by which time 'overfitting' occurs, which is detected by testing (validating) the model on an independent set of samples. Stepwise regression is similar, except that, when a new *X* variable is added, all variables are re-evaluated before continuing, until no further changes result in improvement to the overall fit (Shenk *et al.*, 1981). Errors are usually lower than for step-up regression, as more combinations of *X* variables are evaluated.

Alternative calibration methods include principal-component regression (PCR) (Cowe and McNicol, 1985), partial least-squares regression (PLS) (Martens and Naes, 1989) and artificial neural networks (Naes *et al.*, 1993). In PCR, the original spectral data are compressed into a small number of independent components, or scores, which are used in the regression equation instead of the spectra themselves. This overcomes the problem of intercorrelation between spectral data points. Typically, a model containing between five and ten principal components gives satisfactory predictions. PLS is similar to PCR, except that PLS uses the laboratory reference data (*Y*) as well as the spectral data (*X*) in computing the factors used in regression. This often results in better predictions. A variation of this procedure, called modified PLS (MPLS), is frequently used in forage calibrations and involves standardizing the variables in the regression after each factor is calculated (Shenk and Westerhaus, 1991).

There is no 'best' calibration technique to use in all situations. MPLS has

been shown to be superior to multiple linear regression (MLR) (Shenk and Westerhaus, 1991; Windham and Flinn, 1992), but this is not always the case. Time is usually better spent on obtaining high-quality reference data for calibration than on trying endless combinations of data treatments and regression techniques.

Validation: how do you know the calibration is working?

Different approaches to validation of an NIR calibration equation may be needed, depending on the way in which the equation is used. The traditional procedure using MLR is to randomly select samples from the calibration population, omit them from the calibration process and use them as a validation set to test the calibration equation. Error is measured as standard error of prediction (SEP). This test is satisfactory if working with a closed population but is inadequate for open populations, such as in routine testing of widely diverse forage samples. In this situation, a separate, independent set of samples should be used to test the robustness of the equation before it is implemented. Windham *et al.* (1989) have recommended a protocol for this purpose.

An alternative now widely used in PCR or PLS/MPLS regression is cross-validation, where the calibration set is split into several groups and calibration progressively performed on each group until every sample has been used for both calibration and validation. The validation errors are combined into a standard error of cross-validation (SECV) (Shenk and Westerhaus, 1994). It can thus be argued that both calibration and validation are able to be done in one process.

The performance of a calibration equation on sets of validation samples will depend on the degree to which all sources of variation in the validation samples are encompassed in the calibration set. The equation must be monitored over time to ensure that its accuracy is maintained. Also, random samples during routine analysis should be set aside, analysed by the reference method and subjected to a monitoring test such as that recommended by Shenk *et al.* (1989).

Can a calibration equation be too broad?

It is now widely accepted that broad-based NIR calibrations for forage analysis are more useful than those based on separate species. Whilst the latter can be slightly more accurate, they lack robustness and involve more work and expense (Flinn, 1990; Smith and Flinn, 1991). Studies have also found that multi-forage calibrations (e.g. comprising hay, fresh forage, haylage and silage) can be nearly as accurate as those for single products, provided enough samples are present in the calibration population (Shenk and Westerhaus, 1993).

An alternative approach is to derive a local calibration for a new sample set, using samples in a broad-based calibration population which best match the spectra of the new samples and, if necessary, expanding this local calibration with a few of the new samples (Shenk *et al.*, 1993). The local calibrations in this study were found to be more accurate than the broad-based calibrations.

A disadvantage of this procedure is the increased number of equations required in routine analysis, increasing the complexity of the analytical system. Another development involves computing a specific calibration equation for each sample analysed, based on selected similar samples within a large database. Consistent reduction in SEP was found using this technique for forage data sets (Sinnaeve *et al.*, 1994), but it was considered too slow for real-time analysis.

Evaluation of Forages by NIR Spectroscopy: a Brief History

Measurement of forage quality by NIR was first reported by Norris *et al.* (1976). In what has become a classic paper, the authors found standard errors of 0.95, 3.1, 2.5, 2.1 and 3.5%, respectively, for crude protein (CP), neutral-detergent fibre (NDF), acid-detergent fibre (ADF), lignin and dry-matter digestibility (DMD) *in vitro*, measured in a diverse range of forage samples. This paper stimulated wide interest and a great many investigations soon began across the world to extend and refine the use of NIR spectroscopy in forage analysis. A significant achievement was the adoption of NIR as an official method by the Association of Official Analytical Chemists for the measurement of CP and ADF in forages (Barton and Windham, 1988).

The successful use of NIR to measure the major organic components of forages has been demonstrated by many workers, and was reviewed extensively by Murray (1993). As well as protein and fibre fractions, water-soluble carbohydrates have been measured successfully by NIR in perennial ryegress (Parnell and White, 1983) and total non-structural carbohydrates in lucerne roots (Brink and Marten, 1986) and tropical grasses (Brown *et al.*, 1987).

Introduction of alternative sampling devices for NIR instruments has allowed the analysis of fresh (undried) and unmillled forages (e.g. silage and fresh grass) by NIR spectroscopy (Shenk, 1992). Although errors are often slightly higher for components such as CP, ADF, NDF and DMD *in vitro* than on dried, ground samples, this is balanced by the ability to scan much larger samples, the avoidance of compositional losses and changes due to oven-drying, and a major reduction in analysis time and cost, due to no sample preparation being necessary (Sinnaeve *et al.*, 1994; de la Roza *et al.*, 1996; Kennedy *et al.*, 1996). Park *et al.* (1996) have also reported that NIR spectroscopy can be used for the prediction of fermentation characteristics and biological parameters on undried silages.

A major limitation in the use of NIR spectroscopy for forage analysis is the inadequacy of many conventional laboratory reference methods. The problem was described succinctly by Murray (1988) when he stated that 'we are using 19th century chemistry to calibrate 20th century technology'.

The Role of NIR Spectroscopy for Forage Evaluation

Predicting the digestibility in vivo *of forages*

The use of NIR spectroscopy to predict digestibility *in vivo* has been widely reported by several research groups, including work on mixed forages by Lindgren

(1983) and on grasses by Robert *et al.* (1986). In most cases, NIR spectroscopy provided a better prediction of digestibility than traditional laboratory procedures. In the UK, the most important development was reported by Barber *et al.* (1990). For grass silage, they showed for the first time that NIR spectroscopy was able to predict the organic-matter digestibility (OMD) *in vivo* more accurately than a range of commonly used laboratory methods. This work used a stepwise MLR of eight first- and second-derivatized spectral terms. A summary of the results obtained after validation of the various relationships is shown in Table 15.1.

The work of Barber *et al.* (1990) highlighted the fact that most traditional relationships that had been developed for predicting digestibility from various laboratory methods were, strictly speaking, calibrations and had never been exposed to the validation procedure necessary for NIR spectroscopy calibrations. Recently, De Boever *et al.* (1996) have also shown for grass silages made in Belgium that NIR spectroscopy provided the best calibration relationship for OMD *in vivo* with a variance accounted-for value (R^2) of 0.79, compared with values of 0.68, 0.64 and 0.53 for cellulase digestibility, rumen-fluid digestibility and acid-detergent lignin determinations, respectively.

NIR calibrations have also been successfully developed for prediction of the digestibility and metabolizable energy (ME) content of other forages including grasses and cereal straws. Givens *et al.* (1992b) showed that the metabolizable energy (ME) content of mixed-growth grasses could be predicted by a five-term NIR calibration equation and that this technique was equal to the best traditional laboratory method (pepsin–cellulase). One of the main advantages of the five-term NIR spectroscopy equation was that it did not discriminate between spring, summer and autumn grasses or between grasses harvested in different years.

Gordon *et al.* (1998) have recently reported on an extensive study that compared a range of sample preparation methods and NIR spectral model types, using fresh grass silage, for predicting OMD *in vivo* and voluntary intake. They compared a total of eight sample preparation/NIR scanning methods involving three extents of comminution, two liquid extracts and scanning via either an external probe (1100–2200 nm) or an internal cell (1100–2500 nm). In addition, all log 1/*R* spectral data were studied using three modelling techniques, together with a range of data transformations. A summary of the main findings is presented in Table 15.2.

Table 15.1. Comparison of NIR spectroscopy with several laboratory methods for predicting the organic matter digestibility *in vivo* of grass silage (after Barber *et al.,* 1990).

	Validation statistics			
Method	R^2	SEP	Slope	Bias
NIR – 8-term	0.76	2.6	0.93	−0.79
In vitro OMD	0.64	3.6	0.89	−1.85
Pepsin–cellulase	0.40	4.7	0.71	2.33
Acetyl bromide lignin	0.14	5.3	0.48	1.18
Modified acid-detergent fibre	0.20	5.1	0.52	−0.59

SEP, standard error of prediction (%).

Table 15.2. Analysis of variance of standard error of prediction data for comparisons of sample preparation methods and spectral model type for predicting organic-matter digestibility *in vivo* and voluntary intake of fresh (undried) grass silage (after Gordon *et al.*, 1998).

	Voluntary intake (g kg^{-1} $W^{0.75}$)	OMD *in vivo* (g kg^{-1} OM)
Spectral model type (means across sample method)		
MPLS	6.57	38
PCR	6.68	41
MLR	7.37	40
SEM	0.123	0.9
Significance	$P < 0.05$	$P < 0.06$
Silage sample preparation method (means across spectral model type)		
Scanned by external NIR probe		
Intact, lightly packed in open tray	7.88	41
Intact, lightly packed in tube	5.97	36
Coarsely chopped, lightly packed in tube	6.86	34
Finely milled, lightly packed in tube	6.69	33
Scanned by internal NIR cups		
Coarsely chopped, lightly packed in NIR cup	6.73	29
Finely milled, lightly packed in NIR cup	6.14	26
Expressed liquor, 5 drops on viscous sample cup	6.82	54
Extracted eluent, 5 drops on viscous sample cup	8.37	62
SEM	0.349	1.4
Significance	$P < 0.01$	$P < 0.001$

MPLS, modified partial least squares; MLR, multiple linear regression; PCR, principal-components regression; SEM, standard error of the mean.

For predicting OMD *in vivo*, they showed that the fine-cup method of preparation was the best, although, for voluntary intake, it came second to the intact tube. However, the authors highlight the fact that, when compared on the basis of SECV (valid for MPLS and PCR models), both OMD *in vivo* and intake predictions were always best using the fine-sample cup method. Overall, these findings support the overall concept that enhanced comminution of forages prior to NIR analysis is an important element in improving the accuracy of predictions of both OMD *in vivo* and intake.

Prediction of voluntary intake of forages

In many ruminant production systems, energy intake from forage is more influenced by voluntary dry-matter (DM) intake than by the energy concentration of the forage DM. Accordingly, there have been many attempts to predict intake from

various attributes describing forage composition (for a review, see Minson, 1982), although there are often many non-forage factors that may influence voluntary intake, including the amount of supplementary feed given and animal and environmental factors.

It was proposed by the Agricultural and Food Research Council (AFRC, 1991) that NIR spectroscopy could be one of the most promising areas for predicting the voluntary intake of grass silage, since the spectra contain information on the entire chemistry of the feed. In fact, NIR spectroscopy has been successfully used to predict the intake of mainly non-fermented forages. The early work of Norris *et al.* (1976) and the more recent studies of Ward *et al.* (1982) and Flinn *et al.* (1992) are summarized in Table 15.3. The work of Flinn *et al.* (1992) was unusual in that it involved the use of an MPLS regression, where the NIR spectral data of faeces were related to forage intake, intake having been originally calculated from the *n*-alkane dosing procedure of Mayes *et al.* (1986).

Recent studies by Park *et al.* (1997) and Steen *et al.* (1998) have shown that NIR spectroscopy can also accurately predict the voluntary intake of grass silage by cattle. Park *et al.* (1997) measured the DM intakes of 136 grass silages, using 192 individually fed beef cattle (range 45 to 113 g DM kg^{-1} $W^{0.75}$), and showed that an MPLS spectra relationship could achieve a prediction accuracy (SECV) of ±5.05 g DM kg^{-1} $W^{0.75}$ (R^2 cross-validation 0.78). Steen *et al.* (1998) showed NIR spectroscopy to be superior to some 35 other measurements of silage quality, digestibility *in vivo* and rumen DM degradability *in situ*. Of the other measurements, the next best relationships with intake were obtained with electrometric titration (R^2 0.53), digestibility *in vivo* (R^2 0.30) and DM degradability *in situ* (R^2 0.28).

The fact that NIR spectroscopy seems to offer considerable potential for predicting voluntary intake of forages suggests that such studies should be extended to examine such effects as concentrate supplementation and the impact of feeding forage mixtures.

Table 15.3. Prediction of voluntary dry-matter intake of forages by NIR spectroscopy.

Forage type	Number of measurements	Intake (g kg^{-1} $LW^{0.75}$ day^{-1})		NIR prediction		Reference
		Species	Range	R^2	SEP	
Mixed	76	Sheep	39.7–114.3	0.62	7.8	Norris *et al.* (1976)
Grazed pasture	21	Cattle	52.6–112.3	0.72	9.6	Ward *et al.* (1982)
Grazed pasture	80	Sheep	430–1458*	0.80	140	Flinn *et al.* (1992)

* Organic-matter intake $animal^{-1}$ day^{-1}; LW, live-weight.
SEP Standard error of prediction.

Prediction of new aspects of energy value of forages

It is now becoming clear that the unifying concept of digestibility or energy value is inadequate and, in particular, it is now recognized (AFRC, 1992) that it is important to distinguish between the energy in the forages that is available for rumen microbial growth (fermentable energy) and that which is directly available to the host animal. The importance of estimating fermentable energy is because it represents the portion of dietary energy available for microbial protein synthesis, which in many situations represents at least 0.7 of the total α-amino nitrogen available to the host animal.

Of particular importance for ensiled forages is the fact that they may contain substantial amounts of lactic acid and volatile fatty acids (VFA), which provide little or no fermentable energy (AFRC, 1992; Deaville and Givens, 1998b). Thus, an important future role of NIR spectroscopy would be to predict the concentrations of the individual or total fermentation acids (see Abrams *et al.*, 1988; Sinnaeve *et al.*, 1994). An alternative approach to the problem has been reported by Snyman and Joubert (1992), who showed that fermentation acids in sorghum silages could be adequately predicted by NIR spectroscopy if the acids were first converted to their corresponding salts before microwave-drying. Recently, Deaville and Givens (1996a) (Table 15.4) showed that an MPLS model of the NIR spectra obtained from 800 fresh (undried) silages could be used to predict lactic acid and VFA.

The prediction of silage fermentation acids represents an approach to estimating fermentable energy content by difference. An alternative approach would be a direct prediction of fermentability. The gas-production technique, originally described by Menke *et al.* (1979) and modified by, for example, Cone *et al.* (1996), is a method that has the potential to measure fermentability more directly, including fermentation rate. Recent integrated studies on forages, using both the gas-production technique and NIR spectroscopy, by Herrero *et al.* (1996) showed that it was possible to derive reasonable NIR predictive relationships for cumulative gas volumes produced between 6 and 48 h of incubation, although rates of gas production could not be predicted.

Table 15.4. Estimate of fermentation acids in silages by NIR spectroscopy (Deaville and Givens, 1996a).

	Calibration		Validation	
Fermentation acid	R^2	SEC	R^2	SECV
Lactic acid	0.73	4.24	0.72	4.37
Acetic acid	0.69	1.72	0.66	1.80
Propionic acid	0.59	0.47	0.56	0.49
n-Butyric acid	0.87	0.82	0.86	0.87

SEC, standard error of calibration (g kg^{-1} fresh weight); SECV, standard error of cross-validation (g kg^{-1} fresh weight).

For most forages, the cell-wall fraction represents the major fermentable energy-yielding substrate. However, the rumen degradability/fermentability of cell walls is very variable. Generally, there appear to be relatively few estimates of the extent and rate of degradation of forages, although some *in vitro* estimates of cell-wall digestion dynamics of grass silages have been presented by Foulkes (1993) and Deaville and Givens (1996b). The latter authors also showed that NIR spectroscopy gives considerable scope for predicting potential and effective cell-wall degradability, although the rate of degradation and the lag phase could not be predicted (Table 15.5). As proposed by Herrero *et al*. (1996), the failure of NIR spectroscopy to predict dynamic values may relate to the exponential nature of the models currently used, thereby altering the distribution and range of the parameters and thus preventing a good fit by the NIR calibration approaches, which are all multivariate and linear.

Prediction of new aspects of protein value of forages

Many reports in the literature show the use of NIR spectroscopy to predict the CP content of forages and other feeds. However, for ruminant animals, protein quality is more accurately described by estimating three aspects of protein digestion in the rumen: the immediately soluble fraction, the insoluble but degradable fraction and the rate of degradation of the degradable fraction (AFRC, 1992). These measurements are normally carried out using the *in situ* technique, which not only requires the use of specially modified animals, but is also slow and expensive.

For 19 previously frozen fresh grasses, Waters and Givens (1992) demonstrated that it was possible to derive NIR calibrations for the nitrogen degradation values derived *in situ*, although overall the calibrations were relatively weak and the shortage of samples prevented any validation. Better results were obtained when the population was split into primary growths and regrowths, although the population sizes were very small.

Since a very high but variable proportion (0.45–0.75) of the nitrogen present in grass silage is water-soluble, and hence soluble in the rumen, the ability to predict nitrogen solubility by NIR spectroscopy would be a significant improvement over simply predicting total nitrogen or CP concentration. Abrams *et al.* (1988)

Table 15.5. Prediction of *in vitro* cell-wall degradability characteristics of grass silage (n = 113) by NIR spectroscopy (Deaville and Givens, 1996b).

Degradability parameter	Mean	Calibration		Cross-validation	
		R^2	SEC	R^2	SECV
Potential degradability (%)	73.3	0.73	3.76	0.66	4.27
Effective degradability (%)	53.8	0.83	2.42	0.70	3.21
Rate of degradation (h^{-1})	0.07	0.50	0.01	0.36	0.01
Lag phase (h)	2.49	0.32	0.81	0.17	0.89

SEC, standard error of calibration; SECV, standard error of cross-validation.

showed that with various fresh (undried) silages (DM content 394 ± 137 g kg^{-1}), the solubility and insolubility of nitrogen in buffered sodium chloride solution could be estimated by a six-term multiple stepwise regression (MSR) calibration of second-order derivatized log 1/*R* spectral data (Table 15.6). Recent work (E.R. Deaville and D.I. Givens, unpublished) with fresh (undried) grass silage showed that an MPLS calibration had an ability to predict the soluble nitrogen and ammonia nitrogen concentrations.

Use of NIR Spectra to Monitor Forage Digestion

Most of the initial NIR calibrations were derived by MLR without any inherent knowledge about which regions of the NIR spectrum contained the most information in relation to forage digestibility. Reviews of the literature (Clark and Lamb, 1991; Deaville and Baker, 1993) relating to the prediction of forage digestibility by NIR spectroscopy indicates that several wavelength regions regularly appear as being the most important. Table 15.7 presents a brief summary of some of the reports.

Table 15.6. Estimation of nitrogenous constituents in silage by NIR spectroscopy (after Abrams *et al.*, 1988).

Nitrogen fraction	Mean value (g kg^{-1} fresh weight)	SD	Calibration statistics	
			R^2	SEC
Total nitrogen	9.55	4.49	0.98	0.70
Soluble nitrogen	3.48	1.99	0.92	0.58
Insoluble nitrogen	5.97	2.70	0.98	0.35

SD, standard deviation; SEC, standard error of calibration.

Table 15.7. Primary and secondary NIR spectroscopy wavelength segments used to predict forage digestibility.

Forage	Predicted term	Wavelength (nm)		Reference
		Primary	Secondary	
Mixed	DMD *in vivo*	2266	1662	Norris *et al.* (1976)
Grass	ME	1668	1364	Lindgren (1983)
Grass silage	Lignin	1658	2286	Murray *et al.* (1987)
Hays	DMD *in vivo*	1326	2266	Coelho *et al.* (1988)
Hays/grass	ME	1666	1690	Lindgren (1988)
Grass silage	OMD *in vivo*	2266	1662	Kridis (1989)
Grass	ME	1658	2280	Givens *et al.* (1992b)
Cereal straw	OMD *in vitro*	1662	ns	Guzmán *et al.* (1996)

DMD, Dry-matter digestibility; ns, not stated.

Table 15.7 shows that the spectral regions around 1650–1670 and 2260–2280 nm consistently appear as the most important regions associated with digestibility. Interestingly, the wavelength segments associated with gas production (Herrero *et al.*, 1996) indicated that the primary wavelength is in the same region for digestibility, located between 1664 and 1696 nm. Subsequently, by sequentially subtracting spectra associated with the residues of *in situ* bag incubations in the rumen, Givens *et al.* (1992a) demonstrated that, in cereal straws, the 1650 and 2254 nm regions related to the indigestible fractions of the forage. Deaville *et al.* (1992) went on to show that these regions related to the most indigestible fractions of the plant cell wall and Deaville and Givens (1998a) have shown that these regions are also important in relation to the rumen degradation of grass silage, maize silage and fresh grass. Other findings (Russell *et al.*, 1989; Deaville and Givens, 1998a) indicate that these regions relate to lignin bonding and this gives further confidence that regions of the NIR spectrum have biological meaning. Further aspects of this have also been discussed by Deaville and Baker (1993).

Minerals and Antinutritional Factors

Mineral analysis of forages by NIR appears unlikely, as minerals do not absorb energy in the NIR region. However, correlations between minerals and other organic components allow reasonable NIR calibrations to be obtained in some cases. In a study involving a wide range of minerals in various forages, Clark *et al.* (1987) concluded that accurate NIR analysis was limited to calcium (Ca), phosphorus (P), potassium (K) and magnesium (Mg), which were found to have coefficients of variation (CV) ranging from 10 to 20%. The CV values for other minerals generally exceeded 20%. Smith *et al.* (1991) obtained a calibration for Mg in perennial ryegrass with a CV of 16% and concluded that NIR could be a useful tool for preliminary screening of ryegrass lines for Mg in a breeding programme. Shenk and Westerhaus (1994) concluded that mineral analysis by NIR can be useful if the alternative is to assume an average value from a feed-composition table.

NIR has also been used to screen various forages for antinutritional factors/secondary plant products. These are normally present in small quantities but may be detectable if they affect NIR spectra, again by means of secondary correlations. Examples include total alkaloids and condensed and total phenolics.

Conclusions

NIR spectroscopy has revolutionized the analysis and nutritional characterization of forages. This is extremely important since, worldwide, human food production from ruminant animals is highly dependent on efficient utilization of a diverse range of forage sources. The developments in NIR spectroscopy have allowed for the rapid evaluation of forages thus allowing for rapid, strategic and economic decisions to be made regarding appropriate supplementation or forage upgrading. Current evaluation systems for describing the nutritional characteristics of forages and other feeds for ruminants are undergoing very substantial changes in many

parts of the world, with greater emphasis being placed on estimating the nutrient supply to the animal rather than the all-embracing values of energy and protein. Such approaches will undoubtedly require more complex methods of evaluation and it seems likely that they will only be put into widespread practice if they can be predicted by NIR spectroscopy.

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16 NMR and Other Physicochemical Techniques for Forage Assessment

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Introduction

The use of high-resolution (HR) spectroscopic techniques (e.g. high-field nuclear magnetic resonance (NMR), mid-infrared (MIR), Raman spectroscopy and pyrolysis mass spectrometry (PyMS)) are finding increased usage in forage assessment. These techniques, as opposed to low-resolution (LR) spectroscopic techniques (e.g. near-infrared (NIR) and ultraviolet (UV) spectroscopy), generally provide more specific and detailed information of a primary nature. The trade-off for the gain in resolution is often the sacrifice of time, increased cost and the requirement to use more skilled operators. However, the employment of HR techniques is frequently necessary when the LR techniques fail to provide the desired information or require verification.

UV spectroscopy can be an excellent primary source of information, if the information sought is related to a single or isolated chromophore. However, forages tend to contain a multitude of chromophores, making such analyses difficult. NIR spectroscopy is generally not a source of direct information but an indirect or secondary technique, requiring independent calibration by a primary technique, using chemometric approaches (Kowalski, 1977; Martens *et al.*, 1991). The primary technique is normally some form of gravimetric analysis for moisture, protein, lignin or acid-detergent fibre (ADF) (AOAC, 1997). Although this approach is sufficient and cost-efficient for many needs, the dependence on proximate analyses limits the effectiveness of NIR spectroscopy (Preston *et al.*, 1997).

To date, the focus of the HR techniques has been directed towards providing a route to primary molecular structures. If the HR spectroscopic results can be linked to the LR techniques or be made more cost-effective themselves, the accessibility to more reliable results should be greatly enhanced. The use of chemometrics with any of the techniques can also increase their utility (Bro *et al.*, 1997). Since anatomy and chemical composition have combinatory effects on the digestibility of forages, additional benefits can be realized by the integration of any of these spectroscopic with microscopic techniques.

 Forage Evaluation in Ruminant Nutrition (eds D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed)

NMR Spectroscopy

Both solution and solid-state NMR have been found to be powerful tools for examination of forages and their residues. True solution NMR studies are limited to extractable components that are soluble in suitable deuterated NMR solvents. If the isolated material is pure, a wide array of single and multidimensional experiments may be employed to unequivocally assign the molecular structure and solution confirmation of a molecule (Bruch, 1996). Solid-state NMR is well suited for examining the intact structure of forage materials, without requiring extraction. It is especially suited for the *in situ* study of forages and their residues. However, it is more limited than solution NMR in the variety of experiments that can be performed and the line widths of signals are broader (i.e. show lower apparent resolution).

New experiments, which lie somewhere in the realm between the solution and solid methods, are now in vogue. These are experiments in which all or part of the sample is made mobile by hydration. The carbon-13 (^{13}C) version of this experiment permits separation of component spectra based on an increased separation of relaxation times, due to selective hydration (Newman, 1992). The proton (^{1}H) version of this experiment is characterized by relatively narrow line widths and permits greater use of multidimensional experiments (Gil *et al.*, 1997). These experiments show potential application to studies on forages, but have not yet been employed for that purpose.

Solution NMR spectroscopy

Solution NMR spectroscopy continues to benefit from the development of superconducting magnets with increased field strengths. Commercially available systems, with field strengths of 18.5 tesla (T) (800 MHz for protons), are now being delivered. This is a significant increase in field strength over the previously available highest fields of 11.5 and 14 T (500 and 600 MHz). This means the possibility of even greater signal dispersion and sensitivity and the ability to unravel even more complex polymeric structures, such as those habitually encountered in plant materials used as forages.

Examples of the effective use of the currently available solution NMR technology are provided in studies directed at determination of the specific involvement of hydroxycinnamic ester moieties in restricting the availability of cell-wall polysaccharides of forage grasses to ruminant digestion. Several hydroxycinnamic oligosaccharide esters have been isolated and their structures have been subjected to detailed characterization by solution NMR. Figure 16.1 shows examples of such compounds that are currently known. These compounds have been isolated from bagasse (Kato *et al.*, 1983, 1987, 1990), barley straw (Mueller-Harvey *et al.*, 1986), bamboo (Ishii and Hiroi, 1990a, b; Ishii *et al.*, 1990; Ishii, 1996), coastal Bermuda grass (Hartley *et al.*, 1990; Himmelsbach and Hartley, 1993; Himmelsbach *et al.*, 1994) and maize (Kato and Nevins, 1985).

The characterization of compound 3b in Fig. 16.1 provides a good example of how solution NMR can be used for unequivocal structural assignment of these

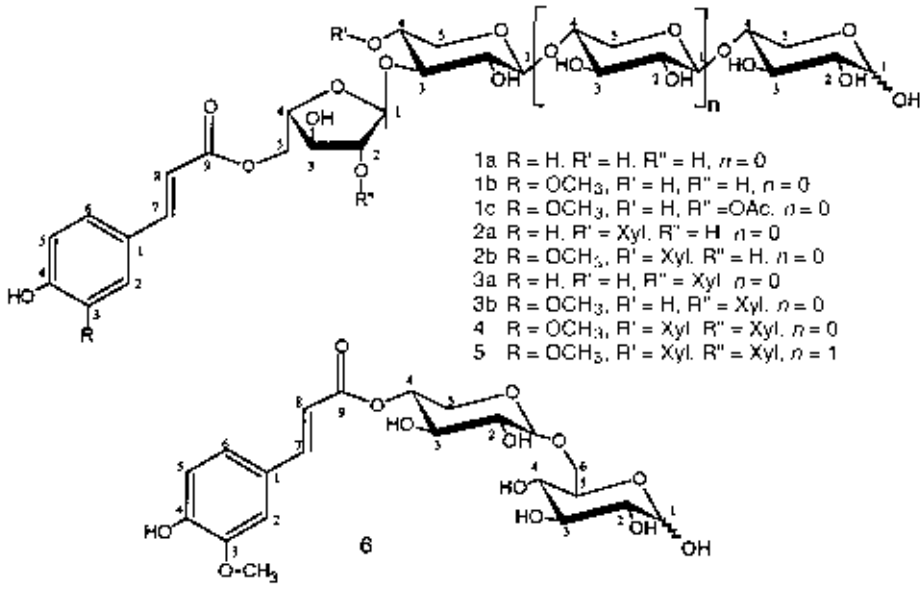

Fig. 16.1. Hydroxycinnamic oligosaccharide esters isolated from grass cell walls. Primary esters: (1a) *O*-[5-*O*-((*E*)-*p*-coumaryl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (1b) *O*-[5-*O*-((*E*)-*p*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (1c) *O*-[5-*O*-((*E*)-*p*-feruloyl)(2-*O*-acetyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (2a) *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-((*E*)-*p*-coumaryl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (2b) *O*-β-D-xylopyranosyl-(1→4)-*O*-[(5-*O*-((*E*)-*p*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (3a) *O*-[5-*O*-((*E*)-*p*-coumaryl)(*O*-β-D-xylopyranosyl)- (1→2)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (3b) *O*-[5-*O*-((*E*)-feruloyl)(*O*-β-D-xylopyranosyl)-(1→2)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (4) *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-((*E*)-feruloyl)(*O*-β-D-xylopyranosyl)-(1→2)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; (5) *O*-[5-*O*-((*E*)-feruloyl)(*O*-β-D-xylopyranosyl)-(1→2)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)—D-xylopyranose. (6) Secondary ester: *O*-[4-*O*-((*E*)-feruloyl)-β-D-xylopyranosyl]-(1→6)-D-glucopyranose.

compounds. The process is begun by first assigning the proton NMR spectrum. This assignment is straightforward for the phenolic acid portion of the molecule, but requires a more sophisticated approach for the carbohydrate portion, due to the many overlapping signals. The assignment of the carbohydrate portion of the proton spectrum can be accomplished most easily by use of the one-dimensional (1-D) analogue of the 2-D {^{1}H, ^{1}H} homonuclear Hartmann-Hahn (HOHAHA) or total correlation spectroscopy (TOCSY) experiment (Davis and Bax, 1985). This involves the selective irradiation of a known position, such as the anomeric proton

of each residue. This generates edited subspectra of each of the monosaccharide components. Then the simple one-bond proton–proton scalar coupling system is worked through to assign the proton signal for each unit. Knowing the proton assignments, the corresponding ^{13}C spectrum can be assigned by employing an experiment such as the inverse detected (^{1}H) one-bond {^{1}H, ^{13}C} heteronuclear multi-quantum correlation (HMQC) experiment (Bax and Subramanian, 1986). The results of this type of experiment are shown in Fig. 16.2.

After both the ^{1}H and ^{13}C spectra have been assigned for the individual subunits, the linkages between them can be found by employing an experiment such as the inverse-detected long-range {^{1}H, ^{13}C} heteronuclear multiple-bond connectivity (HMBC) experiment (Bax and Summers, 1986; Bax and Marion, 1988). The circled cross-peaks, shown in Fig. 16.3, establish the linkages between the subunits

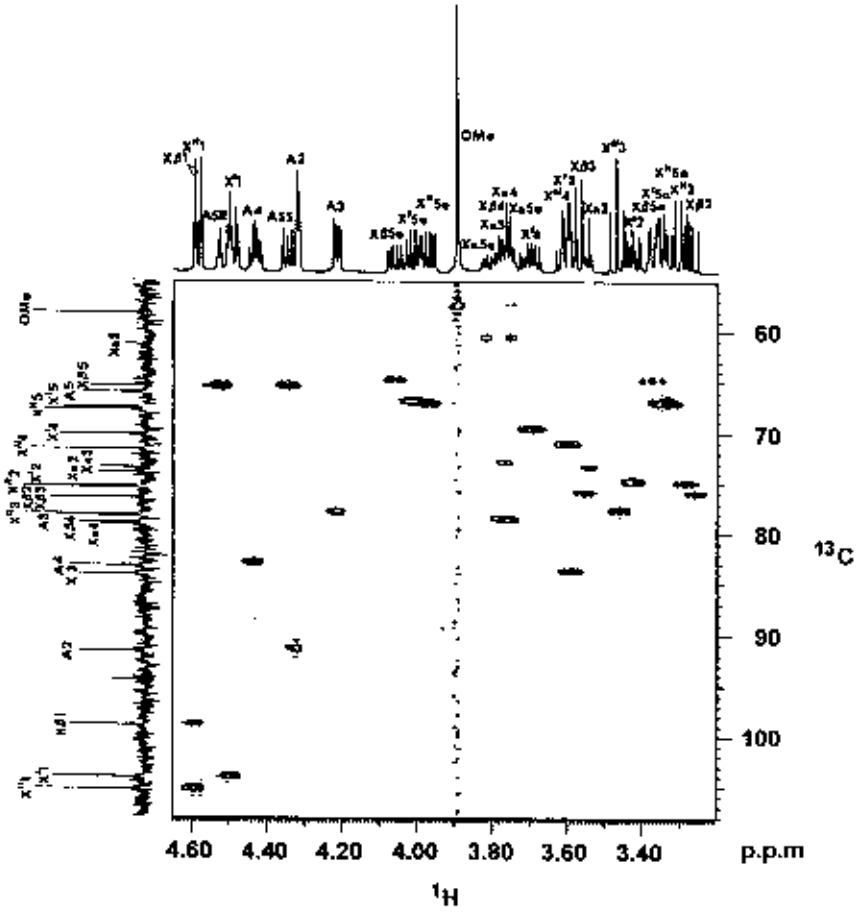

Fig. 16.2. Results of the inverse-detected one-bond HMQC experiment conducted on 5 mg of compound 3b (Fig. 16.1) obtained at 500 MHz for ^{1}H and 125 MHz for ^{13}C in a 20% d_6-acetone/D_2O solution at 25°C.

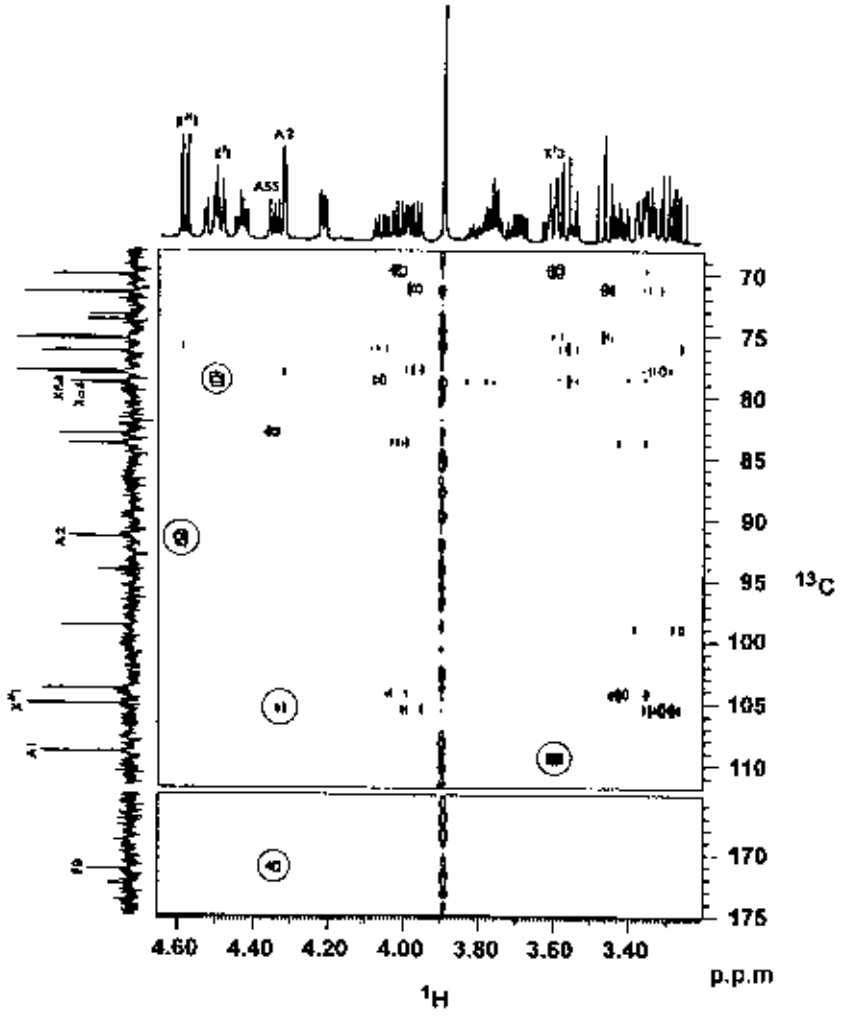

Fig. 16.3. Results of the inverse-detected long-range HMBC experiment conducted on compound 3b (Fig. 16.1) obtained at 500 MHz for ^{1}H and 125 MHz for ^{13}C in 20% d_6-acetone/D_2O solution at 25°C.

of 3b (Fig. 16.1). It shows that the ferulic acid is linked through an oxygen attached to the carbonyl carbon at F9 to the carbon at the 5 position (A5S proton shown) of the arabinofuranoside. This arabinofuranoside, in turn, is linked to two different xylopyranoside units at the 1 (A1) and 2 (A2) positions. At the 1 position, it is linked to the 3 position (X′3) of a xylopyranoside, which is further linked at its 1 position (X′1) to the 4 position of the reducing terminal xylopyranose, which displays signals for both α and β anomers (Xα4, Xβ4).

The actual solution conformation of the molecule in the NMR tube can be established by another 2-D experiment, the phase-sensitive {^{1}H,^{1}H} rotating-frame Overhauser enhancement spectroscopy (ROESY) experiment (Bax and Davis, 1985). The ROESY results are shown (Fig. 16.4b), compared with a {^{1}H,^{1}H} double-quantum filtered correlation spectroscopy (DFQ-COSY) experiment (Fig. 16.4a) (Aue *et al.*, 1976). The circled peaks in the ROESY contour map indicate the through-space interunit interactions. These do not occur in the DFQ-COSY. The stronger the signals, the closer the nuclei lie to each other, in space.

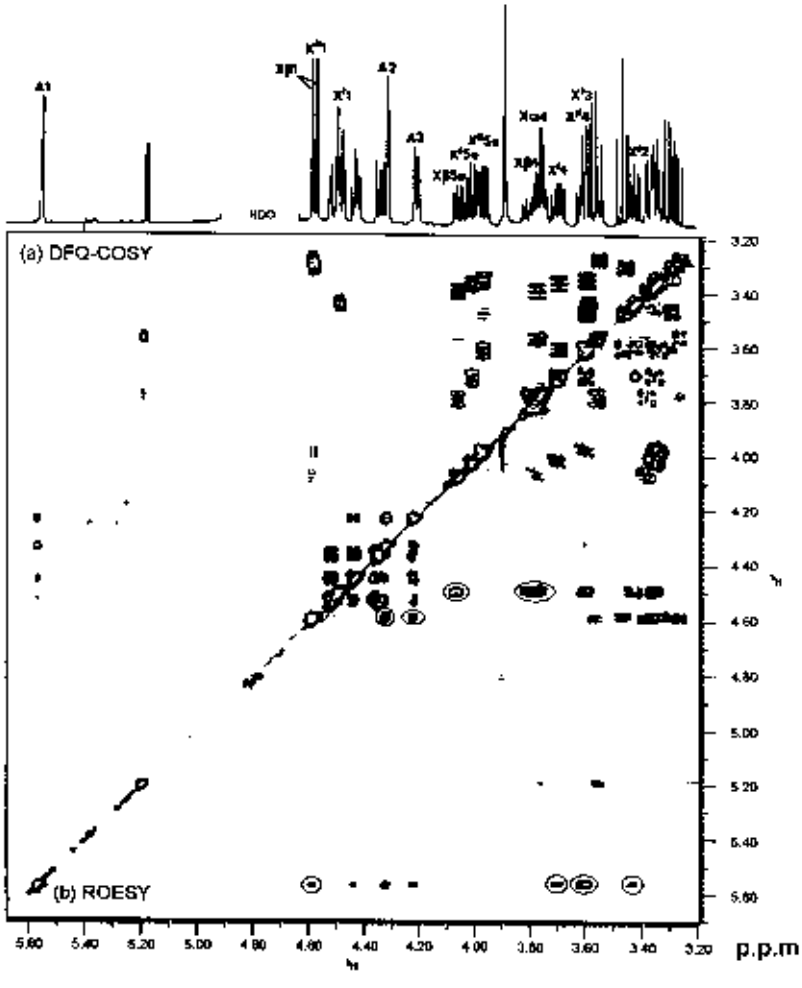

Fig. 16.4. {^{1}H,^{1}H} correlation plots of 3b (Fig. 16.1): (a) DFQ-COSY and (b) ROESY experiments. Both were obtained at 500 MHz for ^{1}H. The circles in the ROESY indicate the through-space interunit interactions.

In addition, the angle of rotation of the hydrocinnamic acid in relation to the arabinofuranoside can be estimated by analysing the three-bond coupling constants ($^3J_{HH}$) made between the proton attached to the A4 carbon and each of the two protons attached to the A5 carbon (Hoffmann *et al.*, 1992). The final conformation can then be expressed as a population of rotamers. In this case, the rotamer population was *trans* = 0.52, gauche$^-$ = 0.38 and gauche$^+$ = 0.10. These energy-minimized structures are shown in Fig. 16.5. Although these conformations will be fixed in the solid-state matrix of the cell walls of forage and not free to rotate, as in solution, this does give some idea of what the preferred (minimum-energy) conformation might be. The solid-state conformation may be critical to how these compounds are incorporated into the rest of the cell wall.

Solution NMR has also been used to provide insight as to how the hydroxycinnamic acids linked to hemicellulosic polysaccharides are incorporated into the cell wall. It has been shown that [2+2] cyclo-addition dimers can be formed in a solid-state photochemical reaction, yielding α-truxillic acids from compounds 1a and 1b (Fig. 16.1) (Hartley *et al.*, 1990). Proton NMR, along with mass spectrometry (MS), has been used to verify that the hydroxycinnamic acids all prefer to couple with a head-to-tail orientation towards each other (Morrison *et al.*, 1992). In the case of the ferulate polysaccharide esters, the 8 position of the hydroxycinnamic

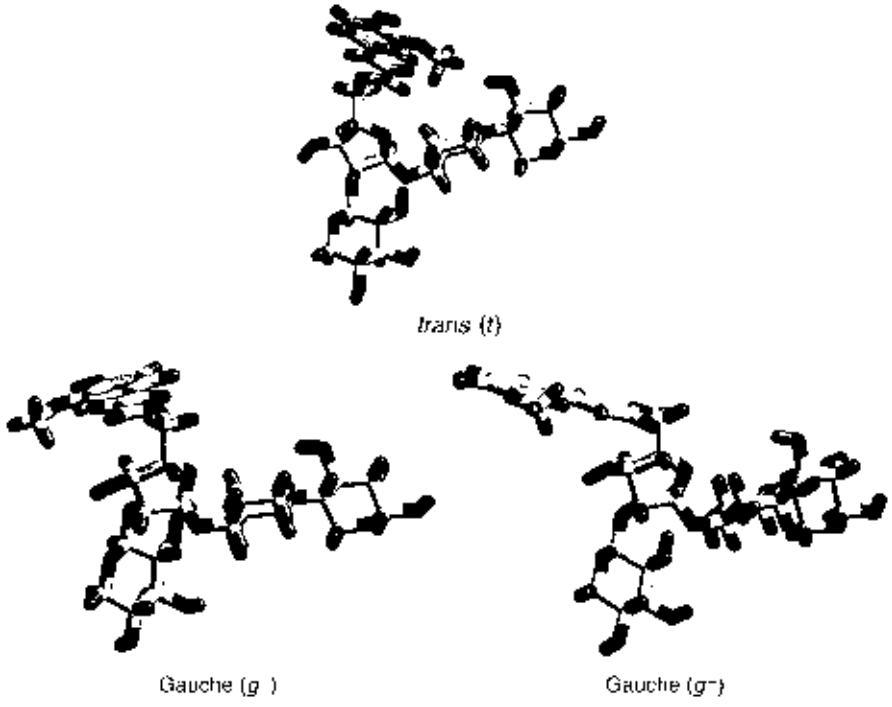

Fig. 16.5. The three minimum energy-staggered rotamers of 3b (Fig. 16.1) that describe its solution conformation in a 20% d_6-acetone/D_2O solution at room temperature.

acid has been shown (by the HMBC experiment) to couple to the β (8) position of both guaiacyl and syringyl alcohol monomers (Ralph *et al.*, 1995). The ferulate esters are also known to form dehydrodiferulates (Harris *et al.*, 1980) and possibly link to core lignin by ether bonds (Jung and Ralph, 1990), although this has not been verified by NMR spectroscopy.

Solution ^{13}C NMR has additionally been used to compare isolated forage lignins and show that warm-season grass forages differ from cool-season in that they incorporate *p*-coumaryl units (Himmelsbach and Barton, 1980). The solution ^{13}C NMR spectra of two representative spectra of these two classes of forages are shown in Fig. 16.6. The incorporation of *p*-coumaryl units in the warm-season forage (Fig. 16.6a) is indicated by the signals labelled '2,6H' and '3,5H'. It has been shown, from solution ^{13}C NMR spectra of bagasse, that treatment of lignin isolated from warm-season grasses with alkali releases most of the *p*-coumaryl units (Fernandez, 1990). This indicates that most of the *p*-coumaryl units exist as acids esterified to lignin and the remainder are esterified to arabinoxylans. This esterification to lignin has been confirmed, using the HMBC experiment, in isolated maize lignin, as compared with synthetic materials. Further, it has been revealed by this experiment that *p*-coumaric acid is extensively incorporated into the α and γ positions of

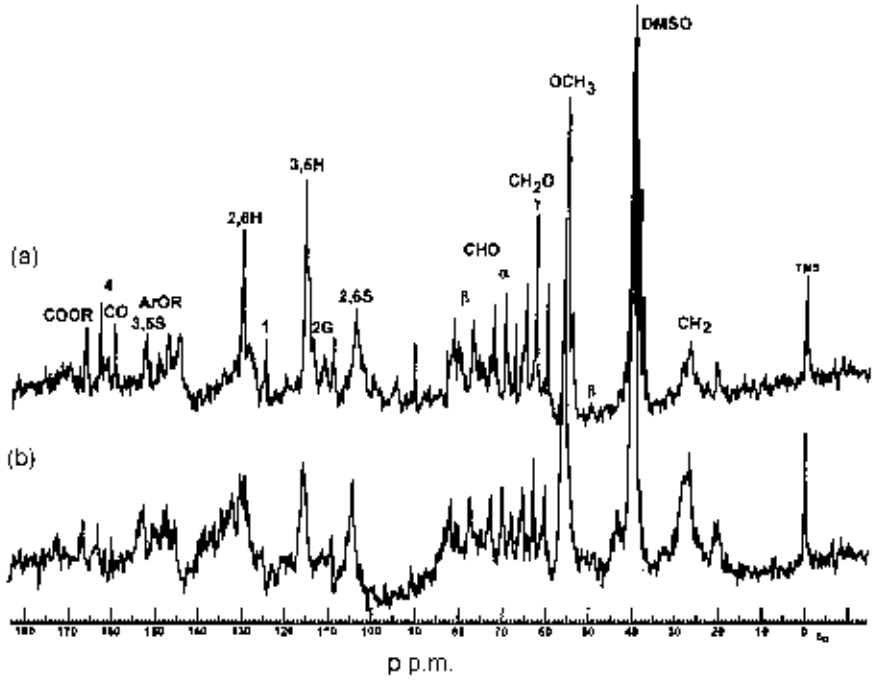

Fig. 16.6. Solution ^{13}C NMR spectra at 25 MHz of (a) Coastal Bermuda grass (warm-season grass) and (b) Kentucky-31 tall fescue (cool-season grass) lignins in d_6-DMSO and referenced to internal TMS. H, 4-hydroxycinnamyl (*p*-coumaryl); G, guaiacyl; S, syringyl; α, β and γ, 7, 8, and 9 positions, respectively, of aliphatic three-carbon side-chains off aromatic rings of lignin precursors.

other lignin subunits (Ralph *et al.*, 1994). It has been suggested (based on this NMR evidence) that *p*-coumaric acid initially occurs esterified to arabinoxylans (see Fig. 16.1) during primary wall development, but is later incorporated by esterification into lignin.

The use of 2-D NMR experiments, for the unambiguous assignment of large polymeric structures, like lignin, has been rather limited because of the poor resolution in the proton dimension. This problem has been somewhat overcome by the use of 3-D solution NMR spectroscopy. An example of this is provided by the use of the 3-D HMQC-HOHAHA experiment, which separates the ^{1}H spectrum into two dimensions and the ^{13}C spectrum into the third dimension. This has been effectively employed to verify that the signal at 50 p.p.m. in the ^{13}C NMR spectrum in an acetylated ^{13}C-enriched lignin is that of the β carbon in a β-1 linkage (Kilpeläinen *et al.*, 1994). An efficient protocol is to assign as many as possible of the signals by 2-D NMR spectroscopy and handle those that are still ambiguous by applying 3-D NMR spectroscopy. The inability of solution NMR spectroscopy to accommodate anything but isolatable and readily soluble materials, such as these, is quickly realized and other methods are required to study larger, more complex materials or intact structures.

Solid-state NMR Spectroscopy

Solid-state NMR spectroscopy can overcome many of the limitations of solution NMR spectroscopy and is ideally suited to the study of intact forages or their residues. Unlike solution NMR spectroscopy, a higher field is not always better. This is especially true for carbonaceous materials, if one is only interested in obtaining isotropic chemical-shift information. Higher fields generate spinning side bands, which must often be spun out at higher speeds. Achieving higher spinning speeds has practical limits and can cause selective loss of some signals. The most popular technique for obtaining solid-state ^{13}C spectra has been that of cross-polarization/magic-angle spinning (CP/MAS) (Schaefer and Stejskal, 1979).

Figure 16.7 shows the use of ^{13}C CP/MAS NMR spectroscopy in an attempt to discern the gross differences between a warm- and a cool-season grass. The only readily apparent difference, at this level, is the presence of additional signals in the region of 25–40 p.p.m. and increased carbonyl intensity at ~175 p.p.m. in the cool-season grass fescue (Fig. 16.7b) over that of the warm-season grass (Fig. 16.7a). This would appear to indicate that there is more protein present in the cool-season grass.

Modifications of the basic solid-state ^{13}C CP/MAS NMR experiment can often be more effectively used to study forages. An example of such a modification is the interrupted decoupling experiment. This experiment, also called the delayed-decoupling experiment, is employed to selectively observe non-protonated carbons (Opella and Fry, 1979). This has been used to detect differences in normal versus brown midrib (*bmr*) mutant lines in sorghum (Akin *et al.*, 1986) and pearl millet (Morrison *et al.*, 1993). The mutant lines are characterized by the brown coloration in the mid-vein of their leaves or stems and are generally more digestible by ruminants than the normal lines. By employing the interrupted decoupling experiment,

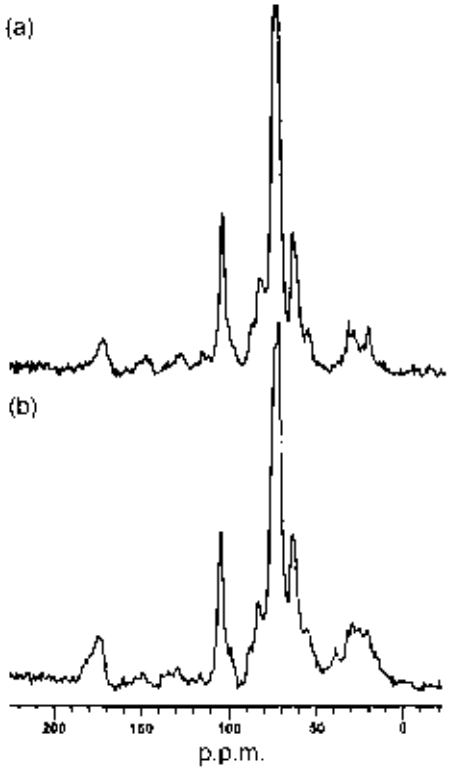

Fig. 16.7. Solid-state ^{13}C CP/MAS NMR spectra at 50 MHz of: (a) Coastal Bermuda grass (warm-season grass) and (b) Kentucky-31 tall fescue (cool-season grass). Spectra were externally referenced to the aromatic signal in hexamethylbenzene, set at 132.3 p.p.m.

a change in lignin structure can be directly detected in the intact forage. Thus, the isolation of the lignin is not required in order to obtain the desired information. A comparison of the results of using the basic CP/MAS and interrupted decoupling experiments is shown for pearl millet in Fig. 16.8. The loss of the signal intensity at 154 p.p.m., due to decrease of syringyl units, can barely be detected in going from the normal to the mutant (Fig. 16.8a and b, respectively) using the standard CP/MAS experiment. However, using the interrupted decoupling experiment, this difference is readily apparent (Fig. 16.8c and d). It also shows that the experiment is not effective when molecular motion averages ^{1}H-^{13}C interactions of proton-bearing carbons. Thus, signals also appear for some protonated carbons, the aliphatic CH_3 in lipids (~22 p.p.m.) and the aromatic OCH_3 (~56 p.p.m.) in lignin. Fortunately, these signals do not interfere with others and the aromatic OCH_3 actually helps in providing confirming information for the decrease in syringyl units.

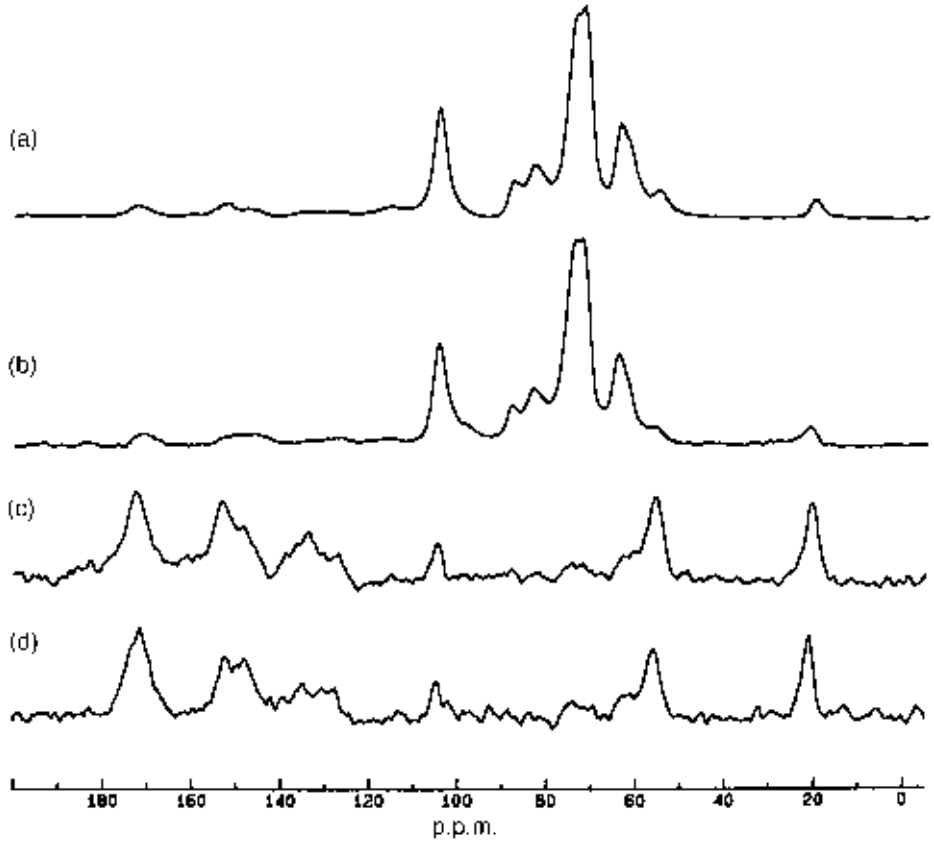

Fig. 16.8. Solid-state ^{13}C NMR spectra at 75 MHz of pearl millet rind tissue: (a) CP/MAS of normal line; (b) CP/MAS of mutant line; (c) CP/MAS with interrupted decoupling of normal line; (d) CP/MAS with interrupted decoupling of mutant line.

Solid-state ^{13}C CP/MAS NMR spectroscopy was used, in the same study (Morrison *et al.*, 1993), to show that treatment with 1 mol l^{-1} sodium hydroxide (NaOH) removed almost all of the phenolics from the *bmr* mutant rind tissue but was not effective in doing so in the normal tissue. It required treatment with 4 mol l^{-1} NaOH to remove phenolics from the normal rind tissue of pearl millet. This indicated that the phenolics in the mutant were ester-linked, rather than ether-linked as in the normal line. This suggests that lignin polymerization had not occurred to a large extent in the mutant and this could account for the greater digestibility of *bmr* mutants by ruminants.

One of the most effective uses of solid-state ^{13}C CP/MAS NMR spectroscopy for the study of forages is the investigation of the nature of the indigestible residues that remain after rumen microbial digestion. It has been used to characterize the residues from both *in vitro* (Akin *et al.*, 1993b) and *in sacco* digestion (Himmelsbach *et al.*, 1988).

Figure 16.9 shows the spectra of intact residues from a legume (lucerne (alfalfa)) and a cool-season grass (cocksfoot (orchardgrass)) after 3 × 72 h *in vitro* digestions by rumen bacteria. Thus, this represented the material remaining after all potentially bacterially degradable material had been removed. The spectra were compared by setting the total integrated area under the trace to 100 and determining the contribution of the areas of each of six specified regions to the total. This provides a semi-quantitative means by which to compare the relative chemical composition of the residues.

The solid-state ^{13}C CP/MAS NMR spectra of residues resulting from neutral-detergent treatment of mixed silage containing 280 g kg^{-1} dry matter (DM) crude fibre after *in sacco* digestion are shown in Fig. 16.10. The samples had originally been subjected to digestion in the rumen of a cannulated steer and removed after 0 (dipped), 48 and 336 h time periods. These time periods represent no digestion, typical digestion and completed digestion. From these spectra, it can be readily seen that carbohydrate is digested over time by the decrease in the signals *c*. 75 and 105 p.p.m. in going from 0 to 336 h. Furthermore, there appears to be no preference for amorphous over crystalline carbohydrates, due to the nearly equal loss of signals at 84 and 89 p.p.m., respectively. Lignin and lipids appear not to be digested. In fact, there is a slight increase in aromatic signals (115–165 p.p.m. region) and in hydrocarbon CH signals (20–30 p.p.m.), which are indicative of these components. There appears to be a loss of one type of carbonyl (CO), as indicated *c*. 175 p.p.m. This may represent the loss of digestible protein.

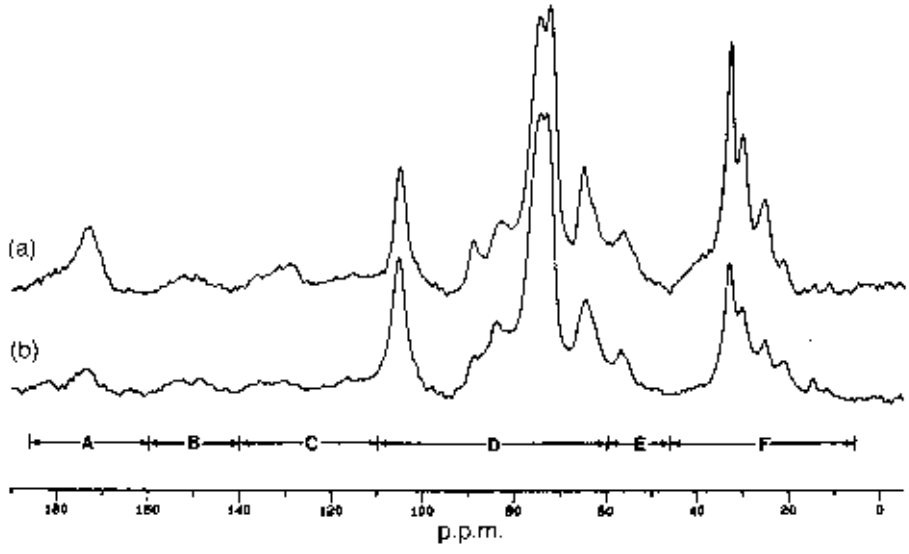

Fig. 16.9. Solid-state ^{13}C CP/MAS NMR spectra at 75 MHz for digested residues of: (a) lucerne and (b) cocksfoot grass. Region A, carbonyls; B, oxygenated aromatics (phenolics); C, non-oxygenated aromatics (phenolics); D, carbohydrates; E, methoxyl; and F, lipids.

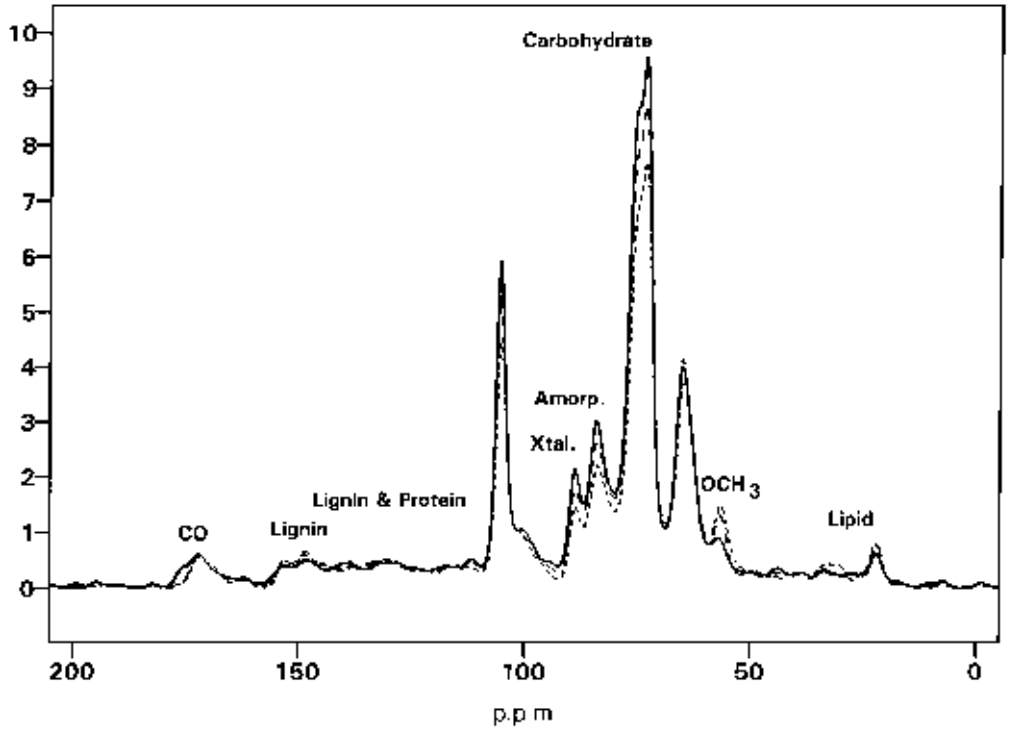

Fig. 16.10. Solid-state ^{13}C CP/MAS NMR spectra at 75 MHz of neutral detergent residues (NDRs) from *in sacco* digestion of a 28% crude-fibre mixed silage after: —— 0 h (dipped in digesta), –––– 48 h and -------- 336 h digestions.

Other Spectroscopic Techniques

Other spectroscopic techniques have been used in attempts to obtain similar information to that obtained by NMR spectroscopy. Diffuse-reflectance infrared Fourier-transform spectroscopy (DRIFTS), in the MIR region, performed on the same samples as were used in the study on *in sacco* residues (Himmelsbach *et al.*, 1988) are shown in Fig. 16.11. Comparison of the MIR spectra reveal, even more clearly, the digestion of carbohydrate. This is shown by the decrease in O-H stretch bands (~3000–3600 cm^{-1}) and C-O mixed-mode heavy-atom vibrations (~900–1200 cm^{-1}). Like the NMR spectra, the MIR spectra show that there is no digestion of lignin or lipids. Again, the indications for lignin or phenolics (1265 and 1505 cm^{-1} bands) actually show a slight increase as digestion proceeds. One indicator for lipids, the C-H stretch at 2850 cm^{-1}, is overshadowed by the loss of similar bands due to carbohydrate, but a sharpening of the shoulder at this position is evident. The other indicator for lipids is the carbonyl (CO) band at 1750 cm^{-1}. It shows the most dramatic change, indicating that lipids are not digested.

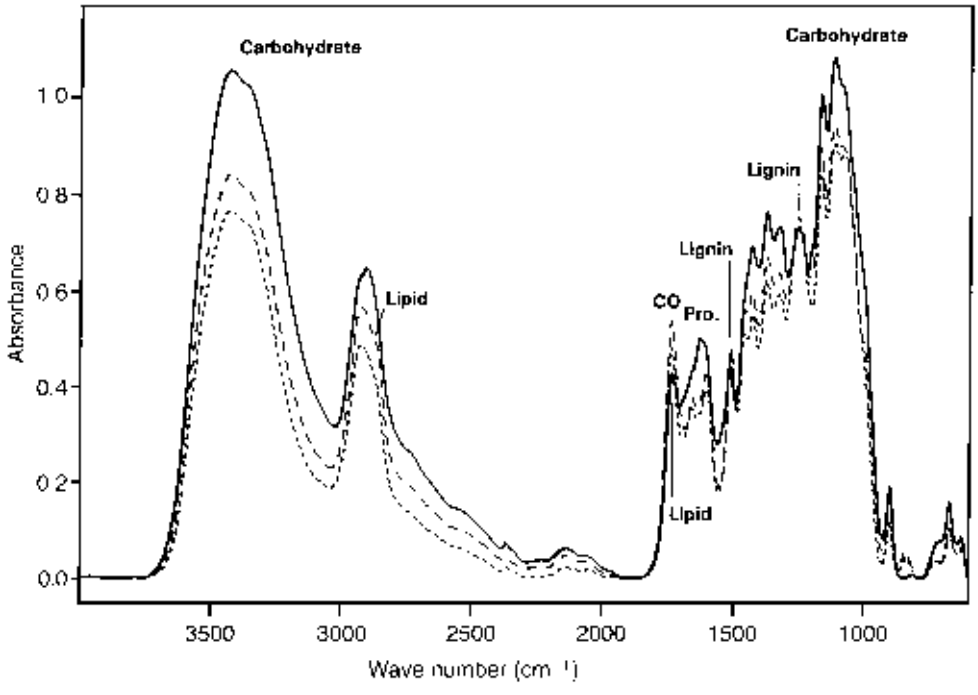

Fig. 16.11. DRIFTS MIR spectra of NDRs from *in sacco* digestion of a 28% crude-fibre mixed silage after: ——— 0 h (dipped in digesta), – – – –48 h and --------- 336 h digestions. All spectra are baseline-corrected.

The Fourier-transform (FT) Raman spectra of these same samples, taken using an NIR laser source, are shown in Fig. 16.12. The baselines in these spectra have been corrected to remove fluorescence effects. The loss of carbohydrate is not as evident in the Raman spectra as in the MIR or NMR spectra. However, the bands in the 900–1200 cm^{-1} region do decrease in intensity as digestion proceeds. The biggest difference in the Raman spectra is the response to aromatic components. This is to be expected, since the Raman active vibrations must be accompanied by a change in polarizability (Colthup *et al.*, 1990) and aromatic compounds contain highly polarizable bonds. Small differences in the phenolic (lignin or hydroxycinnamic acid) content of the residue, barely detectable in the solid-state NMR and MIR spectra, are revealed as large differences in the Raman spectra at ~1600 cm^{-1}. Lipids are only detectable by a sharpening of the shoulder for C-H stretch bands at 2850 cm^{-1}, as in the MIR. The carbonyl band gives only a small response in the Raman, compared with the MIR. The bands in the ~1200–1500 cm^{-1} region cannot be unequivocally assigned to any single component, but could be associated with an initial loss in protein with digestion at 48 h and a reassociation of microbial protein by 336 h of digestion.

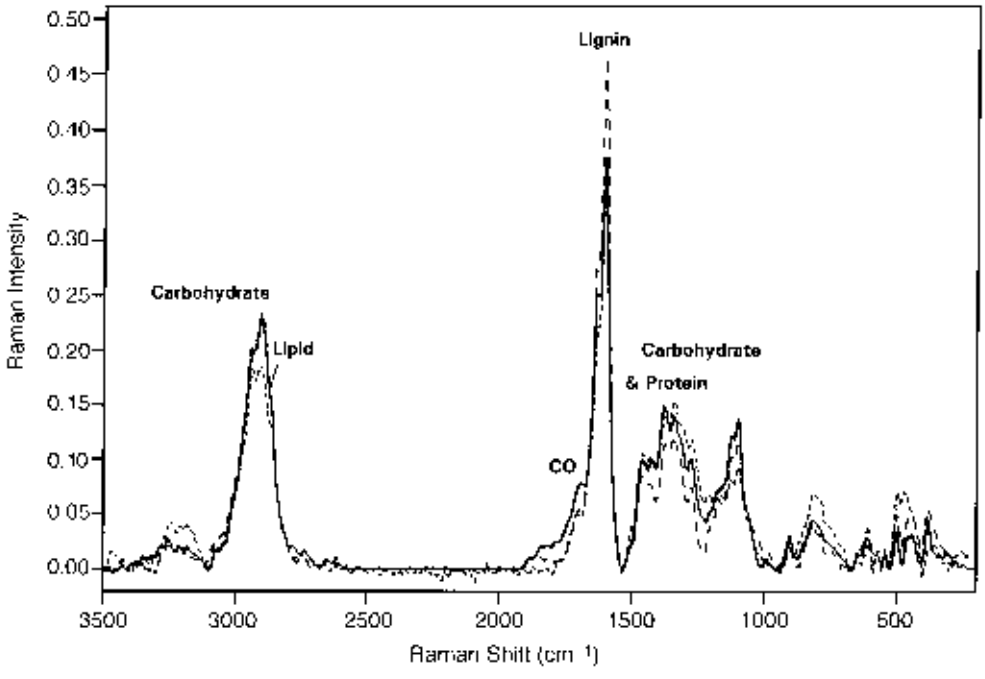

Fig. 16.12. NIR-FT-Raman spectra of NDRs from *in sacco* digestion of a 28% crude-fibre mixed silage after: ——— 0 h (dipped in digesta), –––– 48 h and ---------- 336 h digestions. All spectra baseline-corrected, to remove fluorescence effects.

Diffuse-reflectance NIR spectra of the same samples are shown in Fig. 16.13. Here the base lines are corrected to remove the slope, which is mainly due to the influence of an intense O-H stretch band that occurs in the MIR region and somewhat due to particle-size effects. These are normally corrected with the use of derivatives, but linear baseline removal was done in the same manner as for the other spectral methods for purposes of comparison. Even though NIR spectroscopy is an LR technique, the same basic differences in the intact samples can be detected. The loss of carbohydrates is best observed in the band centred around 2100 nm. The response for lignin or phenolics, at 2260 nm appears to be on a par with that observed in the MIR spectra. The response to aromatics is greatest in the Raman spectra. The responses for lipid, at 1725, 2300 and 2340 nm, are at about the level observed in the Raman and NMR spectra, which is not as good as that observed with the MIR (carbonyl band). Changes in protein are not observable in the NIR spectra of these samples; however, the locations where these bands should appear are shown in Fig. 16.13.

Pyrolysis mass spectrometry (PyMS) has also been employed effectively to examine compositional differences in forages. When low-energy (16 eV) electron impact (EI) is used, phenolic components can be examined. When even softer ionization of pyrolysis products is used, such as chemical ionization (CI),

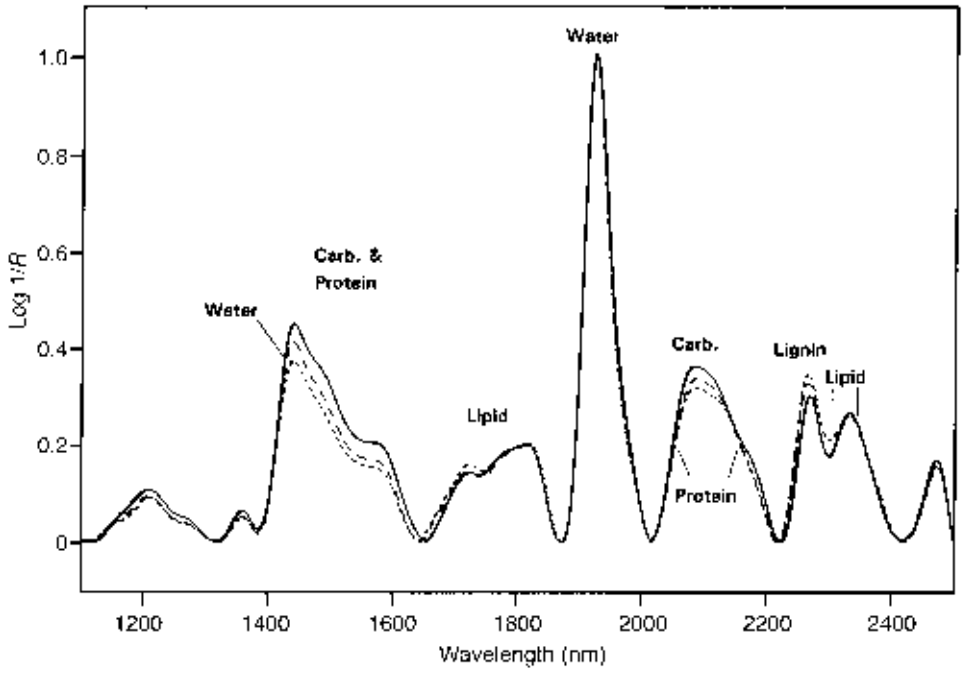

Fig. 16.13. NIR spectra of NDRs from *in sacco* digestion of a 28% crude-fibre mixed silage after: ——— 0 h (dipped in digesta), –––– 48 h and --------- 336 h digestions. All spectra baseline-corrected and normalized around the water band.

carbohydrates can be emphasized. An example of the use of PyMS is provided in the examination of Coastal Bermuda grass and Kentucky-31 tall fescue, using EI PyMS (Fig. 16.14; Morrison *et al.*, 1991). Specific markers were found for *p*-coumaric and ferulic acid esters at 120 and 150 mass-to-charge ratio (*m*/*z*), respectively. The marker for *p*-coumaric acid showed greater abundance in Coastal Bermuda grass. This is in agreement with the NMR spectroscopic results on lignin already discussed. In addition, PyMS results revealed that (after sequential ozone and base treatment) all traces of phenolics could be removed from the Kentucky-31 tall fescue residue but not from the Coastal Bermuda-grass residue. This suggested that all cell-wall carbohydrates were more available in Kentucky-31 tall fescue and could explain the greater rumen digestibility of this forage after treatment with ozone.

Microspectroscopy and Imaging

Morphological, anatomical and chemical factors all potentially contribute to limiting ruminant digestibility of plants (Akin and Chesson, 1989). Thus, being able to study chemical factors within anatomical compartments at specific morphological stages is a highly desirable goal.

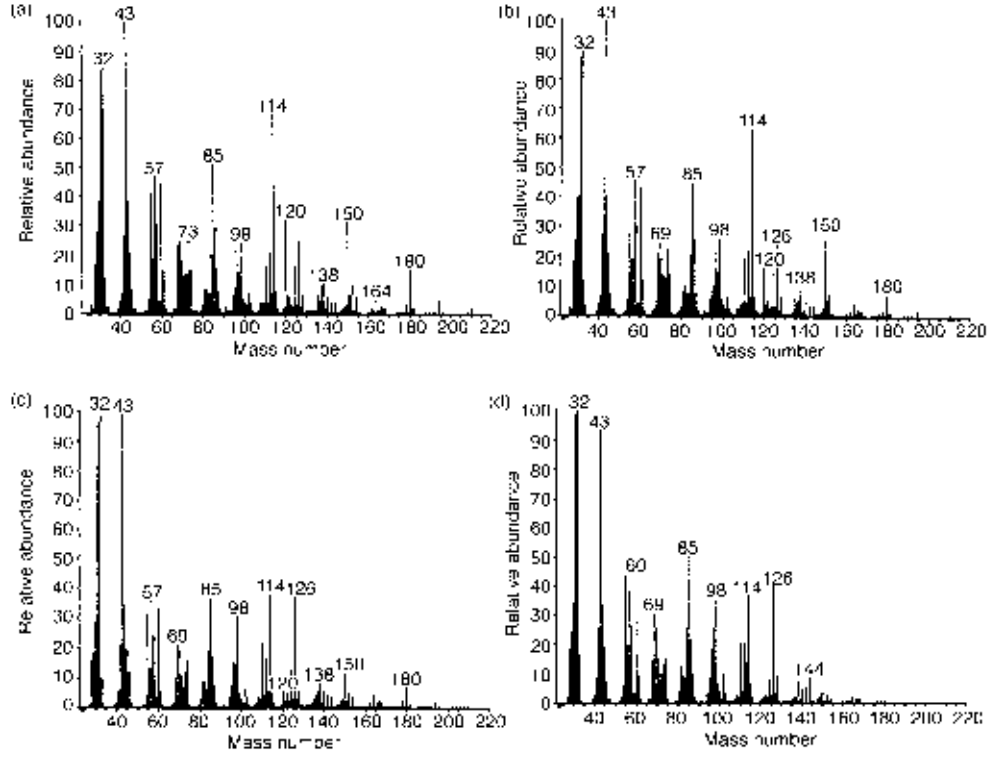

Fig. 16.14. PyMS, electron impact (16 eV) fingerprint of Coastal Bermuda grass and Kentucky-31: (a) and (b) cell walls, respectively, and (c) and (d) chemically treated residues, respectively.

UV spectroscopy has been employed in conjunction with microscopy, called UV microspectroscopy or microspectrophotometry, in an attempt to attain this goal. By restricting the spectra to specific cell types, the interference between compounds that give similar spectral responses can also be reduced. One of the better examples of the use of UV microspectroscopy is its use in the detection of phenolic components in specific cells of forages that show different degradabilities (Akin and Rigsby, 1992). Figure 16.15 shows the UV spectra of the mestome sheath of leaf blades, which is mostly degraded in legumes, partially degraded in cool-season grasses and not degraded in warm-season grasses by rumen microorganisms after 7 days. The important bands in the UV spectra are the absorptions for aromatics, which occur at ~280 nm for condensed lignin or lignin-like compounds and at ~320 nm for those with extended conjugation (e.g. *p*-coumaryl and feruloyl esters). This suggests that, when only condensed lignin or lignin-like structures are present, digestibility is not particularly hindered in this tissue, as in the case of lucerne. When esters are present, digestion is restricted, as with the cool-season grasses (cocksfoot and tall fescue). When both are present to a large extent, then digestion is blocked, as with the warm-season grass Coastal Bermuda grass.

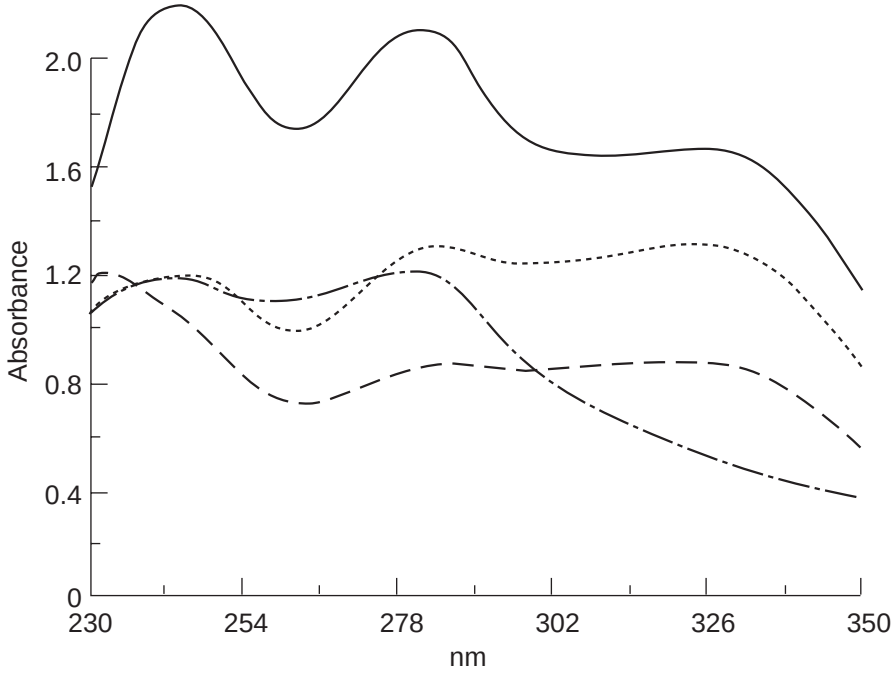

Fig. 16.15. UV absorption spectra of leaf blade mestome sheath of: ——— Coastal Bermuda grass, ------- cocksfoot grass, — — — Kentucky-31 tall fescue and — - — lucerne.

Figure 16.16 shows the UV spectra of the parenchyma-bundle sheath, which is mostly degraded in cool-season grasses and only partially degraded in warm-season grasses. In lucerne, this tissue is totally degraded and thus is not shown. From these spectra it appears that the parenchyma-bundle sheath is essentially non-lignified and the restriction to digestion is provided by the presence of phenolic esters.

The concept of the use of microspectroscopy in the MIR region is also extremely useful. This technique has been around since the 1940s but experienced a resurgence in the 1980s with the development of the FT infrared (Messerschmidt and Harthcock, 1988). The state of the art of this technique has been recently reviewed (Reffner, 1998). An example of the use of MIR microspectroscopy on forages is again provided by pearl millet (Akin *et al.*, 1993a). It has been used to compare the level of phenolics found in the vascular bundle and pith of normal and *bmr* mutant tissue. Figure 16.17 shows that the band for lignin (~1510 cm^{-1}) is less intense in the vascular bundle of the mutant than in the normal line and that the pith showed an even lower intensity than the rind tissue. This was shown to be consistent with the digestibility of these tissues.

An example of the use of MIR imaging on forages is provided by an investigation of a leaf vascular bundle of Coastal Bermuda grass (Wetzel *et al.*, 1998). In this approach, a synchrotron light source was used to enhance the signal/noise ratio and a spatial resolution of ~6 μm was obtained. Here the phenolics, lipid and protein of

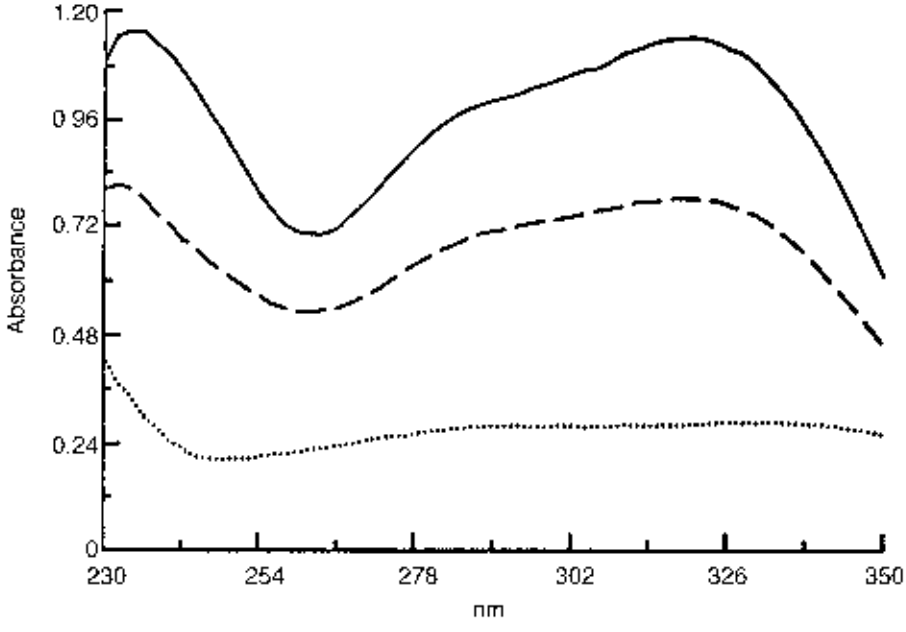

Fig. 16.16. UV absorption spectra of leaf-blade parenchyma bundle sheath of: —— Coastal Bermuda grass, – – – – – cocksfoot grass and ····· Kentucky-31 tall fescue.

the vascular bundle were imaged at 1509, 1550 and 1469 cm^{-1}, respectively. The greatest concentration of phenolics was found in the sclerenchyma and parenchyma-bundle sheath (which included the mestome sheath). Lipids appeared to be located in the epidermal tissue and protein nearby. It is apparent that MIR microspectroscopy is capable of locating more chemical constituents than UV microspectroscopy, but it is limited by its spatial resolution in discriminating between anatomical structures.

Raman microspectroscopy has been around as long as MIR microspectroscopy but has been more difficult to routinely access. Raman imaging was introduced as the Raman microprobe in 1975 (Delhaye and Dhamelincourt, 1975). Like MIR imaging, it has seen a resurgence for many of the same reasons (Turrell and Corset, 1996). However, the development of the FT-Raman confocal systems (Brenan and Hunter, 1995) or systems based on acousto-optic tunable filter or liquid-crystal tunable filter sources (Morris *et al.*, 1994) and charge-coupled device (CCD) detectors have made this a more viable method. Spatial resolution can be achieved on the order of 1 μm and spectral resolution is nearly equivalent to that in MIR. This technique is not very applicable to the study of forage materials, which contain highly fluorescing compounds, such as chlorophyll. However, it is applicable to the study of residues. It would be especially useful in mapping the location of lignin in digested residues, although it has not yet been used for this purpose.

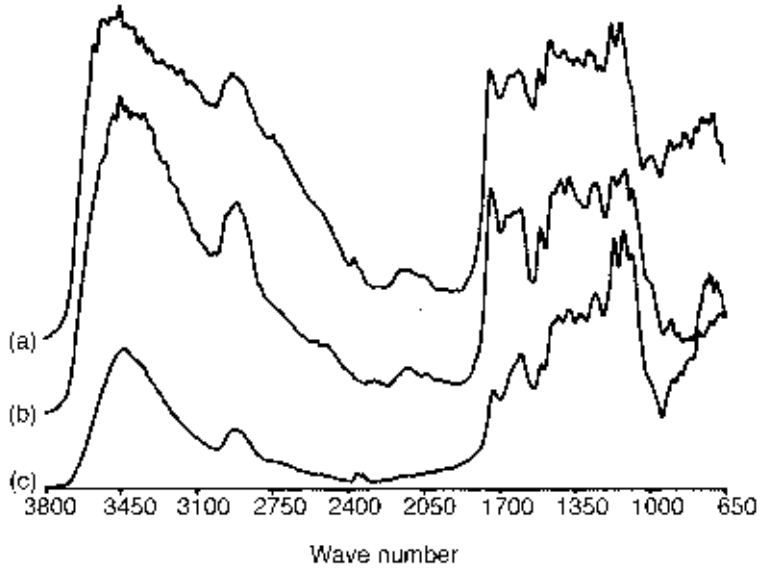

Fig. 16.17. MIR microspectroscopy of cell walls in pearl millet stem: (a) normal rind (sclerenchyma and vascular tissue); (b) vascular bundle within the pith of *bmr* mutant; (c) normal pith.

NIR imaging is just starting to be explored extensively and most MIR imaging systems can be converted to work in the NIR spectral region. Some commercial NIR imaging systems, which are optimized for the NIR, are just becoming available (Treado, 1995) and will make this technique more accessible. Its main advantage is that it can be used to analyse samples that are totally absorbing, which is a situation that can be expected to be encountered in studies on forages. NIR imaging represents a compromise between MIR and Raman imaging with regard to spatial and spectral resolution. It generally has higher spatial resolution but presents difficulties in assigning spectral features, due to severe spectral bands overlapping. Although most of the recent applications of NIR imaging have been in the medical arena, its use is beginning to become more widespread in other biological areas (Dempsey *et al.*, 1996). No applications of NIR imaging to forages could be found, but this technique certainly lends itself to their study, as NIR spectroscopy has already done.

NMR imaging or magnetic resonance imaging (MRI) can also be used to obtain chemical imaging information. This technique has been restricted to solution studies in plants and much of the work is at low spatial resolution, but it can yield information that is inaccessible by other microscopic techniques (Blümich and Kuhn, 1992). The combination of solution 2-D {^{1}H, ^{1}H} correlation spectroscopy with chemical-shift imaging NMR in an experiment called correlation-peak imaging (CPI) is an example of the state of the art. It has been performed on a plant seedling to reveal the locations of soluble carbohydrates and amino acids (Metzler *et al.*, 1995). The ^{1}H image obtained at 500 MHz clearly shows the vascu-

lar bundles, pith parenchyma and cortex parenchyma. The phloem and xylem within the vascular bundles can also be distinguished, with a spatial resolution of ~24 μm. Separate images of each component can be obtained by selecting individual cross-peaks in the 2-D correlation spectrum. Sucrose shows its highest intensity in the vascular bundles and lowest in the cortex parenchyma. The anomers of glucose can be imaged separately and show their highest intensity in the pith parenchyma. Glutamine/glutamate were located in a ring covering the cortex parenchyma. Lysine and arginine were observed in the vascular bundles. Although this technique only works in solution and has lower spatial resolution than the UV and MIR microspectroscopic imaging techniques, it is a step closer to providing complete imaging of plant tissue.

PyMS can also be used for microspectroscopy but is a destructive technique. It has been used for the characterization of cuticle, parenchyma, sclerenchyma and vascular-bundle tissue for the determination of polysaccharide/lignin ratios and detection of *p*-coumaryl acid and truxillic acid groups in forages (Boon, 1989).

Conclusions

The physicochemical techniques that are available to assess forages, just in the form of spectroscopy, are overwhelming. Not all have been touched on here. NMR is exceptionally specific and even sensitive to minute conformational effects but has practical limitations in its use. Each technique discussed has its advantages and disadvantages. Even with all of the advances over recent years, no technique is the panacea for all assessment problems. It is up to investigators to pick and choose those techniques that will best satisfy their particular needs. However, it does appear that, in order to make significant advances in the understanding of the underlying causes of differential digestibility of forages, some form of spectroscopic imaging needs to be utilized. This is necessary in order to embrace the impact of synergistic effects of the morphological, anatomical and chemical factors that influence nutrient availability.

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17 Trace-mineral Status of Forages

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Important trace elements

Of the 16 trace elements currently recognized as being essential for farm livestock (Mertz, 1987), only eight are likely to be of practical significance, as deficiencies of the others occur only rarely, if ever. Those regarded as essential are cobalt (Co), copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), iodine (I), selenium (Se), chromium, molybdenum (Mo), fluorine, lithium, silicon, vanadium, nickel, arsenic and lead (Pb). Future research will probably provide evidence for the extension of this list. Those of most practical significance are Co, Fe, Cu, Zn, Mn, I, Se and Mo, with the last-named being more important for its interaction with Cu and sulphur (S), rather than in its own right. Other elements, eg. calcium (Ca) and cadmium (Cd) can interact with some of the essential trace elements, such as Cu, Zn and Mn and adversely affect their absorption or availability.

Forage as a Supplier of Trace Elements

Many classes of livestock are solely or mostly dependent for all their nutrients on the quality of the forage available to them, either in its natural state or conserved as hay or silage. Hemingway *et al.* (1968) reported that 90% of hays and 62% of silages provided insufficient Cu to meet the needs of dairy cattle. In Latin America, some 43–60% of forage samples were deficient in Co and Cu and 75% deficient in Zn (McDowell and Conrad, 1990). In Latvia, 85% of analysed forages had low concentrations of Cu, Mn, Zn and Co, with both Cu and Co being some 50% below requirements (Strikauska *et al.*, 1994). Forage species vary widely in the range and quantity of trace elements they provide and this in turn is affected by soil type and condition, its acidity or alkalinity and the stage of maturity of the forage itself. Fertilizer application may also affect trace-element concentration.

 Forage Evaluation in Ruminant Nutrition (eds D.I. Givens, E. Owen, R.F.E. Axford and H.M. Omed)

Factors Affecting the Copper Status of Forage

Species

Early work in the UK showed much higher Cu concentration in herbs and weeds (10.8–16.6 mg kg^{-1} dry matter (DM)) than in grasses (4.0–8.2 mg kg^{-1} DM) grown as mixed swards (Thomas and Thompson, 1948). Legumes were higher in Cu than grasses but lower than herbs and weeds (Thomas *et al.*, 1952). Minson (1990) confirms that temperate legumes generally contain more Cu than temperate grasses (7.8 vs. 4.7 mg kg^{-1} DM), except when soil Cu is low, when there is no difference (Beck, 1962). In contrast, tropical legumes contain less Cu than tropical grass (3.9 vs. 7.8 mg kg^{-1} DM). Significant species differences in herbage Cu concentration have been reported from Kenya, where kikuyu grass had the highest and Rhodes grass the lowest concentration (Jumba *et al.*, 1995). Also in Kenya, Maskall and Thornton (1989) recorded higher levels of Cu in grass species than in browse plants. However, Mo levels in all plants reached relatively high values and its availability appeared to increase in the more acid soils near lakesides, leading to a risk of Cu deficiency in the grazing ruminant livestock. Species differences are not always consistent between experiments, probably because of the presence of other confounding factors.

Genotype

Large differences were recorded between cultivars of *Trifolium subterranean* which were unaffected by soil Cu concentration. In temperate grasses, large differences were found between cultivars of *Lolium multiflorum* (84%), *Festuca arundinacea* (57%) (Montalvo-Hernandez *et al.*, 1984) and *L. perrene* (24 and 25%) (Forbes and Gelman, 1981; Montalvo-Hernandez *et al.*, 1984). Cultivars of *Dactylis glomerata* varied by 100% in one study (Montalvo-Hernandez *et al.*, 1984) but not at all in another (Forbes and Gelman, 1981).

Plant parts

Leaves of temperate grasses have 35% more Cu, on average, than the stems, but this is affected by age, with little difference between immature stem and leaf (Davey and Mitchell, 1968). Grass flower-heads have similar Cu contents to the leaves. Temperate legumes have higher leaflet Cu than stem Cu, but there is no such difference in tropical legumes (*Lablab purpurea*) (Hendricksen and Minson, 1980). Sheep tend to be more selective grazers than cattle and should therefore benefit from the higher Cu content of the leaves. Occasionally, this can prove to be a hazard rather than a benefit, particularly with breeds susceptible to Cu poisoning (see p. 358).

Stage of growth

Maturity leads to a decrease in the Cu content of forage because of a decline in the proportion of leaf present and a lowering of the Cu content of the stem. Young oat plants (*Avena sativa*) contained 9.4 mg Cu kg^{-1} DM, but fell to 3.2 mg kg^{-1} DM at the milk-ripe stage (Piper, 1942; Burridge, 1970; MacDonald and Wilson, 1980). Similar findings have been reported by many other workers for other grasses (Minson, 1990) and legumes. Moorland plants, including flying bent, deer hair, white bent and draw moss, fell from a mean of 11.1 to 5.7 mg kg^{-1} DM between May and September. On the other hand, Thomas *et al.* (1945) found little seasonal change in Cu concentration of heather samples, which ranged between 11.9 and 14.2 mg kg^{-1} DM. Increasing age from 2 to 10 years also had no effect. Trace-element deficiencies in livestock tend to be rare where heather is prevalent. MacPherson (1967) recorded a decline in the crude-protein content of maturing herbage from 289 to 88 g kg^{-1} DM which was paralleled by a fall in herbage Cu from 10.6 to 5.5 mg kg^{-1} DM.

Soil fertility

If total Cu content of soil is low, forage Cu levels will be low, but they may also be low on soils of normal Cu content but with a high proportion unavailable (Alloway and Tills, 1984). Reduced availability of Cu can be due to the formation of Cu–organic-matter complexes, e.g. peat soils and mineral soils with > 10% organic matter. Many references relate forage Cu to soil type (Lee, 1951; Cunningham, 1960; Alloway and Tills, 1984; Cornforth, 1984) or parent material (Fraser, 1984; Berrow and Ure, 1985).

Fertilizer nitrogen

Nitrogen (N) fertilizer applied to regularly cut cocksfoot and perennial ryegrass swards reduced forage Cu levels, possibly as a result of soil Cu depletion. If soil-available Cu was high, fertilizer N had no effect on the Cu content of star-grass (Rudert and Oliver, 1978), cocksfoot and tall fescue (Reid *et al.*, 1967a). On soils of low Cu status, phosphatic fertilizers can reduce subterranean clover Cu levels by up to 50%, apparently through a growth-stimulation effect, which consequently dilutes the Cu content (Reddy *et al.*, 1981). Other workers have reported no effect of phosphatic or potassic fertilizers on forage Cu contents (Hemingway, 1962; Reith *et al.*, 1964).

Liming

This has generally been found to have little direct effect on forage Cu concentration (Fig. 17.1), but it is nevertheless important in the aetiology of Cu deficiency in sheep on improved pastures, due to a change effected on the forage Cu:Mo ratio,

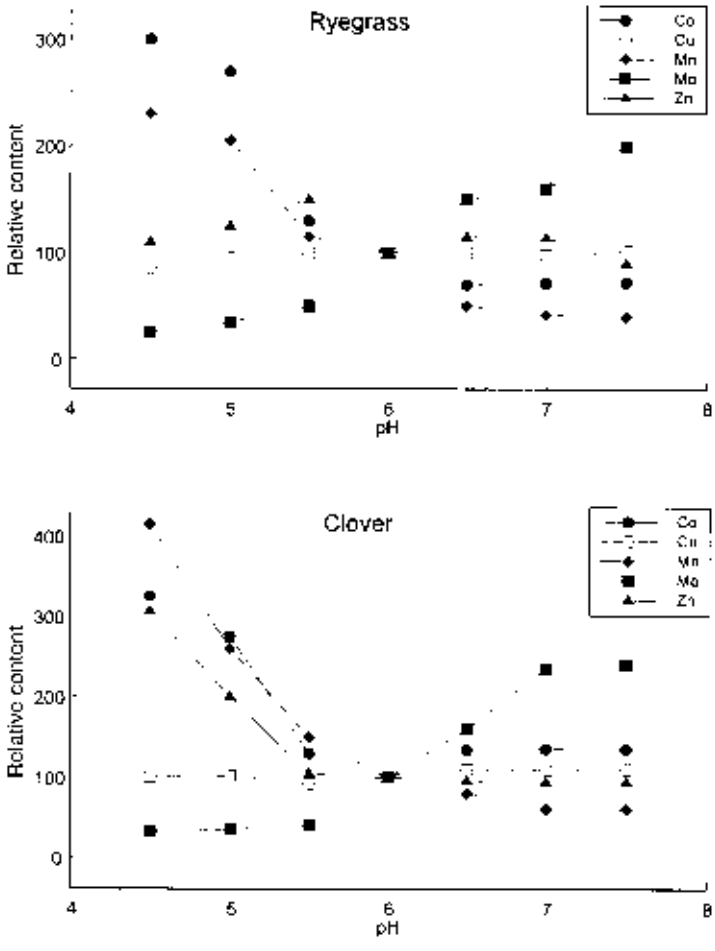

Fig. 17.1. Relative contents of trace elements in ryegrass and clover separated from mixed herbage. Contents in mg kg^{-1} DM at pH 6.0 are equal to 100 and are as follows: Co 0.03; Cu 3.1; Mn 51; Mo 2.7; and Zn 23.

as the more alkaline conditions following liming favour increased Mo uptake (Fig. 17.1). Heather Cu concentrations fell from 12.5 to 11.1 mg kg^{-1} DM following the application of 5 t ground limestone ha^{-1} (Thomas *et al.*, 1945). Stewart (1951) recorded a higher incidence of sway-back on limed pastures, but could not show any effect of treatment on forage Cu. MacPherson (1967) reported that liming at rates up to 4 t ha^{-1}, which gave a range in soil pH from 5.5 to 7.0, had no effect on herbage Cu content.

Climate

In north-eastern Brazil, herbage Cu concentrations ranged from 1.33 to 4.63 mg kg^{-1} DM in the dry season and only from 1.56 to 2.57 mg kg^{-1} DM in the wet season. Herbage Mo concentrations ranged only from 0.51 to 0.54 mg kg^{-1} DM (Sousa *et al.*, 1989). Other Brazilian workers (Pott *et al.*, 1989) considered Cu deficiency a possibility in grazing cattle at the start of the rainy period.

Factors Affecting the Cobalt Status of Forage

Co concentrations in forage range from < 0.01 to 1.26 mg kg^{-1} DM (Beeson, 1950), due mainly to differences in soil Co. Forage legumes generally contain more Co than grasses (Andrews, 1966). Lucerne and clover had from 0.3 to 0.4 mg Co kg^{-1} DM compared with 0.08 mg kg^{-1} DM in grasses grown on the same soil (Latteur, 1962). In Virginia, legumes were reported to contain from 0.06 to 0.48 mg kg^{-1} DM and grasses 0.02 to 0.24 mg kg^{-1} DM (Price and Hardison, 1963). Legumes and grasses both had low and similar concentrations when grown on low-Co soils in Florida (Kretschmer *et al.*, 1954) and New Zealand (Andrews, 1966). Occasionally, lower concentrations have been reported in legumes than in grasses (Winter *et al.*, 1977). On soils with relatively high Co concentrations, little variation in the Co content of different legumes has been reported.

Effect of species

Perennial ryegrass would normally have more Co than timothy. While differences between different grasses and legumes are inconsistent, they mostly follow the order: red clover > white clover > perennial ryegrass > Italian ryegrass > cocksfoot > meadow fescues > timothy (Voss and MacPherson, 1977). These authors state that hill species, such as heather and white bent, contain relatively high Co concentrations. Following upland reseeding and land-improvement schemes, the risk of Co deficiency increases, as relatively Co-poor grasses, such as timothy and ryegrass, replace the natural sward. Indeed, timothy, whether home-grown or imported, has often formed the basis of experimental diets designed to provide low concentrations of the element.

Genotype

There are not many published data available on the effect of genotype on forage Co content. In a study of cultivars of cocksfoot, perennial ryegrass and white clover grown on low-Co soil, no significant differences were found between cultivars of different species or between the forage species (Forbes and Gelman, 1981).

Plant parts

Fleming and Murphy (1968) reported more Co in leaves than in flowering heads and more in flowering heads than in stems of grasses. Handreck and Riceman (1969) found that Co was concentrated at the margins of the leaflets of legumes and at the base and the extreme tips of the leaf blades of grasses.

Stage of maturity

Increasing maturity sometimes, but not always, leads to a decrease in Co content. Fleming and Murphy (1968) and Fleming (1970) recorded lower Co concentrations in perennial ryegrass with maturity, as did Whitehead and Jones (1969) in sainfoin and red clover. These latter, however, reported no consistent changes in lucerne or white clover, confirming similar findings by Rossiter *et al.* (1948) for subterranean clover.

Soil fertility

Low herbage Co is generally associated with soils of low available Co content. Soils derived from Old Red Sandstone and granitic parent material tend to be inherently low in the element, as do soils on limestone parent material. High soil Mn is known to reduce Co uptake by forages (Adams *et al.*, 1969; Norrish, 1975).

Waterlogging of soils allows the release of Co from the soil minerals into soil solution, so that forage on poorly drained soils can have up to seven times more Co than that from well-drained soils. If herbage yield is increased by additional moisture, there may be no effect on Co concentration.

Fertilizer N has been variously reported to have no effect on forage Co concentration (Stewart and Holmes, 1953; Wright and Lawton, 1954; Mudd, 1970), to lower it (Percival *et al.*, 1955; Reith *et al.*, 1964, 1983) and to increase it and the Co status of lambs grazing on it (Voss and MacPherson, 1977). Increased Co concentrations following N fertilization have also been reported for a mineral soil (Klessa *et al.*, 1988) and a peat soil (Reith *et al.*, 1983). Explanations for these varied responses include clover dieback following N application and a dilution effect on herbage Co when growth rate exceeded rate of Co uptake (Reith *et al.*, 1964; West, 1981). Mills and MacPherson (1982) relate the different responses to different soil Co levels. N fertilizer reduces uptake of Co on deficient and borderline status soils and on naturally freely drained soils of whatever status. Klessa *et al.* (1988) suggest the following factors are involved: soil availability; acidification by ammonium fertilizers; and ratio of ammonium (NH_4)-N : nitrate (NO_3)-N taken up by the roots and its effect on the pH of the rhizosphere.

Liming and soil pH

After soil drainage, soil pH is the next most important determinant of Co availability (West, 1981). Decreased Co concentrations following liming have been fre-

quently reported (Ekman *et al.*, 1952; Hill *et al.*, 1953; Wright and Lawton, 1954; Percival *et al.*, 1955; Archer, 1970). Mitchell (1972) reported that an increase in pH of one unit from 5.4 to 6.4 resulted in a halving of the Co concentration of mixed pasture. Others have reported that increasing pH above 6.0 has little further effect (Wright and Lawton, 1954; McLaren *et al.*, 1987; Klessa *et al.*, 1988) (Fig. 17.1). A greater incidence of Co deficiency in grazing livestock occurs on naturally occurring high-pH soils. Finch and Rogers (1979) stated that Co deficiency was more likely on soils formed on limestone and Mitchell (1954) explained the high prevalence of Co deficiency on the machair pastures of the Western Isles by the abundant supply of calcium carbonate ($CaCO_3$) from sea shells. Not only does liming reduce availability by causing greater adsorption on to the various exchange sites, resulting in the precipitation of various Co salts, but higher pH also encourages the establishment of better-quality grasses of lower Co content.

Seasonal changes

No definitive trend can be asserted, but a tendency for concentrations to be higher in spring and autumn has been noted (Voss and MacPherson, 1977), but not in all studies (Andrews, 1966; Fleming, 1970). Silage cut in late spring had higher Co concentrations than hay cut in midsummer (Reith *et al.*, 1983). This can probably be accounted for by a decreased leaf:stem ratio as the herbage matured. Heavy grazing pressure may prevent maturation and maintain pasture at an early physiological growth stage and thus with a higher Co concentration. Repeated cutting maintained Co concentration at almost double that of uncut grass by mid-July, except for clover and timothy. Voss and MacPherson (1977), however, found lower Co concentrations in plots repeatedly cut to simulate grazing. The sporadic nature of seasonal variation is due both to the type of growing season and stocking density. When grass is plentiful, the grazing livestock will not crop it to the same extent, thus allowing maturation to occur. On the other hand, when grass growth is poor or the stocking density is high the grass will remain at a young growth stage throughout the growing season.

Factors Affecting the Selenium Status of Forage

Plants differ markedly in Se concentration ranging from very high concentrations of > 300 mg Se kg^{-1} DM in accumulator plants grown on Se-rich soils to < 0.05 mg Se kg^{-1} DM in normal herbage grown on Se-deficient soils. Although forage species commonly used for grazing livestock will have lower Se levels than accumulator plants, they can still contain toxic concentrations (e.g. *Poa pratense*, 33 mg kg^{-1} DM, and *Medicago sativa*, 40 mg kg^{-1} DM) when grown on seleniferous soils (Hamilton and Beath, 1963). Although knowledge of the toxic effects of Se predates that of its essentiality, it has since been discovered that the detrimental effects of Se deficiency far outweigh those of its toxicity. Areas of Se deficiency have been identified in Australia, New Zealand, China, the USA, many parts of Africa (e.g. Zaïre), much of continental (particularly northern) Europe and the UK

(Gardiner *et al.*, 1962; Gardiner and Gorman, 1963; Long and Marshall, 1973; Ammerman and Miller, 1975; Young *et al.*, 1977).

Effects of species

In New Zealand, grasses contain more Se than white clover at all levels of soil and fertilizer Se (Davies and Watkinson, 1966). Tropical legumes also have lower Se concentrations than associated grasses (Long and Marshall, 1973). No difference was recorded between timothy and white clover on a range of Se-deficient soils in Canada (Gupta and Winter, 1975), between lucerne and grasses in Denmark (Gissel-Nielsen, 1975) or between subterranean clover and associated grasses in Australia (Caple *et al.*, 1980). Ehlig *et al.* (1968) found twice as much Se in lucerne as in timothy, cocksfoot or brome grass. Of the different grass species sweet vernal had the highest concentration followed by Yorkshire fog, bent grass and perennial ryegrass.

Plant parts/stage of maturity

Neither appears to have had many data generated about it. Cocksfoot spikelets had twice as much Se as the leaf fraction, with the leaf sheath and stem intermediate (Davey and Mitchell, 1968). Ehlig *et al.* (1968) recorded that stage of growth had little effect on the Se content of lucerne.

Soil fertility

Forage Se is directly related to the Se content of the soil and the parent material from which it is derived (Robinson and During, 1961; Watkinson, 1962; Kubota *et al.*, 1967; Andrews *et al.*, 1968; Gupta and Winter, 1975; Doyle and Fletcher, 1977). Forage Se tends to be low on sandy soils and lower on mineral upland soils than on organic moorland soils. In Australia and New Zealand, nutritional muscular dystrophy (NMD) is often associated with lush pasture and superphosphate application. It may be due to a higher proportion of legumes, which are lower in Se than grasses, and to a stimulation of growth diluting the Se content (Westerman and Robbins, 1974).

Climate

Annual changes in mean temperature can affect the Se content of forage, e.g. *A. sativa* forage grown at a mean temperature of 14°C contained 0.012 mg Se kg^{-1} DM, compared with 0.042 mg kg^{-1} DM at a mean temperature of 19°C (Lindberg and Lannek, 1970). Areas with higher rainfall may also have a lower Se content in forage – 0.026 compared with 0.036 mg Se kg^{-1} DM, with annual mean rainfall figures of 250–375 and > 750 mm, respectively. Seasonal variations in concentration

have been reported from Denmark (Gissel-Nielsen, 1975), where a maximum concentration of 0.20 mg kg^{-1} DM in March fell to a minimum of 0.02 mg kg^{-1} DM in June and September. No such changes were found over the same time period in moorland pasture in Norway (Garmo *et al.*, 1986).

Factors Affecting the Iodine Status of Forage

Low values (0.09 mg kg^{-1} DM) have been recorded for the I concentration of forage grown on sandy soil in the Netherlands (Hartmans, 1974), but much higher values (0.42 mg I kg^{-1} DM) for forage grown in New Zealand (Johnson and Butler, 1957). Forages grown under the same conditions showed species differences (Johnson and Butler, 1957; Alderman and Jones, 1967; Hartmans, 1974), but the ranking order between studies was not always consistent, possibly because different cultivars were used in each comparison. Different cultivars of perennial ryegrass, prairie grass and white clover exhibited large differences in I concentration (Butler and Glenday, 1962; Alderman and Jones, 1967; Rumball *et al.*, 1972). I concentration tends to be higher in leaves (0.24 mg kg^{-1} DM) than in stems (0.10 mg kg^{-1} DM) (Blom, 1934). Concentrations of I tend to be high early in the growing season, but these quickly fall away (Alderman and Jones, 1967).

Soil fertility

I concentrations in forage tend to be lowest on sandy soils and river clays low in organic matter and highest on soils of the younger marine clays and peats. Fertilizer N reduced I concentration in cocksfoot, perennial ryegrass and timothy, but with no difference between species. This effect was greater on younger herbage than on the more mature. N fertilizer also increased the concentration of cyanogenetic glucosides, thereby effectively increasing I requirement, so that it exercised a doubly detrimental effect (Rudert and Oliver, 1978). No consistent seasonal trends have been reported in I concentration.

Factors Affecting the Zinc Status of Forage

Minson (1990) quotes a mean Zn concentration of 36 mg kg^{-1} DM in 719 samples of forages in the world literature; 11% of these had concentrations below 20 mg kg^{-1} DM – a potentially deficient amount. The Pennsylvania State Testing Service analysed 17,000 forage samples over 5 years. The mean Zn concentration was 29 mg kg^{-1} DM (range: 3–300 mg kg^{-1} DM) (Adams, 1975). In Louisana, the mean concentration reported was 27 mg Zn kg^{-1} DM (Kappel *et al.*, 1985), with an even lower mean value of 22 mg kg^{-1} DM recorded for British Columbia (Miltimore *et al.*, 1970). These differences are possibly due to differences in soil Zn, forage species and stage of maturity.

Effect of species

Tropical forages were slightly higher (2 mg kg^{-1} DM) in mean Zn content than temperate ones (Minson, 1981). Although the overall difference was not great, the risk of values below 20 mg Zn kg^{-1} DM was doubled. In general, grasses tend to contain less Zn than legumes. This difference was confirmed when grass and clover were grown together (Metson *et al.*, 1979). The difference increased at higher levels of soil Zn (Gladstones and Loneragan, 1967). Different grass species grown at the same site exhibit large differences in Zn concentration, but the ranking order is not consistent and indeed may be totally reversed (Gomide *et al.*, 1969; Perdomo *et al.*, 1977).

Maximum differences found between genotypes of perennial ryegrass and prairie grass were 21 and 136%, respectively (Butler *et al.*, 1962; Rumball *et al.*, 1972). Minson (1984) recorded only a 10% difference in Zn concentration among five species of *Digitaria* (Pangola and Woolly Finger Grass). Legume species have been found both to vary little in Zn concentration (Loper and Smith, 1961; Whitehead and Jones, 1969) and to vary considerably (Gladstones and Loneragan, 1967).

Plant parts

The most recently formed parts of cocksfoot have the highest Zn concentration, with the leaf blades usually higher than the stems or leaf sheath (Davey and Mitchell, 1968). Fleming (1963) recorded a mean Zn concentration in the leaf blades of cocksfoot, meadow fescue, perennial ryegrass and timothy of 20 mg kg^{-1} DM compared with a mean of 15 mg kg^{-1} DM for their stems. The flowering heads of the grasses were considerably higher at 36 mg Zn kg^{-1} DM. White-clover flowering heads and leaf plus petiole had similar concentrations of 40 and 42 mg Zn kg^{-1} DM, respectively, but stems were much lower, at 12 mg kg^{-1} DM.

Stage of growth

Although it would be anticipated that, as plants mature, their Zn concentration would decline, due to an increasing proportion of stem, results have not always confirmed this. Gladstones and Loneragan (1967) did find that Zn concentration fell with increasing maturity in 24 different forages and was unaffected by level of soil Zn. No such differences were recorded by Whitehead and Jones (1969) or by Gomide *et al.* (1969). Fleming and Murphy (1968) found a high Zn concentration in the first spring of a trial with mixed temperate grasses, but in the following year Zn concentrations remained constant throughout. No change in Zn concentration with maturity was found for Bermuda grass (Miller *et al.*, 1964), tall fescue (Reid *et al.*, 1967a), hyacinth bean (Hendricksen and Minson, 1985) or white clover (Whitehead and Jones, 1969).

Soil fertility

Raising soil Zn effectively increases Zn concentration in forage (Brown *et al.*, 1962; Winter and Jones, 1977). High applications of fertilizer N have increased Zn concentrations in Bermuda grass and perennial ryegrass by around 12 mg kg^{-1} DM (Miller *et al.*, 1964; Hodgson and Spedding, 1966; Large and Spedding, 1966). If ammonium sulphate was used as the source of N the resulting increased acidity could account for the improved Zn absorption. More normal rates of N fertilization (up to 500 kg ha^{-1} $year^{-1}$) had no consistent effect on forage Zn content of giant star-grass (Rudert and Oliver, 1978), tall fescue (Reid *et al.*, 1967a) or cocksfoot (Reid *et al.*, 1967b). Extremely high levels of lime, which raised soil pH from 4.9 to 6.8, resulted in a lowering of the Zn concentration of Bermuda grass from 37 to 28 mg kg^{-1} DM. In Scotland, increasing soil pH had a much greater impact on lowering Zn concentration in clover than in ryegrass grown on the same site (Fig. 17.1). There is little available information on the effect of phosphorus (P) and potassium (K) fertilizers, but they appear to exercise little influence (Reid *et al.*, 1967a).

Factors Affecting the Manganese Status of Forage

Forage manganese concentrations range markedly from 1 to 2670 mg kg^{-1} DM. The mean value on a worldwide basis was 86 mg kg^{-1} DM, with only South Australia (24%) (Piper and Walkley, 1943) and Scotland (8%) (Hemingway *et al.*, 1968) having a reasonable proportion of samples with concentrations of < 20 mg Mn kg^{-1} DM. New Zealand had the highest mean concentrations, at 166 mg kg^{-1} DM (Smith and Edmeades, 1983).

Effect of species

Where Mn concentrations are not above 60 mg kg^{-1} DM, grass and legumes grown on the same site have similar levels. Above 60 mg kg^{-1} DM, however, grasses tend to have considerably higher values than legumes (Hemingway, 1962; McNaught, 1970; Reay and Marsh, 1976: Metson *et al.*, 1979). Of the grass species, cocksfoot and red-top bent tend to have the highest Mn concentrations, with brome grass and tall fescue intermediate and meadow grass and timothy the lowest. Maize silage contains only half as much Mn as grass silage. White clover has higher Mn concentrations than lucerne but some lupins were found to have much higher concentrations, thought to be due to their deep-rooting capability (Gladstones and Loneragan, 1970).

Plant parts

No very consistent pattern emerges. Similar levels have been found in leaf and stem (Fleming, 1963) for cocksfoot, but Mitchell and Davey (1968) reported twice as much Mn in leaf blade as in the sheath and stem. In six species, Mn in leaf was

higher than in stem, but in meadow fescue and perennial ryegrass the reverse situation prevailed (Fleming, 1963). Generally, stage of growth has little effect on Mn concentration, but small increases (Beeson and MacDonald, 1951) and decreases (Thomas *et al.*, 1952) have been recorded in a range of legumes. This variability in response may be attributable to differences in availability of soil Mn.

Soil fertility

Mn follows Fe as the second most abundant of the essential elements in the earth's crust. Its availability is inversely related to soil pH. Thus the highest Mn concentrations are found in forages growing on acid soil. Whitehead (1966) reported a decrease in the Mn content of forage from 702 to 127 mg kg^{-1} DM following the liming of acid soil. Large falls in Mn concentrations in both ryegrass and clover with increasing soil pH were also recorded in Scotland (Fig. 17.1). Poor drainage also leads to higher Mn concentrations. The use of ammonium sulphate as a N fertilizer reduced soil pH and led to an increase in mixed-pasture Mn content from 31 to 63 mg kg^{-1} DM (Hemingway, 1962) and in six tropical grasses from 121 to 164 mg kg^{-1} DM (Gomide *et al.*, 1969). Ammonium nitrate applications had little effect on soil pH and had no effect on Mn concentration in cocksfoot (Reid *et al.*, 1967b) or star-grass (Rudert and Oliver, 1978).

No consistent trend in seasonal Mn concentrations has been reported (Minson, 1990).

Factors Affecting the Iron Status of Forage

Iron is naturally well supplied by forages, and deficiencies of the element in grazing livestock are unlikely to occur normally but may result from blood loss due to heavy parasitic infestation or some other cause of haemorrhage. The mean concentration in UK grasses has been reported as 103 mg kg^{-1} DM (range 73–154 mg kg^{-1} DM) (Whitehead, 1966). Legumes would generally contain more Fe than grasses (Kabata-Pendias and Pendias, 1992). Forage Fe concentration is affected by changes in soil conditions, by climate and by stage of growth. Grass grown on soils derived from serpentine had Fe concentrations ranging from 2127 to 3580 mg kg^{-1} DM (Johnson and Proctor, 1977), but values above 300 mg kg^{-1} DM would normally reflect soil contamination of the sample, rather than intrinsic Fe. Such adventitious Fe is important in the context of its impact on Cu availability to the ruminant, and consequently care should be exercised in minimizing the level of any such contamination in grass silage and fodder crops.

What Affects Trace-element Availability

This section deals with those factors that influence the availability of the trace-element content of forages to animals and not those affecting the plant content itself. Table 17.1 details the threshold concentrations for desirable, marginal and

Table 17.1. Threshold concentrations of trace elements in forage for ruminants.

			Cattle	Sheep
Cu	Desirable	(Mo < 1.0)	> 10.0mg kg^{-1} DM	> 5.0mg kg^{-1} DM
	Marginal	(Mo > 3.0)	> 10.0	> 5.0
	Deficient	(Mo > 3.0)	<10.0	< 5.0
Co	Desirable		> 0.11	> 0.11
	Marginal		0.05–0.07	0.07–0.10
	Deficient		< 0.05	< 0.07
Se	Desirable		> 0.10	> 0.10
	Marginal		0.03–0.05	0.03–0.05
	Deficient		< 0.03	< 0.03
I	Desirable (normal diet)		0.50	0.50
	(goitrogenic diet)		2.0	2.0
Zn	Desirable		50	50
	Marginal		20–40	30–50
	Deficient		< 20	< 30
Mn	Desirable		25	25
Fe	Desirable		> 30	> 30
	Calves (< 150 kg)		100	
	Cows in late pregnancy		40	

NB Because of the extreme susceptibility of some sheep breeds to Cu toxicity, it is possible, even with forage Cu levels within the normal range of 8–15 mg kg^{-1} DM, for poisoning to occur if forage Mo is also low (0.2–0.8 mg kg^{-1} DM). Critical Cu : Mo ratio = 20 or greater.

deficient levels of these elements in forages. These perhaps somewhat arbitrary thresholds have been based on the Agricultural Research Council (ARC, 1980), Jumba *et al*. (1996) and the author's own experience and delineate where normal production, some loss of tissue trace element status possibly associated with subclinical deficiency and frank deficiency respectively might be anticipated. Productive responses to supplementation would generally be anticipated in the deficient, occasionally in the marginal and not at all in the desirable ranges. Interactions between elements are frequently critical in the ability of a forage to meet the trace-element requirements of ruminants, and a small change in the concentration of one element can mean a swing from deficiency to excess in the supply of another.

Copper

Its availability to the animal is governed mainly by interactions with Mo, S and Fe. Suttle (1981) produced prediction equations for the availability of Cu in grass, grass silage, hay, cereals and brassicas. Even when within their normal ranges, both Mo and S exercised marked effects and, as the relationships are logarithmic, initial small increases in concentration exercise the greatest inhibitory effects. The equations used to predict Cu availability (ACu) are as follows:

Grass	ACu (%) = 5.72 − 1.297S − 2.785$\log_e$Mo + 0.227(Mo × S)
Silage	ACu (%) = 10.6 − 6.65$\log_e$S
Hay	ACu (%) = 8.9 − 0.70$\log_e$Mo − 2.61$\log_e$S
Brassicas	ACu (%) = 0.042 − 0.0096Mo × S

Fresh grass has the lowest available Cu, followed by silage, hay, brassicas and cereals. S alone had the greatest influence on silage Cu availability, while both Mo and S were in operation in fresh grass and in hay, but to a much lesser extent. These equations have proved to be helpful in the diagnosis of Cu deficiency in the field over the past two decades and have been adopted by the extension services with considerable success. Some anomalies do, however, occur, as, for example, the development of hypocupraemia in kale-fed cattle, despite its theoretically high available Cu (Bray and Hacker, 1981), and, at the other end of the scale, the occurrence of copper poisoning in sheep while grazing herbage of normal copper concentration but very low Mo content and despite a relatively high S concentration of 4 g kg^{-1}. The presence of a highly susceptible breed, Texel, undoubtedly contributed to the fatal outcome (MacPherson *et al.*, 1997). The Cu from animal-derived feedstuffs is twice as available as that from plant sources (Baker and Ammerman, 1995).

A high Fe content in spring pasture was blamed by Jarvis and Austin (1983) for Cu deficiency in grazing cattle, but, although individual animals showed clinical signs, no overall growth retardation was recorded. Concentrations of Fe > 300 mg kg^{-1} DM are reported to have a deleterious effect on Cu availability; Suttle and Peter (1985) effected a 33% decrease in sheep given 800 mg Fe and fed on a semi-purified diet, while McFarlane *et al.* (1991) recorded Cu deficiency in cattle grazing pasture on rendzina soils high in Fe. Forages are frequently contaminated with soil Fe and this must be taken into account when estimating Cu availability. As sway-back occurred on pastures grown on calcareous soils, it was thought that Cu availability might be adversely affected by dietary Ca, but this has not been substantiated. High levels of Zn can inhibit Cu uptake and protect against its toxic effects (Bremner *et al.*, 1976), but such high values are likely to be rare in practice. The presence of high levels of Cd in sewage sludge and in superphosphate fertilizers affects Cu absorption and reduces placental transfer.

Cobalt

The availability of dietary Co has not got the same significance as the other essential trace elements, as the requirement by the animal is not for the element itself but for a preformed Co compound, vitamin B_{12}, which is synthesized by microorganisms in the rumen and then absorbed lower down the gastrointestinal tract. This microbial production of the vitamin is vulnerable to Co deficiency, to a decreasing roughage : concentrate ratio and also to greater or lesser production of inactive vitamin B_{12} analogues at the expense of 'true' vitamin $B_{12.}$ (Gawthorne, 1970; Sutton and Elliot, 1972; Hedrich *et al.*, 1973). Sheep fed lucerne hay apparently retained more Co than those fed timothy hay (Looney *et al.*, 1976). If the Co concentration in the rumen fluid falls below 5 ng ml^{-1}, the rate of vitamin B_{12} syn-

thesis will be insufficient to meet the animal's needs (Smith and Marston, 1970). The production of vitamin B_{12} in the rumen is relatively inefficient, as is its absorption from the small intestine. Opposing findings have been recorded with respect to the efficiency of vitamin B_{12} production in the rumen during deficiency and sufficiency of Co supply. Elliot *et al.* (1971) found 3 and 10%, respectively, while Smith and Marston (1970) found 13 and 3%. Absorptive efficiency of synthesized vitamin B_{12} has been variously quoted at values ranging from 5–20% (Smith and Marston, 1970; Hedrich *et al.*, 1973) to 8–38% (Rickard and Elliot, 1978).

Selenium

In forages, Se tends to occur as protein-bound selenomethionine, with smaller amounts of selenocysteine, and both appear to be well used by ruminants. Cantor *et al.* (1975) reported that Se of plant origin is more readily available than that of animal-derived feeds and this was confirmed by Douglass *et al.* (1981). The bioavailability of Se varies widely, both between and within foods of plant and animal origin (Mutanen, 1986). Organic Se appears to be more readily absorbed from the digestive tract than inorganic (Pehrson, 1993). Pehrson *et al.* (1989) found that selenomethionine was about twice as active as inorganic Se in increasing glutathione peroxidase activity in erythrocytes of heifers. Molnár *et al.* (1997) reported that selenomethionine was more efficiently incorporated into the muscle of sheep than sodium selenite, but that there was no difference in organ tissue. In a study in rats, Whanger and Butler (1988) found increased tissue Se retention from selenomethionine, rather than from selenite, particularly in muscle and brain. Selenomethionine can be incorporated directly into body proteins and stored in this form and can thus inflate bioavailability estimates based on body retention (Henry and Ammerman, 1995). There tends to be a greater excretion in urine of inorganic Se (Aspila, 1991), but the reverse is true of milk, where higher concentrations follow organic supplementation.

Se and vitamin E metabolisms have long been linked, due to their complementary roles as biological antioxidants. Consequently, a good supply of either nutrient was able to make good or at least to mask to some extent a deficiency of the other. However, Se is now known to have several other roles in the body unlinked to vitamin E, so there is a fundamental requirement for a dietary supply of the element that cannot be met by high concentrations of the vitamin. The minimum necessary Se concentration in the presence of high vitamin E levels in forages has not, to my knowledge, been established, but it would be unwise to rely on concentrations below the deficiency threshold of 0.03 mg kg^{-1} DM (Table 17.1) being adequate.

Iodine

Dietary I is apparently well absorbed by ruminants, mostly from the abomasum (Miller *et al.*, 1974; Price, 1989), but its incorporation into the thyroid may be reduced by low dietary Co and Mn concentrations (Underwood, 1977). However,

the main inhibitor of I availability/absorption and thyroid hormone synthesis is the presence of goitrogens. Brassicas contain the vinylthio-oxazolidine type of goitrins (Hetzel and Maberly, 1986), while white clovers contain cyanogenetic glycosides (Flux *et al.*, 1956). Grasses, however, appear to be largely free of goitrogenic activity. According to Underwood (1981), in times of a diminished I supply, the thyroid can clear the element from the blood more rapidly and utilize it in hormone synthesis more efficiently. Through its presence in the deiodinase enzymes, Se is required for the conversion of tetraiodothyronine to tri-iodothyronine and may thus influence I bioavailability (Arthur *et al.*, 1988). The processes of ploughing, drainage and liming involved in the improvement of upland pasture have been implicated in a reduction in their capacity to retain I in a bioavailable form (Lidiard, 1995). Miller and Ammerman (1995) conclude a recent review on I availability by saying that little is known about its bioavailability from plant sources.

Zinc

There is a lack of information on the physiological availability of Zn from forages to ruminants. In monogastrics, Zn from animal-derived protein concentrates is more available than that from plant sources, due to its combination with phytate and fibre (Underwood, 1981). High Ca can inhibit Zn absorption, particularly in pig and poultry diets and the ratio of phytate $\times$ Ca:Zn is claimed to be a better predictor of Zn availability than the phytate:Zn ratio *per se* (Fordyce *et al.*, 1987). Other elements that adversely affect Zn availability include Cu (although the reverse effect is more potent), Cd and Pb in high concentrations. Some, but not all, chelators can improve Zn bioavailability – their efficacy is related to their stability constants (Vohra and Kratzer, 1964).

Manganese

Absorption of dietary Mn by all domestic livestock appears to be poor and is adversely affected by high concentrations of Ca, P and Fe. Excess P appears to be a greater inhibitor of dietary Mn than Ca and it appears to exercise its effect at gut level (Wedekind and Baker, 1990a, b). Bremner (1970) reported that only 10% of Mn in sheep rumen was in a soluble form, compared with about 50% of dietary Mn being water-soluble. Other workers have shown that no more than 1% dietary Mn is absorbed (Sansom *et al.*, 1976; Vagg, 1976) by adult cattle. Most of the data relating to Mn bioavailability have been obtained from studies with poultry, but Henry (1995) states that results suggest that it is similar for both poultry and sheep. Excess Mn is removed by the liver and excreted in the bile.

Iron

Not much is known about the availability of forage Fe to ruminants other than that it is affected by the solubility of the chemical form, e.g. ferric oxide Fe is signifi-

cantly less available to calves and sheep than more soluble forms, such as ferrous sulphate and ferric chloride (Morris, 1987). However, as little Fe is lost from the body, the normal content of the grazed herbage is sufficient to supply all that the animal requires.

How to Remedy Low Status

There are a multiplicity of methods by which trace-element deficiency in ruminants grazing forage or consuming conserved products made from it may be remedied. It would require a review in its own right to assess correctly the best options for each situation. All that can be done here is to list the range of methods available and possibly highlight the author's preference or prejudice with respect to some of them.

For animals at grass in non-extensive situations, it is possible to treat the ground with the appropriate trace element(s) and raise herbage concentration(s) sufficiently to meet the need of the grazing livestock. This works well for Co, where a single treatment of 2 kg ha^{-1} can boost herbage concentrations to normal for 3 or 4 years. New Zealand and Finnish experience has shown that Se treatment can be effective in raising herbage and crop Se status sufficiently to correct deficiencies of the element in livestock and humans. Application at a rate of 1 kg ha^{-1} provided protection for 2 years (Aspila, 1991; Watkinson, 1991). Se treatment has not often been attempted in the UK, but there is no reason why it should not operate successfully here. With Cu, however, while there is a big initial boost in concentration, it is not maintained and long-term effects tend to be marginal and generally insufficient on their own to correct a deficiency.

In extensive and rough-terrain grazings, such applications are either uneconomic or impracticable and treatment or prevention of deficiencies has to rely on the provision of free-access minerals. These may be provided loose in a covered trough, to prevent weathering, or in the form of blocks or mineral licks. To encourage optimum usage, they are best positioned close to watering points and one block or trough should be available for every 25–40 cows. The major snag with this method is the variable intake, ranging from nil to an oversupply. This may have to be tolerated if the alternative of individual animal treatment is inconvenient to the nature of the enterprise. Where cattle and sheep are grazing side by side, care needs to be exercised with respect to Cu, as sheep are highly susceptible to excess intakes of the element, whereas cattle are relatively tolerant.

Where handling facilities exist and individual treatments can be administered successfully, there are again a wide variety of possibilities. These include oral treatments of various descriptions and parenteral-injectable preparations, and many of these were reviewed by Allen and Moore (1983) and MacPherson (1983). On the basis that the fewer treatments that are required per annum the better, only such long-efficacy products will be alluded to, unless some other consideration becomes paramount. Various boluses, from the traditional Co one to the more recently developed soluble glass products, provide suitable vehicles for the correction of deficiency in grazing ruminants. These can be either single-element treatments or various combinations of two or more elements and, in some cases, vitamins as well,

as in the Agrimin all-trace bolus. These can be effective for 6–8 months at grass and can provide all the necessary trace elements for a complete grazing season, at least in these northern climes, at a single handling. One of the possible drawbacks with the traditional bullets was the risk of their forming an insoluble coating of $CaCO_3$ and being thus rendered ineffective. This was possible because these boluses did not erode from the surface but the active ingredient, which only formed a very small proportion of the total weight, leached out through the interstitial spaces. With the soluble-glass bolus, the whole surface is continually eroding and this is also true of the exposed end of the Agrimin all-trace bolus. Where a traditional bolus is being used in a situation where coating is a problem, it can be overcome, to some extent, by the use of a second bolus or an inert grub screw. Constant abrasion of the two boluses in the reticulum prevents build-up of the coating. Regurgitation is another hazard of this form of treatment but, where care is taken in the administration of the bolus, it should not be too serious. Another treatment that is effective over a whole grazing period is copper oxide wire, which is administered in a gelatin capsule, which dissolves rapidly, allowing the short pieces of wire to become trapped by the intestinal villi, where the acid conditions dissolve some of the Cu which is then absorbed and stored in the liver (MacPherson, 1984). Injectable preparations, which use barium selenate as the source of Se, have also been shown to be long-lasting (MacPherson *et al.*, 1988), being efficacious in cattle for up to a year and in ewes for up to 18 months.

Administration of trace elements via the water-supply is feasible and can be done by means of a plumbed-in metering device or by the addition of trace-element pellets placed in a filter tube at the bottom of water troughs. In order for this method of supplementation to be successful, there must be no access to untreated sources of water, such as streams or springs.

For housed animals, the method of choice is by supplementation of the feed with the required amounts of the appropriate trace elements. This can be achieved by incorporation into a complete diet or into the concentrate portion of it or by dissolving the required elements and spraying them over the forage portion of the ration. These methods ensure that all animals get their requisite intake of these essential nutrients by eliminating the possibility of individuals selecting not to consume the minerals provided.

A matter of some controversy in recent years has been the debate over the relative merits of inorganic versus organic or chelated minerals. Such products include bioplexes, where the minerals are bound to proteins or yeast, and chelates and metalosates, where the mineral is bound to a protein, part of a protein or to a specific amino acid, such as lysine. In theory these organically bound products should be protected against breakdown in the rumen, thus preventing the formation of unavailable chemical compounds. The protected compounds then progress further down the digestive tract and are absorbed. Great claims are made for these new formulations with respect to improved performance and fertility and enhanced resistance to infectious challenge, such as mastitis. To date, however, these claims have not been substantiated by solid scientific evidence; in studies comparing the treatments, when used at the same rate, no productive benefit has been recorded for the complexed minerals and they have the added disadvantage of being considerably more expensive. A situation of high forage Mo would be one where Cu pro-

teinate products would be expected to be beneficial if they could successfully protect the element from forming insoluble compounds with Mo in its passage through the rumen. However, in my own experience, one company would not support a trial in just such a situation because they felt it might present too severe a test of their product's efficacy. Similarly, claims are made for the efficacy of Se yeast products, but many much cheaper alternatives apparently work highly effectively. At present, the best approach to this problem would appear to be to use the considerably cheaper traditional products first and only if these fail to resort to the appropriate bioplex or chelate.

Further Research Needed

As trace-element deficiencies are widespread in forages throughout the world and as some exist now in areas of improved pasture where they did not prior to the improvement, there would appear to be ample scope to improve the trace-element content of the productive forages by selective breeding and to such an extent as to render fertilization or direct animal supplementation unnecessary. In tandem with this could be a selective breeding programme to produce lines of animals that would be less susceptible to the effects of low or deficient intakes. This has been at least begun with the Scottish Blackface breed, where a flock has been separated into 'low'- and 'high'-line animals with respect to their ability to absorb Cu and to resist the development of sway-back in their lambs (Wooliams *et al.*, 1986). Breeding for less susceptibility to Cu poisoning should also be feasible for vulnerable breeds, such as the Texel.

Definitive studies could be undertaken to determine whether chelated minerals do have advantages over inorganic sources and, if they do, to identify the situations where these would be of sufficient economic benefit to justify their use. Better diagnostic criteria would be helpful to identify economically responsive situations more accurately than is currently possible. A reliable indicator for Co deficiency in cattle remains a priority for future research.

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18 Major Minerals in Forages

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Important Major Minerals

Undernutrition is commonly accepted as one of the most important limitations to grazing livestock production. The lack of sufficient energy and protein is often responsible for suboptimum livestock production. However, numerous investigators have observed that ruminants sometimes deteriorate in spite of an abundant feed supply. Mineral imbalances (deficiencies or excesses) in soils and forages have long been held responsible for low production and reproductive problems among grazing ruminants in the tropics. Wasting diseases, loss of hair, depigmented hair, skin disorders, non-infectious abortion, diarrhoea, anaemia, loss of appetite, bone abnormalities, tetany, low fertility and pica are clinical signs often suggestive of mineral deficiencies throughout the world.

The seven major minerals supplied by forages are calcium (Ca), chlorine (Cl), phosphorus (P), magnesium (Mg), potassium (K), sodium (Na) and sulphur (S). Each of these minerals has been found to be deficient for grazing livestock under specific conditions, with the exception of Cl (McDowell, 1985, 1992, 1997). Deficiencies of Cl in animals in general have been unequivocally observed only on specially purified or concentrate diets. There are few forage Cl concentrations reported, and those that are available indicate adequacy of the element. Cl will not be discussed further in this review. The importance of the remaining macrominerals is as follows.

Calcium and phosphorus

Clinical signs of borderline Ca and P deficiencies are not easily distinguishable from those of other deficiencies. An inadequate intake of Ca may cause weakened bones, slow growth, low milk production and tetany (convulsions) in severe deficiencies. Signs of P deficiencies are not easily recognizable, except in severe cases,

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when fragile bones, general weakness, weight loss, emaciation, stiffness, reduced milk production and chewing of wood, rocks, bones and other objects may be noticed. Abnormal chewing of objects may also occur, however, with other dietary deficiencies. A number of tropical countries (e.g. South Africa, Argentina, Brazil and Senegal) have reported death from botulism as a result of bone-chewing (McDowell, 1992). In areas of Piaui, Brazil, an estimated 2–3% of approximately 100,000 cattle die annually of botulism.

For grazing livestock, the most prevalent mineral-element deficiency throughout the world is lack of P (McDowell, 1976; Underwood, 1981). In a review (McDowell *et al.*, 1984), P deficiency was reported in 46 tropical countries of Latin America, South-East Asia and Africa. P deficiency is more prevalent for tropical than for temperate grazing regions. It is also known that in tropical grasses there is very little or no difference in P concentration between species in a given region (Long *et al.*, 1970; Minson, 1990). Many reports from tropical regions of the world, dating back to the early part of the century, have revealed the beneficial effects of P supplementation on overall performance. Reports of improved weight gains by P-supplemented cattle have been summarized for various world regions (McDowell, 1985). From Brazil, Moraes *et al.* (1982) related supplemental P to daily gains of growing cattle. Treatments of 0, 4.3, 6.7 and 8.4 g of P per day resulted in daily gains of 216, 379, 465 and 564 g, respectively. From Peru, Echevarria *et al.* (1973) obtained weight gains of 0.59 kg day^{-1} in steers supplemented with dicalcium phosphate and 0.27 kg for controls. Bolivian cattle gained 96.4 kg in 338 days when receiving a source of P, versus 79.4 kg for controls (McDowell *et al.*, 1982). Estevez-Cancino (1960) reported increased milk production (up to 24%) with bone-meal supplementation in farms that had P-deficient forages.

The most devastating economic result of P deficiency is reproductive failure, with P supplementation dramatically increasing fertility levels in grazing cattle from many parts of the world. In 2-year observations of 200 South African cattle, calf crops increased from 51% in control cattle to approximately 80% for cattle supplemented with bone-meal or other P sources (Theiler *et al.*, 1928). Latin American countries reporting increased fertility rates resulting from P administration include Bolivia, Brazil, Colombia, Panama, Peru, Uruguay and Venezuela, and from Asia, the Philippines and Thailand (McDowell, 1997). From this review, averaging 18 cattle reports on the effect of supplementation on calving percentage, those receiving P supplement averaged a 73.3% calf crop versus 51.3% for controls (McDowell, 1997; Rojas and McDowell, 1997).

From Zimbabwe (Ward, 1968) and northern Australia (Lamond, 1970), the effects of undernutrition on fertility were apparent. With few exceptions, lactating cows were not calving 2 years in succession. In P-deficient areas, if a calf is produced, cows may not come into a regular oestrus again until body P levels are restored, either by feeding supplementary P or by cessation of lactation.

Compared with P inadequacies, Ca deficiency is rare in grazing cattle, with the exception of cows lactating large quantities of milk or those grazing on acid, sandy or organic soils in humid areas where the herbage consists mainly of quick-growing grasses devoid of legume species (Underwood, 1981). Although Ca deficiency can easily be produced in young, growing animals and lactating dairy cows fed native forages supplemented with concentrates, the deficiency is infrequently reported in grazing beef cattle, even during lactation.

Magnesium

The practical importance of Mg is its relationship to the serious metabolic disorder grass tetany (hypomagnesaemia). Grass tetany is a complex ruminant metabolic disorder that is affected by forage species and mineral composition, soil properties, fertilizer practices, season of the year, temperature, animal species, breed and age.

Clinical signs of hypomagnesaemia in ruminants include reduced appetite, increased excitability, profuse salivation and convulsions. For adult ruminants, milk yield is usually reduced and the animals are nervous. In more severe cases, affected cows may avoid the rest of the herd, walk with a stiff gait and lose their appetite. In the most severe stage, the animal collapses to the ground with continuation of the tetanic muscular spasms. Death occurs after the collapse stage of tetany if the animal does not receive medical treatment.

Grass tetany is a serious problem of grazing ruminants in many parts of the world. Susceptibility to grass tetany is increased in older ruminants because of decreased ability to mobilize skeletal Mg with increasing age. Grass tetany generally occurs during early spring or a particularly wet autumn, among older cattle grazing grass or small-grain forages in cool weather. Clinical tetany is endemic in some countries, affecting only a small proportion of cattle (1–2%). However, individual herds may report tetany as high as 20%. Females are mainly affected, with cattle more susceptible than sheep or goats. This is especially a problem with grazing beef cattle, where a first indication of grass tetany may be finding one or more dead animals. Excellent and extensive reviews concerning hypomagnesaemic tetany are cited by Rendig and Grunes (1979) and Underwood (1981).

Occurrence of tetany is seen where ruminant production is highly developed, high-quality pastures are available and high-yielding, quick-maturing stock is raised. Incidence of hypomagnesaemic tetany occurs in most European countries, North America, Australia, South Africa and New Zealand. Some of the reasons for fewer reports of grass tetany in tropical regions include the prevalence of the condition during cooler temperatures (8–14°C) and, for many tropical countries, the generally low productivity of pastures and of herds and lack of fertilization of pasture with nitrogen (N) and K.

Sodium

The initial sign of Na deficiency is a craving for salt (NaCl), demonstrated by the avid licking of wood, soil and sweat from other animals, and drinking water. Cattle deprived of salt may be so voracious that they often injure each other in attempting to reach salt. A prolonged deficiency causes loss of appetite, decreased growth, unthrifty appearance, reduced milk production and loss of weight. More pronounced signs of Na deficiencies include shivering, incoordination, weakness and cardiac arrhythmia, which can lead to death.

Natural forages low in Na have been reported in numerous tropical countries throughout the world. French (1955) concluded that NaCl is the most needed nutrient for livestock throughout East Africa. According to Sütmoller *et al.* (1966), the insufficiency of Na is the most widespread mineral deficiency in the Amazon

Valley of Brazil (~ 3.5 million square kilometres). From Bolivia, steers receiving salt had significantly higher average weights than unsupplemented controls – 385 versus 370 kg, respectively (McDowell *et al.*, 1984). In Zambia, Walker (1957) reported that the daily feeding of 28 g of NaCl resulted in a marked increase in weight gains for grazing steers. Australian workers (Murphy and Plasto, 1973) noted dramatic responses in beef cows supplemented with salt.

Na deficiency is most likely to occur: (i) during lactation, due to secretion of Na in milk; (ii) in rapidly growing animals; (iii) under tropical or hot semi-arid conditions, where large losses of water and Na occur in the sweat and where pastures are low in Na; and (iv) in animals grazing pastures heavily fertilized with K, which depresses herbage Na levels. Even after prolonged severe deficiency, NaCl levels secreted in milk remain high. Thus, lactating animals suffer most from lack of salt in the diet.

Potassium and sulphur

There are fewer reports of K and S deficiencies under natural grazing conditions than of P, Ca, Na and Mg. K deficiency for ruminants results in non-specific signs, such as slow growth, reduced feed and water intake, lowered feed efficiency, muscular weakness, nervous disorders, stiffness, decreased pliability of hide, emaciation, intracellular acidosis and degeneration of vital organs.

There are very few confirmed reports of K deficiency for ruminants grazing exclusively forages. One report from Nigeria indicated clinical manifestations of K deficiency in 27 cattle raised in the traditional semi-nomadic herding system, which consumed forages analysed as containing 2 g K kg^{-1} dry matter (DM) (Smith *et al.*, 1980). Clinical signs quickly disappeared after administration of K. The main reason for lack of widespread K deficiency, even when forages contain less than the requirement, is probably due to other nutrient deficiencies of forages. Mature tropical forages are often deficient in energy, protein, P, Na and a number of trace elements. It is likely that a K deficiency will not be expressed as long as there are other nutrients that are even more deficient.

Signs of S deficiency have been described as loss of weight, weakness, lacrimation, dullness and death. In S deficiency, microbial protein synthesis is reduced and the animal shows signs of protein malnutrition. S is required for protein formation, and the depressed appetite of animals eating low-S feed is due to a protein deficiency. Low levels of S have been found in young regrowths of *Digitaria decumbens* and feeding an S supplement has increased voluntary feed intake by 28% (Rees *et al.*, 1974). In relation to animal requirements, temperate forages are generally adequate in S compared with tropical plants.

Forage as a Supplier of Major Elements

Forage macromineral concentrations

Frequently, livestock producers, particularly in tropical countries, do not supplement grazing livestock with minerals, with the possible exception of salt. Grazing

livestock must therefore depend largely upon forages to supply their mineral requirements. However, often forages cannot completely satisfy each of the mineral requirements for grazing animals. Table 18.1 summarizes the mineral concentrations of 2615 Latin American forages (McDowell *et al.*, 1977). Borderline or deficient levels of macroelements were noted for many entries: Mg, 35%; P, 73%; and Na, 60%. For K, 84.9% of samples were high (> 8 g kg^{-1}) in the mineral and for S there were no analyses.

Most often, tropical forages contain less macrominerals than species grown in temperate regions. A number of publications from different tropical regions of the world have well documented the inadequacy of tropical forages in meeting the mineral requirements of grazing ruminants (French, 1955; McDowell, 1976, 1985, 1997; Underwood, 1981). Table 18.2 evaluates forage macromineral concentrations from one African and six Latin American countries. The majority of forages from the seven countries were low to deficient in Ca, P, Na and Mg; in addition, the majority of forages from two of seven countries were low in K.

Calcium

Reviewing the world literature on forage Ca (1263 samples), Minson (1990) reported that 31% of forages were low in Ca (< 3 g kg^{-1} DM). A similar proportion of 1123 samples of forage collected in Latin America (McDowell *et al.*, 1977) and 103 Caribbean grass samples (Devendra, 1977) contained less than 3 g kg^{-1} DM

Table 18.1. Mineral breakdown and concentrations of 2615 Latin American forages (g kg^{-1}, DM).*

Element	No. of entries	Requirements of ruminants†	Level of nutrients in forages	Low level	Higher level
Calcium	1123	1.8–8.2	Concentrations	0–3	Over 3
			Percentage of total entries	31.1	68.9
Magnesium	290	1–2	Concentrations	0–2	Over 2
			Percentage of total entries	35.2	64.8
Phosphorus	1129	1.8–4.8	Concentrations	0–3	Over 3
			Percentage of total entries	72.8	27.2
Potassium	198	6–8	Concentrations	0–8	Over 8
			Percentage of total entries	15.1	84.9
Sodium	146	0.6–1.8	Concentrations	0–1	Over 1
			Percentage of total entries	59.5	40.5

* Latin American Tables of Feed Composition (McDowell *et al.*, 1977).

† Requirements (DM) based on national research and agricultural research councils for various ruminant species (McDowell, 1992, 1997).

Table 18.2. Percentage of forage minerals below critical concentrations needed by ruminants.*

Mineral level	Critical level based on ruminant needs† (g kg^{-1} DM)	Dominican Republic (1)	Bolivia (2)	Colombia (3)	Guatemala (4)	Malawi (5)	Nicaragua (6)	Venezuela (7)
Ca	3	24	57	100	71	13	80	97
P	2.5	83	100	92	57	75	67	98
K	6–8	0	1	15	13	57	20	84
Na	0.6	78	100	100	88	97	80	84
Mg	2	33	64	56	76	31	96	94

* Complete reference citations for each study are given in McDowell (1997).
† Critical levels based on Ruminant National Research and Agricultural Research Councils for various ruminant species (McDowell, 1992, 1997).
1, based on 69 samples (Jerez *et al.*, 1984); 2, based on 84 samples (Peducassé *et al.*, 1983); 3, based on 36 samples (Vargas *et al.*, 1984); 4, based on 84 samples (Tejada *et al.*, 1987a, b); 5, based on 21 samples (Mitimuni *et al.*, 1990); 6, based on 304 samples (Velásquez-Pereira *et al.*, 1997a, b); 7, based on 198 samples (Rojas *et al.*, 1993a, b).

Ca. Temperate forages generally contain more Ca than those grown in the tropics. Hay from Ireland had a mean Ca concentration of 8 g kg^{-1} DM (Wilson *et al.*, 1968), which is similar to that reported by the Pennsylvania State Forage Test Service for over 9500 forage samples grown in 5 years (Adams, 1975). The mean level of Ca was 8.4 g kg^{-1} DM, with mean values of 4 and 11.5 g kg^{-1} DM for grass and legume hays, respectively. Legumes are significantly greater sources of Ca than grasses.

Phosphorus

The mean P concentration of 1823 forage samples from the world literature was 2.9 g kg^{-1} DM (Minson, 1990). This would be higher than the predominantly tropical forages from Latin America, where 73% of 1129 forages contained less than 3 g P kg^{-1} DM (McDowell *et al.*, 1977). From six ranches in northern Mato Grosso, Brazil, forage P averaged 0.8 and 2 g kg^{-1} DM for the dry and wet season, respectively (Sousa *et al.*, 1979). From Costa Rica, 1468 forage samples contained 1.8 g P kg^{-1} DM in the wet season, and 1335 samples averaged 1.1 g P kg^{-1} DM in the dry season (J. Sanchez, personal communication). Table 18.3 illustrates extremely low Ca and P (particularly P) forage concentrations for 11 ranches in Venezuela during the wet and dry seasons. For all farms, the extremely P-deficient farms averaged 0.8 g P kg^{-1} DM in the wet season and 0.6 g P kg^{-1} DM in the dry season.

In the temperate zones, higher levels of P have usually been reported. Forage samples analysed by the Pennsylvania State Forage Testing Service over a 5-year period contained 2.6 g P kg^{-1} DM, with a higher concentration in legumes (3 g kg^{-1} DM) than in grasses (2.2 g kg^{-1} DM) (Adams, 1975).

Magnesium

The Mg content of forage plants is normally higher in legumes than in grasses. Mayland *et al.* (1976) estimated the tetany hazard of several forages and found wheat > oats > barley > rye. Mg levels of wheat were the lowest, whereas those of rye were the highest.

For tropical herbage, Mg concentrations were relatively low, with slightly more than one-third of 288 forages included in the 1974 *Latin American Tables of Feed Composition* containing 2 g Mg kg^{-1} DM or less (McDowell *et al.*, 1977). Forage mineral analysis from a large number of farms in four Latin American tropical countries indicated that a considerable number of forages contained less than 2 g Mg kg^{-1} DM: Bolivia (64%), Colombia (56%), Dominican Republic (33%) and Guatemala (76%) (McDowell, 1985).

Sodium

There are two classes of forage plants, the Na accumulators and the non-accumulators (Youssef, 1988). Na-accumulating forages contain > 2 g Na kg^{-1} DM, provided the soil contains sufficient Na and soil K is not in excess. The non-accumulating forages always contain 2 g Na kg^{-1} DM, even when grown on soils high in Na, and the concentration is almost independent of the level of soil K (Minson, 1990). Forages usually range from 0.07 to 1.2 g Na kg^{-1} DM. The Food and Agricultural Organization (FAO) has published an extensive summary of the composition of tropical feeds (Göhl, 1975). High-Na sources (DM basis) were: salt bush,

Table 18.3. Mean forage calcium and phosphorus concentration (g kg^{-1} DM) from cattle ranches in the states of Monagas and Guarico, Venezuela (adapted from Velásquez, 1979; Faria *et al.*, 1981).*

	Monagas				Guarico			
	Calcium		Phosphorus		Calcium		Phosphorus	
Ranch no.	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season
1	2.6	3.1	0.5	1.0	1.9	3.0	0.1	1.6
2	1.9	1.8	0.8	1.6	1.9	1.7	0.5	1.6
3	1.4	1.2	1.2	0.5	2.4	3.5	0.2	1.3
4	1.7	2.3	0.7	0.8	1.6	2.9	1.8	0.2
5	1.7	2.2	0.7	0.8	1.5	3.3	20.6	2.2
6	0.6	1.6	0.9	0.7	1.2	1.0	1.0	0.2
7	1.6	1.5	0.8	1.0	1.2	1.8	1.0	0.2
8	–	–	–	–	2.1	1.2	1.0	0.4
9	–	–	–	–	3.2	1.4	4.0	0.4
10	–	–	–	–	4.9	2.8	3.0	0.7
11	–	–	–	–	3.8	3.6	1.0	0.3
Overall mean	1.6	1.9	0.8	0.9	2.3	2.4	0.6	0.8
Standard deviation	0.6	0.6	0.2	0.3	1.2	1.0	0.8	0.7

* Each mean is based on four pastures per farm and ten subsamples per pasture.

63.4 g kg^{-1}; creeping salt bush, 46.6 g kg^{-1}; seaweed meal, 29.3 g kg^{-1}; and sunflower meal, 20.9 g kg^{-1}.

Na deficiency is more likely to occur in cattle grazing tropical pasture species, as these plants generally accumulate less Na than temperate species (Morris, 1980). Natural forages low in Na have been reported in numerous tropical countries throughout the world (McDowell, 1985). Forage Na analysed from cattle ranches in northern Mato Grosso, Brazil, during both the wet and dry seasons, was extremely deficient, meeting only between 14 and 30% of the animals' requirements (Sousa *et al.*, 1982).

Potassium

K in growing forage is usually quite high (10–40 g kg^{-1} DM); thus, it had been assumed that grazing livestock consuming primarily a forage diet would receive adequate K. In tropical regions, it is possible that K deficiencies could arise, in view of the decreasing content of this mineral with increasing forage maturity during the extended dry season and the use of urea, which supplies none of this element. Low K values have been reported for forages in Florida, Brazil, Panama, Nigeria, Swaziland, Uganda and Venezuela (McDowell, 1997). In Brazil, average K content (DM basis) of six grasses at 4 weeks was 14.2 g kg^{-1} DM versus 3 g kg^{-1} DM at 36 weeks of age (Gomide *et al.*, 1969). In Florida, a 1979 forage analysis from four regions indicated adequate K in September–October, but five of seven ranches sampled in February–March contained forage K concentrations considerably less than 6 g kg^{-1} DM (Kiatoko *et al.*, 1982). Kalmbacher and Martin (1981) report creeping bluestem (*Schizachyrium stoloniferum*) affected by seasonal patterns and low K concentrations (DM basis), ranging from 3.3 to 5.4 g kg^{-1} with an average of 4.2 g kg^{-1} DM.

Sulphur

S analysis in biological materials is comparatively difficult. For these reasons, there is a scarcity of S analyses, particularly tropical forages. A limited amount of analysis has indicated that many tropical forages contained less than an optimum S concentration of 2 g kg^{-1} DM. S analyses of 10 forage samples from the llanos rangelands of both Colombia and Venezuela were low, ranging from 0.32 to 0.88 g kg^{-1} DM (Miles and McDowell, 1983). From these regions, severe leaching of soils and frequent burning of grasslands led to the assumption that many, if not most, llanos rangeland forages would be found deficient in S. Ruminants consuming large quantities of maize silage would probably receive inadequate S. More than 80% of the maize silages had less than one-half the estimated 2 g S kg^{-1} needed for lactating cows (Miller, 1979). Forages high in S (e.g. > 4 g kg^{-1} DM) combined with molybdenum will bring about copper deficiencies.

Grazing selectivity and forage mineral intake

Selective grazing patterns of livestock in relation to choosing not only particular species of forage but parts of the plant need consideration. The principles of dietary selection are not clearly understood. Nevertheless, there is recorded evidence that

grazing animals tend to select higher protein and more highly digestible fractions from the pasture available (Arnold *et al.*, 1966). In conditions of reduced availability of mineral elements from the soil, the younger leaves of growing plants, which constitute a highly digestible fraction, are often the leaves of lowest content of most elements, which are poorly or only slowly mobilized within the plant. Kincaid and Cronrath (1983) reported that significant portions of Ca and P are associated with the fibrous versus the leaf portion of lucerne (alfalfa) hay and silage. P, Na, K and Mg were all in higher concentration in stems than in leaves of dwarf *Pennisetum purpureum* (Montalvo *et al.*, 1987). Under grazing conditions, however, cattle show a preference for leaves over stems, and leaf consumption can be 46% more than stems of similar digestibility and the animals are capable of selecting a diet of 60% leaves from a sward that has only 20% (Laredo and Minson, 1973; Stobbs, 1976).

What Affects Major-element Status of Forage?

Wide ranges of mineral concentrations in tropical forages have been observed (Reid and Horvath, 1980; McDowell, 1992). The concentration of mineral elements in plants from diverse world regions are dependent upon the interaction of a number of factors, including soil, plant species, stages of maturity, yield, pasture management and climate. As an example, forage mineral content is affected by: (i) plant maturity; (ii) species as well as variety within a species; (iii) management procedures such as grazing or crop removal systems; (iv) fertilization, particularly with K and N; and (v) soil and environmental conditions (McDowell, 1985). Interrelationships with other elements likewise influence forage mineral concentrations. In many tropical countries, high amounts of soil iron (Fe) and aluminium (Al) accentuate P deficiency by forming insoluble phosphate complexes, making P unavailable for plant uptake.

Soils

Soil is the source of all mineral elements found in plants. Most naturally occurring mineral deficiencies in livestock are associated with specific regions and are directly related to both soil mineral concentration and soil characteristics. Of the total mineral concentration in soils, only a fraction is taken up by plants. The 'availability' of minerals in soils depends upon their effective concentration in soil solution (Reid and Horvath, 1980).

Young and alkaline geological formations are more abundant in most elements than are the older, more acid, coarse, sandy formations (Hartmans, 1970). In tropical regions under conditions of heavy rainfall and high temperature, there has been marked leaching and weathering of soils, making them deficient in plant minerals (Pfander, 1971).

Soils derived from basic igneous rocks usually have higher readily soluble contents of the important elements than do corresponding soils from acid igneous or metamorphic parent materials. The amounts of readily soluble elements are generally smaller in coarse-textured than in fine-textured soils.

Soil, drainage and pH

The soil content of an element would seem the most important limitation. However, availability factors, including soil pH, texture, moisture content and organic matter, are probably more often the limiting factors rather than soil mineral content (Williams, 1963). With increasing acidity of soils, there is impaired absorption of Ca, P and Mg (Reid and Horvath, 1980). Elkins *et al.* (1978) found that tall fescue grown in field trials on poorly drained soil had lower concentrations of Mg than fescue grown on well-drained soil.

Forage species and varieties

Mineral intake by animals depends more on the type of plant and level of consumption than on the parent rock from which the soil was derived and on which plants were grown. Large variations in mineral content of different plant species growing on the same soil have been reported (Thompson, 1957; Gomide *et al.*, 1969; Underwood, 1981). *Brachiaria decumbens* nearly doubled its P content when soil P was doubled, but P content of other pasture species, such as *Sporobulus*, did not vary widely (Long *et al.*, 1969). It is a generally accepted view that herbs and legumes are richer in a number of mineral elements than are grasses (Fleming, 1973). From Rhodesia, Jones (1964) reported one variety of *Chloris gayana* (DM basis) to contain 0.3 g Na kg^{-1}, while a second variety grown on the same site had a vastly different Na level of 3.1 g kg^{-1}.

Forage maturity

Variations in mineral content of tropical grasses have been reported to occur with increasing age of the plant. Gomide *et al.* (1969) studied the N, K, P, Ca and Mg content of five tropical grasses of Brazil at different stages of maturity (Table 18.4). It was concluded that, as forages neared maturity, mineral content declined for most minerals, and that K and P could be deficient for grazing cattle. In most circumstances, P, K, Mg, Na and Cl decline as the plant matures (Gomide *et al.*, 1969; Underwood, 1981). Forage Ca concentration is less affected by advancing maturity (Gomide *et al.*, 1969), thereby resulting in a detrimental widening of the ratio of this mineral with other elements (e.g. a wide Ca:P ratio). Stage of maturity is probably the most important factor influencing forage K. Forages harvested at an early stage of maturity among the richer sources of K. Actively growing grass and legumes are usually high in K, containing 10–50 g kg^{-1} DM. However, mature pastures, winter pastures that have weathered or hay that has been exposed to rain and sun can have deficient K levels.

As plants mature, mineral contents decline, due to a natural dilution process (Fleming, 1973) and translocation of nutrients to the root system (Tergas and Blue, 1971). Fleming (1973) concluded that, as photosynthetic areas increase, DM production outstrips mineral uptake, resulting in a decline in mineral concentration. The process of translocation of nutrients is a more serious problem for tropical

Table 18.4. Variation of mineral composition with forage age.

	Forage age (days)	Composition of forage (g kg^{-1} DM)			
		P	K	Ca	Mg
Grass					
Guinea grass	14	1.8	22.4	4.1	2.8
(*Panicum maximum*)*	28	1.4	23.3	3.4	2.3
	42	1.3	28.0	3.4	2.0
	56	1.0	26.4	3.4	1.7
	70	0.8	25.3	3.1	1.4
Molasses grass	14	2.0	23.0	2.8	2.5
(*Melinis minutiflora*)*	28	1.8	23.0	2.7	2.5
	42	1.8	22.0	2.7	2.5
	56	0.6	20.0	2.0	2.0
	70	0.5	17.0	2.0	1.8
Elephant grass	28	3.3	23.8	6.1	4.2
(*Pennisetum purpureum*)*	84	1.5	12.0	3.8	2.8
	140	1.1	3.4	4.3	3.6
Pangola grass	28	1.6	13.2	5.6	3.9
(*Digitaria decumbens*)*	84	1.1	7.4	5.0	3.8
	140	1.2	3.7	6.6	3.9
Jaragua grass	28	2.8	16.8	4.0	4.6
(*Hyparrhenia rufa*)*	56	1.7	6.3	2.0	3.6
	84	1.1	5.7	2.3	5.8
Setaria (*Setaria sphacelata*)†	Hay stage	1.3	–	1.1	1.4
Rhodes grass (*Chloris gayana*)†	Hay stage	1.4	–	1.7	1.5
Legumes:					
Gliricidia	60	1.9	27.5	11.9	4.0
(*Gliricidia sepium*)‡	90	2.1	28.0	17.5	4.0
	120	2.3	25.5	16.9	4.2
Leucaena	21	1.2	25.0	6.3	4.2
(*Leucaena leucocephala*)§	42	1.3	25.0	13.0	2.5
	63	1.0	23.4	12.6	2.5
	84	1.0	22.5	12.1	2.4

* Modified from Gomide *et al.* (1969).
† Modified from Jumba *et al.* (1995).
‡ From Chadhokar (1982).
§ Kabaija and Smith (1989).

versus temperate regions, since freezing conditions in temperate areas will stop translocation, while the movement of nutrients to the root system is continuous throughout the dry season in tropical regions.

Pasture management, forage yield and climate

Pasture management, forage yield and climate influence the species of forage predominating and also change the leaf–stem ratio radically, thereby having a direct bearing on the mineral content of the sward. From India, Whyte (1962) observed that good perennial grasses are 'grazed into the ground' late in the dry season, resulting in disastrous effects on their powers of regeneration and replacement with tougher species of inferior quality. From Africa, uncontrolled, heavy grazing pressure is also causing many palatable genuses, such as *Brachiaria* and *Panicum*, to disappear and to be replaced by highly lignified *Sporobulus* species.

Increased crop yields remove elements from the soil at a fast rate, with the result that deficiencies are frequently found on the most progressive farms. Climate also exerts an effect upon the mineral composition of forages. Reith (1965) notes that the maximum content of major nutrients is obtained at soil temperatures that produce maximum growth, and low temperatures generally produce crops containing lower percentages of N, P, K, Ca and Mg.

Climate limits the potential yield and the magnitude of yield response to mineral additions. Where climate varies markedly between seasons, large between-season differences in nutrient uptake are observed. Digestibility of forages, in general, is reduced due to high temperatures, which stimulate rapid growth and onset of maturity, with decrease of digestibility being 0.8 to 1.0% for each per cent of increase in temperature (Minson and McLeod, 1970; Denium and Dirven, 1975). Under the abundant rainfall and high temperatures of the wet tropics and semi-tropics, soils are often low in soluble minerals (Allman and Hamilton, 1949). Grass tetany predictably occurs primarily in the cool season. Pasture management involving liming and fertilization can usually be extremely beneficial to increasing forage macromineral concentrations. Overuse of N and K fertilizers increases the incidence of grass tetany (Kemp *et al.*, 1961), and K fertilization also dramatically reduces forage Na content (Underwood, 1981).

What Affects Major-element Availability?

There is an extreme lack of data on the biological availability of minerals from forage sources. Availability of Ca and P may vary considerably, according to their chemical combination or physical association with other compounds in feeds. Phytic acid, which is formed from six phosphate molecules combined with myo-inositol, hinders intestinal absorption of P, Ca and other minerals. Phytate P is readily used by ruminants, because they have abundant amounts of microbial phytase in the rumen. Before the rumen is developed, phytase is less utilized. Lucerne meal is quite high in Ca (18 g kg^{-1} DM), but several studies indicate that the Ca is poorly available. The low availability of Ca in lucerne may be owing to the presence of oxalates in lucerne. Ward *et al.* (1979) followed the fate of Ca oxalate. In India, Ca deficiency is reported for cattle fed straw, due to the large amounts of oxalates in this feed. Certain tropical grasses contain high levels of oxalate (Kiatoko *et al.*, 1978).

Biological availability of different sources of Mg for ruminants is considerable.

Peeler (1972) found that the availability of Mg ranged from 10 to 25% in forages and from 30 to 40% in grains and concentrates. Greater Mg availability in concentrates, along with the substantial amounts of grain usually fed to lactating dairy cows, is one of the major reasons why grass tetany is less prevalent among dairy cows than among beef cows in the USA. This would not be the situation in many developing tropical countries, since dairy cows often do not receive concentrates in substantial quantities.

Kemp *et al.* (1961) reported that Mg availability improves with increasing maturity of grasses and may be decreased by heavy K and N fertilization. Likewise, usually Mg in preserved forages is more available than in pastures. From temperate pastures (growing perennial ryegrass and white clover) fed fresh to sheep, apparent availability of Mg, Ca, P, Na and K was found to be 31, 21, 17, 95 and 97%, respectively (Grace, 1972).

Perdomo *et al.* (1977) studied the apparent digestibility in sheep of five minerals at three stages of regrowth for three tropical forages (Table 18.5). Digestibility and retention of Ca, P and Na by sheep tended to decline with maturity, while availability of Mg tended to increase at 56 days of age for the three species. Emanuel and Staples (1990) and Emanuel *et al.* (1991) evaluated mineral availability of six forages cut at 32 to 37 days, using an *in vitro* technique that simulated

Table 18.5. Apparent digestibility of various nutrient elements in three forages harvested at three ages of regrowth and fed fresh to sheep (from Perdomo *et al.*, 1977).

		Age of forage regrowth, days (% digestibility of forage mineral elements)			
Forage	Element	28	42	56	Mean ± standard error
Pangola	Ca	10.9^{b}	-40.0^{a}	23.3^{b}	-1.92 ± 29.4^{e}
	P	43.0^{b}	11.6^{a}	41.4^{b}	$32.00 \pm 10.8^{d,e}$
	Mg	41.4^{a}	32.3^{a}	66.3^{b}	46.70 ± 16.4^{e}
	K	80.6^{a}	80.9^{a}	88.6^{b}	83.4 ± 6.0^{d}
	Na	75.0	68.4	79.8	74.10 ± 7.2^{e}
Guinea grass	Ca	62.6^{c}	8.87^{a}	37.0^{b}	36.20 ± 23.0^{f}
	P	62.4^{b}	20.9^{a}	21.9^{a}	$35.00 \pm 22.3^{d,e}$
	Mg	25.4^{a}	44.5^{b}	56.5^{b}	42.20 ± 15.0^{e}
	K	88.8	87.2	89.2	88.40 ± 2.5^{e}
	Na	69.6	58.5	63.9	64.00 ± 10.1^{d}
Star grass	Ca	58.9^{b}	20.0^{a}	33.1^{b}	37.30 ± 18.2^{f}
	P	63.1^{b}	20.8^{a}	26.6^{a}	36.90 ± 22.1^{e}
	Mg	29.9^{a}	54.3^{b}	57.5^{b}	47.20 ± 16.1^{e}
	K	90.1^{b}	88.6^{b}	18.6^{a}	87.40 ± 3.9^{e}
	Na	62.6	70.0	63.1	65.20 ± 9.1^{d}

Different superscript letters in a row indicate that variations in values are significant for effect of age of regrowth ($P < 0.05$), and different superscript letters in a column for mean ± standard error values for specific elements indicate that variations are significant ($P < 0.05$).

rumen abomasum and intestine mineral release. K release was complete in the rumen. Ranking of minerals based on maximal extent of release was K > Mg > P > Ca. With the exception of K, legumes released more of their minerals than grasses. As was true for Ca, mean P release from legumes averaged across the sites (95%) was greater than the mean P release from grasses (82%) (Emanuel *et al.*, 1991).

Greene *et al.* (1986) performed an experiment to determine if breed or DM intake influenced Mg availability to dry, non-pregnant, mature cows. Hereford, Holstein and Jersey breeds excreted more faecal Mg and absorbed less Mg than Angus and Brahman breeds.

How to Remedy Low Status

Indirect methods of providing minerals for grazing animals include use of mineral-containing fertilizers, altering soil pH and encouraging growth of specific pasture species. Direct administration of minerals to ruminants, through water, mineral licks, mixtures and drenches, heavy pellets and injections, is generally the most economical method of supplementation. Benefits and disadvantages of mineral supplementation methods have been discussed (Underwood, 1981; McDowell, 1992).

Providing mineral-containing fertilizers

Increasing mineral intake of grazing livestock via fertilizers has been reviewed (Reid and Horvath, 1980; Underwood, 1981; Butterworth, 1985; Minson, 1990; McDowell, 1992, 1997; Barnes *et al.*, 1995). Fertilization of grasses and legumes is a practice capable of increasing the mineral level of these plants. However, there are frequently cases in which the plants do not respond to fertilization – i.e. the mineral composition is not increased and, at times, the inverse reaction occurs. As an example, the results obtained for *Panicum maximum*, fertilized or non-fertilized, respectively, cut at 28 days of age, were as follows: 24 and 23.8 g N kg^{-1} DM, 1.4 and 1.5 g P kg^{-1} DM, 23.3 and 24.1 g K kg^{-1} DM, 3.4 and 4.5 g Ca kg^{-1} DM and 2.3 and 2.9 g Mg kg^{-1} DM (Gomide *et al.*, 1969). One frequent response to N fertilization of pastures is a change in botanical composition, with a loss of the legume component and a consequent decline in total herbage concentration of elements such as Ca and Mg. Numerous studies (Reid and Horvath, 1980) have demonstrated a depression of Ca, Mg and Na concentration in herbage with the application of K fertilizers. Fertilizers would not normally be applied to forages for the purpose of increasing plant content of Ca, K, S and Cl. Fertilizer has been used to increase forage P and, to a lesser degree, to elevate plant Na and Mg.

Phosphorus

On sparse P-deficient grazing, the direct-supplementation method is preferred, because the use of phosphate fertilizers involves high transport and application cost and herbage productivity is usually limited by climate or soil problems. The effects of P fertilization on plant mineral uptake are dependent, to a considerable degree,

on the properties of the soil. Whitehead (1966) concluded that fertilization often had little effect on the P concentration of herbage, unless the soil was severely deficient.

With infertile soils, P is frequently the major factor limiting pasture growth and, under these conditions, the regular use of fertilizer may not only increase yield substantially but also double the concentration of P in herbage (Reith, 1973). In Australian studies, superphosphate applications at the rate of 125 kg ha^{-1} doubled pasture yields and increased forage P by some 50% (Underwood, 1981). From pastures grown on soils from Florida, USA, Kirk *et al.* (1970) reported increases in the P content of pangola grass (*D. decumbens*) from 0.7–1 g kg^{-1} DM to 2.5–3 g kg^{-1} DM due to P fertilization.

P fertilization to raise forage P would not be effective for very acid soils, as the fertilizer P would be tied up in the soil and forage levels of the element would be increased only slightly (McDowell, 1985). Australian research (Underwood, 1981) has shown that not only does superphosphate fertilizer increase herbage P but it also results in improved palatability and digestibility of the forage. However, unless there are definite forage-yield increases that can be utilized effectively by grazing herbivores, use of mineral-containing fertilizers is economically prohibitive.

Sulphur

Responses to fertilizer S are widespread in the tropics and have been recorded in 40 tropical countries with 23 different crops (International Fertilizer Development Center (IFDC), 1979). However, responses are from crop-yield studies and have not generally been designed to relate fertilizer-S to plant-S concentrations. Rees *et al.* (1974) reported that applying S fertilizer not only raised the S level of the plant but also increased the yield of DM and the leaf percentage. With S fertilizer, the voluntary intake of *D. decumbens* was increased by 44%.

Sodium

The Na concentration in Na-accumulating forages is related to the ratio of Na to K in the soil and can be increased by applying fertilizers containing Na (Henkens, 1965). The Na concentration in forage species that do not accumulate Na is relatively independent of the level of soil Na. With this group of forages, applying Na fertilizers will not increase the Na concentration in the forage or prevent a deficiency in the animal (Minson, 1990).

Magnesium

Mg fertilizers can significantly increase pasture concentrations. This method of control has limitations on many soil types and usually has to be accompanied by other means of supplying additional Mg. Foliar dusting of pastures with calcined magnesite ($MgCO_3$) before or during tetany-prone periods is one such means that has proved effective, provided it is applied at not less than 17 kg ha^{-1} at not more than 10-day intervals (Rogers, 1979).

Free-choice mineral supplementation

For grazing livestock that receive no concentrates, the major method of receiving supplemental macrominerals is to rely on the free-choice feeding of mineral supplements. The main ingredient for the complete free-choice supplement is common salt. The vast majority of minerals are highly unpalatable; however, animals consume a complete mineral mixture because of the favourable taste and their craving for salt. Contrary to popular belief, livestock do not select minerals on the basis of their physiological needs, but rather by taste of the ingredients (McDowell, 1992, 1997). Even though some animals will either overconsume or underconsume mineral supplements, there is no other practical way of supplying certain mineral needs under grazing conditions. There are definite disadvantages to the free-choice mineral-supplementation method, the major concern being the lack of uniform consumption by animals. Observations indicate that some animals may consume double their needs, while others may eat practically none of a supplement.

Factors that affect the consumption of mineral mixtures have been reviewed (McDowell, 1992, 1997) and are as follows: (i) soil fertility and forage type consumed; (ii) available energy–protein supplements; (iii) individual requirements; (iv) salt content of drinking-water; (v) palatability of mineral mixture; (vi) availability of fresh mineral supplies; and (vii) physical form of minerals.

The characteristics of a good free-choice mineral supplement for cattle are listed in Table 18.6 and recommendations for sheep are available (McDowell, 1996). In order to evaluate a mineral supplement for ruminants, it is necessary to have an approximation of: (i) requirements of the target animals for the essential nutrients; this includes the age of the animals involved, stage of current production or reproduction cycle and intended purpose for which the animals are being fed; (ii) relative biological availability of the minerals in the sources from which they will be provided; (iii) approximate daily intake per head of the mineral mixture and of total DM by the target animals; and (iv) concentration of the essential nutrients in the mineral mixture (Ellis *et al.*, 1988).

Problems concerned with mineral-supplementation programmes in diverse regions include: (i) insufficient chemical analyses and biological data to determine which minerals are required and in what quantities; (ii) lack of mineral consumption data needed for formulating supplements; (iii) inaccurate and/or unreliable information on mineral ingredient labels; (iv) supplements that contain inadequate amounts or imbalances; (v) standardized mineral mixtures that are inflexible for diverse ecological regions; (vi) farmers not supplying mixtures as recommended by the manufacturer (e.g. mineral mixtures diluted 10:1 and 100:1 with additional salt); and (vii) difficulties involved with transportation, storage and cost of mineral supplements.

An oral Mg supplement is of value only during seasonal occurrences of grass tetany (Allcroft, 1961). Unfortunately, many commercial Mg-containing free-choice mineral supplements are often of little value because: (i) they contain inadequate amounts of Mg to protect against tetany during susceptible periods; and (ii) provision of such supplements to normal animals during non-susceptible periods is useless as a prophylactic measure, since additional Mg will not provide a depot of readily available Mg for emergency use. Some producers feed Mg supplements

Table 18.6. Characteristics of a 'good' free-choice cattle mineral supplement (from McDowell, 1992).

1. Final mixture containing a minimum of 60–80 g total P kg^{-1}. In areas where forages are consistently lower than 2 g P kg^{-1}, mineral supplements in the 80–100 g P kg^{-1} range are preferred
2. Calcium : phosphorus ratio not substantially over 2 : 1
3. Provide a significant proportion (e.g. about 50%) of the trace-mineral requirements for Co, Cu, I, Mn and Zn.* In known trace-mineral-deficient regions, 100% of specific trace minerals should be provided
4. Composed of high-quality mineral salts that provide the best biologically available forms of each mineral element, and avoidance or minimal inclusion of mineral salts containing toxic elements. As an example, phosphates containing high F should be either avoided or formulated so that breeding cattle would receive no more than 30–50 mg F kg^{-1} in the total diet. Fertilizer or untreated phosphates could be used to a limited extent for feedlot cattle
5. Formulated to be sufficiently palatable to allow adequate consumption in relation to requirements
6. Backed by a reputable manufacturer with quality-control guarantees as to accuracy of mineral-supplement label
7. An acceptable particle size that will allow adequate mixing without smaller-size particles settling out
8. Formulated for the area involved, the level of animal productivity, the environment (temperature, humidity, etc.) in which it will be fed, and is as economical as possible in providing the mineral elements used

For most regions it would be appropriate to include selenium (Se), unless toxicity problems have been observed. Iron (Fe) should be included in temperate region mixtures but often both Fe and Mn can be eliminated for acid soil regions. In certain areas where parasitism is a problem, Fe supplementation may be beneficial.
Co, cobalt; Cu, copper; I, iodine; Mn, manganese; Zn, zinc; F, fluorine.

about a month before the Mg tetany season to decrease the amount of Mg needed daily during the susceptible period.

Responsible firms that manufacture and sell high-quality mineral supplements provide a great service to individual farmers. However, there are companies that are responsible for exaggerated claims in their advertising. Others produce inferior products of little value or, worse, likely to be a detriment to animal production.

Further Research Needed

Many experiments compare varieties of forages in relation to yield and quality of components (e.g. available energy and protein) or potential for improved forage intakes. A few studies (Ramos-Santana and McDowell, 1993, 1994; Faría-Mármol *et al.*, 1996) have also evaluated mineral concentrations of new tropical forage accessions. However, generally there is little, and usually no, regard for mineral concentrations of new forage varieties taken into account. Forages have even been selected on their ability to grow in environments of low available soil P (e.g.

Stylosanthes in acid, tropical soils), which results in forage exceedingly low in P. In developing new strains and varieties of forages, new research should include mineral concentrations as a component of the 'quality' term.

Other research needs are as follows:

- There is a scarcity of data on the mineral status of vast grazing areas worldwide, particularly in developing countries. Studies are needed which include mineral analyses of forage and animal fluids and tissues to establish which minerals are needed for supplementation.
- Determining the effects of various environmental and production factors upon mineral concentrations of forages, as well as element requirements for different species and classes of grazing livestock under varying conditions.
- It is important to study soil–plant interrelationships as they affect plant mineral concentrations.
- Developing methods to evaluate the mineral status of grazing livestock and biological assay techniques for minerals in both forages and mineral supplements.
- Improved methods of providing supplemental minerals for livestock are needed, particularly for grazing animals, with cost–benefit relationships established.
- To formulate free-choice mineral supplements for various soil types and ecological regions, information on mineral supplement consumption is required.

Cost Implications

Only limited data exist on cost implications of increasing mineral intake by grazing livestock. Providing minerals via fertilizer treatment is economically the method of choice if the treatment improves both yield and mineral composition of herbage. Cost–benefit relationships of fertilizer treatment have generally been restricted as to forage-yield components vs. the value of increased plant minerals.

Under most grazing conditions, increased mineral intake would economically favour direct supplementation. Most studies would conclude that providing direct administration of minerals to livestock as drenches, rumen preparations and injections (practical only for trace elements) is more expensive than providing animals access to a free-choice mineral mixture. Free-choice mineral supplementation is a low-cost insurance with economic returns on mineral investment generally of more than 2:1 in Latin American studies (McDowell *et al.*, 1984) and returns could be as high as 20:1 in some studies (J. de Sousa, personal communication). Berger (1992) calculated that, in a l00-cow herd, preventing the loss from grass tetany of a single cow every 3 years would more than pay the cost of Mg supplementation.

Cost comparisons were made on P supplementation methods for cattle at the King Ranch in Texas, USA, for the following treatments: disodium phosphate in water, bone-meal in self-feeders, fertilized with triple superphosphate and control (Reynolds *et al.*, 1953). The cows that yielded the highest return ha^{-1} after the costs of supplementation had been deducted were those fed disodium phosphate, followed by those on fertilized pasture, those fed bone-meal and the unsupplemented group, the returns being $50.75, $49.93, $42.28 and $34.78, respectively.

The importance of mineral supplementation on overall production of cattle in the llanos regions of Colombia is presented in Table 18.7. Supplemental minerals

Table 18.7. Four-year Colombian study evaluating supplemental minerals.*

Item	Salt only	Complete minerals†
Abortions (%)	9.3	0.75
Births (%) year^{-1}	50.0	67.0
Death losses, birth to weaning (%)	22.6	10.5
Calves weaned of total herd (%) year^{-1}	38.4	60.0
Weaning weight (9 months) (kg)	117.0	147.0
Gain of growing cattle (572 days) (kg)	86.0	141.0
Average gain per day (g)	150.0	247.0
Weaned calf wt (kg year^{-1} cow^{-1})‡	44.9	88.2

* Evaluation of CIAT (1977) report (Miles and McDowell, 1983).
† Evaluation of the complete mineral supplement indicated adequate concentrations of most minerals but suboptimum levels of Zn and Cu, with no added Se and S.
‡ Weaned calf per cent multiplied by weaning weight.
Zn, zinc; Cu, copper; Se, selenium.

dramatically increased all parameters of production. Multiplication of the weaning percentage by the weaning weight gave 88.2 kg of calf produced per cow with complete minerals, compared with 44.9 kg with salt alone. For this study, free-choice mineral supplementation resulted in a return of 15.6 Colombian pesos for every 1 peso invested in the supplement (Miles and McDowell, 1983).

When there is no information available on mineral status for specific regions, then complete ('shotgun') mineral mixtures are warranted. However, with additional information on likely limiting minerals, more economical mixes can be formulated. In Colombia, ten cattle experiments were designed to evaluate feeding complete commercial supplements as against feeding supplements formulated to contain a minimum number of minerals that had previously been established as deficient on the basis of forage and animal-tissue analyses (Laredo *et al.*, 1989). The specifically tailored mineral supplements produced equal production with a 42% cost saving.

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19 Vitamins in Forages

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Vitamins are organic molecules with complex molecular structure and they are essential in minimum quantities for the health, growth and reproduction of ruminants (bovine, ovine and caprine) (Table 19.1). Vitamin deficiency results in a number of metabolic disorders of varying levels of gravity. Forage has the potential to play a significant role in the supply of vitamins to ruminants. However, as will be demonstrated, the vitamin content of forages is highly variable and unpredictable and the production of synthetic vitamins was essential to the advent of intensive livestock production.

Vitamin Requirements of Ruminants

Adult ruminants are different from monogastrics (pigs and poultry) with respect to their dependence on an exogenous supply of vitamins. Synthesis of B-group vitamins (thiamine, riboflavine, niacin …) and vitamin K occurs during the degradation and fermentation of feed ingredients by ruminal microorganisms. Vitamin D is synthesized by the action of ultraviolet radiation on the sterols present in the skin of ruminants, vitamin C is synthesized from C_6 sugars (glucose and galactose) and niacin from tryptophan (if the amino acid is present in excess). It is therefore mainly with respect to vitamins A and E that ruminants have specific dietary dependence.

However, very young ruminants, which do not possess a fully developed rumen and which lack a fully functional rumen microflora, cannot synthesize adequate B-group vitamins. Calves and lambs can therefore suffer from a B-group vitamin deficiency. In this instance, as in monogastrics, they are dependent on an exogenous supply of B-group vitamins.

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Table 19.1. Fat- and water-soluble vitamins with their major functions (adapted from McDowell, 1989).

Names	Functions
Fat-soluble vitamins	
A, retinol	Vision Maintenance of epithelial cells Reproduction Growth
E, α-tocopherol	Biological antioxidant Phospholipid membrane stability Immunomodulation
D, ergocalciferol D_2, cholecalciferol D_3	Phosphocalcium metabolism Growth Reproduction
K	Cofactor in coagulation
Water-soluble vitamins	
B_1, thiamine	Coenzyme in oxidative decarboxylation Role in neurophysiology
B_2, riboflavine	Intermediary in the transfer of electrons in biological oxidation–reduction reactions
B_3, PP factor, niacin	Constituent of coenzymes NAD and NADP in carbohydrate, lipid and protein metabolism
B_5, pantothenic acid	Coenzyme A precursor
B_6, pyridoxine, pyridoxamine	Amino acid metabolism Formation of biogenic amines
B_{12}, cobalamin	Integrity of nervous system Haematopoiesis Growth Gluconeogenesis from propionate
H, $B_{w,}$, biotin	Coenzyme in carbohydrate, lipid and protein metabolism
M, B_c, folacin	Growth Haematopoiesis Maintenance of immune system
Choline*	Synthesis of acetylcholine Component of phospholipids Methyl radical donor Lipotrophic factor
C, ascorbic acid	Collagen biosynthesis Transfer of electrons Oxidation reactions

*Choline is classified as one of the B-complex vitamins.
PP, pellagra-preventing; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate.

Thus, in addition to the standard classification of vitamins according to their solubility in either water (C and B group) or in lipid solvent (A, D, E and K), vitamins for adult ruminants can be divided according to:

- self-supply (via either the rumen or endogenous supply) (K, C, D and B group);
- supply from the feed (A and E).

Different rearing methods and changes in ration formulation may alter the endogenous production of vitamins and result in a need to modify this classification. Animals reared indoors or fed rations rich in concentrates cannot produce sufficient vitamin D and thiamine. Furthermore, there is an increasing need for an exogenous supply of niacin, as, following genetic selection, the increase in productivity of ruminants begins to approach the quantitative limits of ruminal vitamin synthesis. The consumer demand for animal products of high quality is also imposing a need for an increased exogenous supply, particularly of niacin and vitamin E. The supply of niacin to high-producing cows at the beginning of lactation has a tendency to increase milk production and also the fat and protein content of milk (NRC, 1989; Hullár and Brand, 1993). In the nutrition of adult ruminants, the five most important vitamins with respect to an exogenous supply are therefore vitamins A, E, D and, in specific conditions, niacin and thiamine.

The supply of vitamins to ruminants other than from the synthetically produced material is totally dependent on a supply from fresh or conserved forage in the ration, since the concentrate portion of the feed is practically devoid of any naturally occurring vitamins A, E, D or their precursors (Brown, 1953; Machlin, 1984; McDowell, 1989).

Vitamin A or retinol exists only in animal products (McDowell, 1989). However, vegetable material can contain provitamins A (carotenoids), of which 80 forms are known, with β-carotene being the most important. β-Carotene is partially absorbed at the intestinal level, where it is transformed into vitamin A. It has been established that the conversion rate in the bovine is of the order of 400 international units (IU) of vitamin A mg^{-1} β-carotene (McDowell, 1989) (where 1 IU is defined as the biological activity of 0.300 μg of retinol). However, the efficiency of this conversion varies according to the isomer. The all-*trans* form of β-carotene has the highest activity, the neo-B and neo-U forms have only 53 and 38% of all-*trans*-form activity, respectively (Aitken and Hankin, 1970).

Vitamin E is found in eight known forms, four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ) (McDowell, 1989). The α-tocopherol is the most active and is considered true vitamin E.

Vitamin D forms are derived from the ultraviolet irradiation of sterols in animals (D_3 = cholecalciferol) and in vegetables (D_2 = ergocalciferol), the two forms having the same biological potency in ruminants (McDowell, 1989). One international unit of vitamin D activity is defined as the activity of 0.025 μg of vitamin D_3.

The term niacin is used to cover nicotinic acid and nicotinamide, the two compounds possessing the same vitamin activity in ruminants (McDowell, 1989). Thiamine consists of a molecule of pyrimidine and a molecule of thiazole linked by a methylene bridge (McDowell, 1989).

The physiological requirements for these vitamins are difficult to define and can vary appreciably according to the measures used as reference, particularly

whether the criterion is the prevention of deficiency symptoms or the requirement for maximum animal performance. Standard tables of requirements tend to ignore the additional needs that have been identified as a result of different repeated stressors occurring during livestock production (unfavourable rearing conditions, infection, etc.). Present recommendations for ruminants concern vitamins A, E, D and niacin and are greater than the strict physiological needs, since they incorporate a margin of safety in order to ensure that the performance of the animals is not compromised (Table 19.2). The minimal recommendations are given for mean rearing conditions but are increased in accordance with a number of factors, such as the composition of the ration and the performance of the animal (growth, reproduction and milk production). Supply of highly fermentable diets increases the requirements for both thiamine and vitamin A (NRC, 1989). The continued increase in genetic potential of animals as a result of breeding results in a general increase in vitamin requirements.

Forage as a Source of Vitamins

The diet of ruminants is composed essentially of a combination of concentrates (cereals and protein supplements) and forages. Fresh or conserved forages are potential sources of vitamins A, E, D, niacin and thiamine (Aitken and Hankin, 1970; McDowell, 1989). However, although it is relatively easy to list vitamins present in forage, it is more difficult to give precise estimates of the mean values. Levels of vitamins found in forages are highly variable (Table 19.3). The factors that are responsible for this variability, and which include plant origin, climatic conditions, stage of maturity, conservation methods (drying, ensiling, dehydration …) and storage conditions, are described in a later section. It would appear that the minimal attention given to the vitamin content of forages, other than for the vitamins A, E and, to a lesser extent, D, is the result of the lack of literature data concerning the content of niacin and thiamine, since vitamin analysis is not routinely performed on forages.

At first sight, the majority of forages can make a significant contribution to the dietary supply of β-carotene and α-tocopherol to cover the daily recommendations of ruminants (RPAN, 1998; Fig. 19.1). Indeed, only approximately 15% of the forages sampled supplied less than 50 mg of β-carotene or α-tocopherol kg^{-1} dry matter

Table 19.2. Vitamin recommendations for ruminants (RPAN, 1998).

Ruminants	Vit. A (IU $head^{-1}$ day^{-1})	Vit. E (mg $head^{-1}$ day^{-1})	Vit. D (IU $head^{-1}$ day^{-1})	Niacin (g $head^{-1}$ day^{-1})
Dairy cow, lactating	80,000–120,000	100–1,000	15,000–50,000	1–2
Dairy cow, dry	75,000–125,000	500–900	10,000–20,000	0–1
Finishing cattle	40,000–70,000	200–1,500*	4,000–7,000	1–2

* Improvement of meat quality after slaughter.

Table 19.3. Vitamin content of fresh and conserved forages (adapted from Blaylock *et al.*, 1950; Brown, 1953; Keener, 1954; Wallis *et al.*, 1958; Albonico and Fabris, 1958; Hjarde *et al.*, 1963; Hoffmann and Nehring, 1967; Bunnel *et al.*, 1968; Aitken and Hankin, 1970; NRC, 1989).

	β-Carotene (mg kg^{-1} DM)			α-Tocopherol (mg kg^{-1} DM)			D (IU kg^{-1} DM)			Thiamine (mg kg^{-1} DM)			Niacin (mg kg^{-1} DM)		
Forages	*n*	M	SD	*n*	M	SD	*n*	M	SD	*n*	M	SD	*n*	M	SD
Green forages (1)	349	196 (15–606)*	108	86	161 (9–400)	91	25	365 (31–1800)	470	18	4.6 (1.9–8.3)	2.4	5	37 (13–56)	17
Dehydrated forages (2)	16	159 (66–271)	73	12	125 (28–238)	57	4	– (176–617)	–	7	4 (3.8–4.5)	0.5	7	53 (39–64)	9
Silages (3)	50	81 (2–276)	68	4	– (0–310)	–	10	440 (80–866)	311	1	0.1 –	–	2	– (1.1–34)	–
Hays (4)	68	36 (1–162)	34	10	61 (10–211)	62	40	1156 (90–5560)	1161	19	2.7 (0.2–4.5)	1.3	14	28 (6–52)	17

(1) Grasses (cocksfoot, timothy, ryegrass, fescue …) and legumes (lucerne, clover …); (2) lucerne; (3) legume silages (lucerne, clover), grass silages (cocksfoot, ryegrass …) and maize silage; (4) legume hays (lucerne, clover) and grass hays (timothy, ryegrass, fescue, cocksfoot …).
n, Number of samples; M, mean; SD, standard deviation.
* Range.

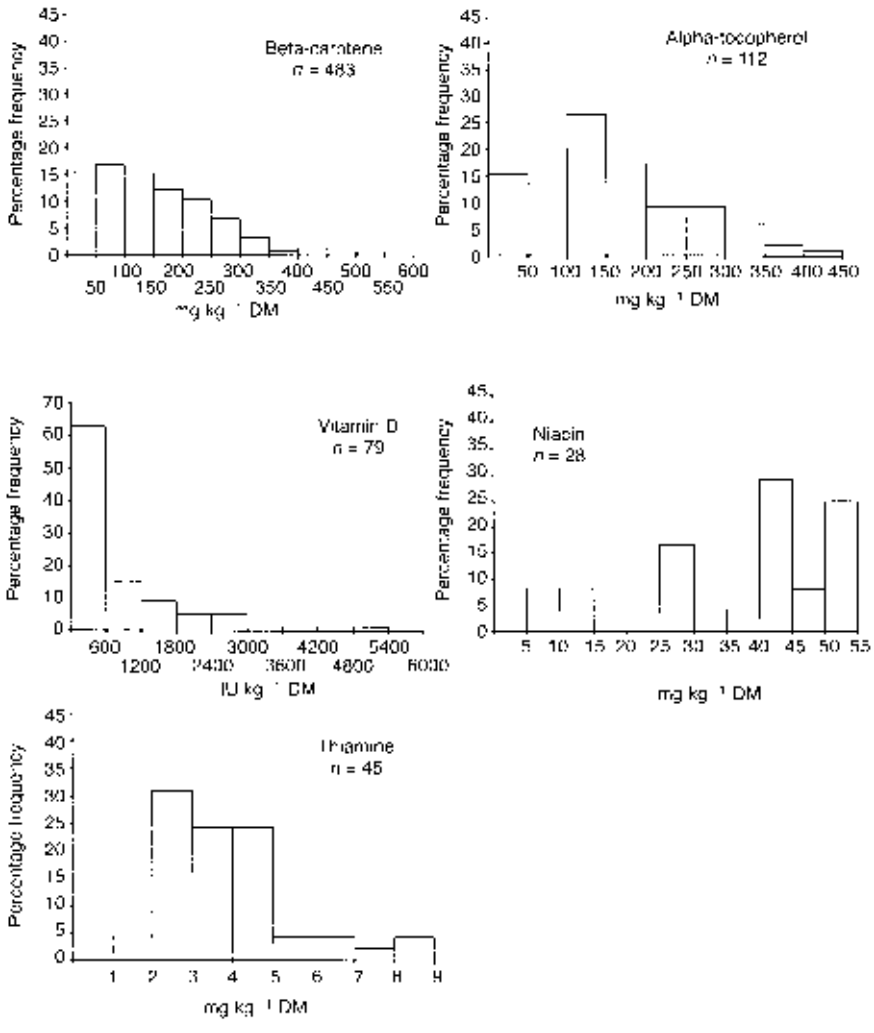

Fig. 19.1. Histograms representing frequency distribution of vitamin content of forages (adapted from Table 19.3). *n* = Number of samples.

(DM), the remaining 85% supplying in excess of 50 mg kg^{-1}. It would appear that forages, in general, are very good sources of the vitamins A and E. However, these data must be viewed with great prudence, due to lack of precision of most analytical methods for vitamins in forages and also to lack of estimates of the bioavailability of β-carotene and α-tocopherol in forages.

Methods of analysis used for feed materials have a tendency to overestimate concentrations of β-carotene and α-tocopherol in a wide range of raw materials (Aitken and Hankin, 1970; Ullrey, 1972; McDowell, 1989). The presence of geometric isomers of all-*trans*-β-carotene that have a lower biological activity results in

an overestimation of the biological activity of β-carotene (Table 19.4). This overestimation is dependent on the forage under consideration (+6–11% for fresh lucerne and *Cynadon dactylon*, +13–17% for the same material dried and +19–33% for dehydrated lucerne).

In most cases, even if the concentration of β-carotene and α-tocopherol in the forage is high, the requirements for vitamins A and E of ruminants cannot be adequately covered by their daily forage consumption (Nadai, 1968; Ferrando and Mainguy, 1970; McDowell, 1989). Between the ingestion of β-carotene and α-tocopherol present in forage and the metabolic use of vitamins A and E, two important steps are involved: the passage of the material through the rumen and intestinal absorption, with, in addition, for carotene its conversion into vitamin A (see later). A number of factors can therefore modify the nutritional supply of vitamins A and E from forage. The level of conversion of β-carotene varies (1 mg of β-carotene = 400 IU of vitamin A in a lactating dairy cow) according to the health status and the quantity consumed. The rate of conversion is reduced as the level consumed increases, and it is generally accepted that this conversion factor for ruminants is only valid at levels of intake corresponding to maintenance (Nadai, 1968; Bondi and Sklan, 1984).

Over 60% of the forages analysed (Table 19.3) had a vitamin D content of less than 600 IU kg^{-1} dry matter (Fig. 19.1), resulting in a severe risk of deficiency in housed animals. Niacin and thiamine, although found in forages, are present in quantities insufficient to cover recommendations (RPAN, 1998). The same caution as was given for vitamins A and E must be exercised in estimating the nutritional supply of vitamins D, niacin and thiamine from forages, in particular the

Table 19.4. Stereoisomers of β-carotene and biological efficiency of lucerne (fresh, dehydrated and sun-cured) and *Cynadon dactylon* (fresh and dried at 65°C).

Forages	Stereoisomers of β-carotene (%)			Biological efficiency of total carotene (% of all-*trans* β-carotene)	References
	Neo-B	All-*trans*	Neo-U		
Fresh lucerne	7	84	9	90	Livingston *et al.* (1966)
	4.6	90	5.4	94	Thompson *et al.* (1951)
	6	84	10	91	Kemmerer *et al.* (1944)
Dehydrated lucerne					
T = 104°C*	27	63	10	81	Livingston *et al.* (1966)
T = 160°C	46	36	18	67	
Sun-cured lucerne hay	12	77	11	87	Thompson *et al.* (1951)
Fresh *Cynadon dactylon*	8	81	11	89	Kemmerer *et al.* (1944)
	5	86	9	92	
Cynadon dactylon dried at 65°C	11	70	19	83	Kemmerer *et al.* (1944)

* Outlet temperature of drier.

sensitivity of different analytical techniques and effects on biological availability (McDowell, 1989).

In conclusion, therefore, although forages are potentially good sources of vitamins, the levels which they may contribute in the diet of ruminants are influenced by a number of factors inherent to both the plant and the animal. This range of influencing factors results in a major lack of precision in estimating the quantity of vitamins available to ruminants from forage.

Factors Affecting the Vitamin Content of Forages

The considerable variability found in the vitamin content of forages tends to suggest that a number of factors contribute to the variability encountered. These include:

- the origin of the plant (family, species and variety);
- climatic conditions;
- stage of maturity of the plant;
- conservation methods (drying, ensiling, dehydration …);
- storage conditions.

The origin of the plant

During the early stages of growth, the grasses and legumes have similar mean levels of β-carotene (approximately 300 mg kg^{-1} DM) (Table 19.5). However, at the stage of flowering and at maturity, legumes are richer in β-carotene than the grasses (Moon, 1939). Thus, the stage of growth does not affect the level of β-carotene of legumes to the same extent as in the grasses (Moon, 1939; Seshan and Sen, 1942a). In a comparison of grasses and legumes at the start of the flowering stage (Table 19.6), Livingston *et al.* (1968c) reported that the leaves had similar levels of carotene, but these levels were four to 11 times higher than the levels found in the stems (Seshan and Sen, 1942a; Olsson *et al.*, 1955; Ramanujan and Anantakrishnan, 1958; Park *et al.*, 1983). However, account must be taken of the fact that, at the start of flowering, the proportion of leaves in legumes (mean 40%) is twice that found in the grasses (mean 19%). Thus, at advanced stages of growth, the legumes are the better source of β-carotene, since the proportion of stem is less than in the grasses (Moon, 1939; Olsson *et al.*, 1955).

Results presented by Olsson *et al.* (1955) also indicate the existence of differences between plant species in terms of β-carotene (Moon, 1939; Seshan and Sen, 1942a; Park *et al.*, 1983). At an early growth stage (1st cycle) (Table 19.7) perennial ryegrass has the lowest level of β-carotene of the grasses (146 mg kg^{-1} DM)(Smith and Wang, 1941) and, of the legumes, white clover has the highest level (438 mg kg^{-1} DM). Thus, certain plant species possess an important capacity to synthesize carotene (Park *et al.*, 1983). During the course of a study, Olsson *et al.* (1955) noted that, at a stage of full bud, red clover had a concentration of β-carotene (206 mg kg^{-1} DM) close to that of lucerne (233 mg kg^{-1} DM) and that,

Table 19.5. Influence of plant origin and stage of maturity in β-carotene and α-tocopherol contents of fresh forages (mg kg^{-1} DM) during the first growth cycle (adapted from Brown, 1953; Hjarde *et al.*, 1963; Hoffmann and Nehring, 1967; Aitken and Hankin, 1970; NRC, 1989).

	β-carotene				α-tocopherol			
Forages and stages	*n*	M	Max.	Min.	*n*	M	Max.	Min.
Grasses								
Vegetative to ear	51	278	606	84	29	253	400	121
Early to end flowering	44	133	258	53	17	98	154	40
Mature	30	59	156	4	7	22	30	9
Legumes								
Vegetative to bud	62	309	552	140	19	129	202	79
Early to end flowering	34	192	488	97	6	116	127	109
Mature	7	130	252	80	–	–		

n, Number of samples; M, mean.

Table 19.6. Variation in carotene content of four grasses and three legumes harvested at early flowering during the first growth cycle (from Livingston *et al.*, 1968c).

Forages	Date harvested	Fraction	% (dry wt)	Carotene (mg kg^{-1} DM)
Grasses				
Fescue	18/6	Leaf	12	460
		Stem	88	101
Timothy cv. Verdant	2/7	Leaf	17	387
		Stem	83	95
Cocksfoot cv. Potomac	20/6	Leaf	21	521
		Stem	79	62
Reed canary grass	18/6	Leaf	26	640
		Stem	74	90
Legumes				
Red clover cv. Lakeland	14/6	Leaf	34	724
		Stem	66	66
Lucerne cv. Saranac	9/6	Leaf	39	629
		Stem	61	75
Birdfoot cv. Empire	1/6	Leaf	42	449
		Stem	58	62

at the stage of end ear, timothy (96 mg kg^{-1} DM) had a lower level than that of cocksfoot (175 mg kg^{-1} DM). Table 19.7 also shows that the growth cycle has little effect on the level of β-carotene in lucerne (Bruhn and Oliver, 1978) and in red clover, but strongly influences that in white clover and the grasses. According to these authors, the differences in level of β-carotene are mainly due to the ratio of leaf to stem in the plant and the regrowth.

Table 19.7. β-Carotene content (mg kg^{-1} DM) of legumes and grasses harvested at an early stage of development several times in the vegetation period, in years 1942–1946 (number of samples = 5) (from Olsson *et al.*,1955).

	First cut			Second cut			Third cut			Fourth cut		
Forages	M	Max.	Min.	M	Max.	Min.	M	Max.	Min.	M	Max.	Min.
Legumes												
Lucerne	263	315	235	239	365	172	272	380	225	293	424	244
Red clover	356	462	270	313	362	256	330	391	253	335	411	245
White clover	438	538	387	269	395	184	268	463	177	422	595	342
Grasses												
Timothy	238	369	133	128	222	63	192	233	119	248	356	142
Cocksfoot*	242	267	217	148	191	104	104	–	–	254	331	177
Perennial ryegrass	146	214	95	161	407	68	250	425	123	207	259	160

* Number of samples = 2 (1943 and 1944).
M, mean.

At early stages of growth, grasses contain nearly twice as much α-tocopherol as legumes (Table 19.5). However, at flowering, the legumes and grasses have approximately the same mean levels of α-tocopherol. Similar to the case with carotene, the ratio of leaf to stem is an important factor in the evolution of the level of vitamin E in forages (Brown, 1953), with the leaves being richer in α-tocopherol than the stems (Brown, 1953; Ramanujan and Anantakrishnan, 1958; Booth, 1964). Variability between species is also evident for α-tocopherol. Cocksfoot (313–362 mg kg^{-1} DM) is a better source of α-tocopherol than fescue and timothy (184–249 mg kg^{-1} DM) at the 20–25 cm stage (Brown, 1953). Furthermore, for thiamine, fescue is superior to brome grass and timothy (3.2 mg and 2.6 mg kg^{-1} DM, respectively) and lucerne is superior to clover (7.5 mg and 6.5 mg kg^{-1} DM, respectively) (Robinson *et al.*, 1948).

Brown (1953) reported approximately 22% variation in the level of α-tocopherol when comparing four varieties of lucerne. Thompson (1949) observed a variation of the order of 30% in the level of β-carotene in nine varieties of lucerne. However, comparing three varieties of red clover, Hjarde *et al.* (1963) found no significant difference in the levels of β-carotene and α-ζ-tocopherol. Vail *et al.* (1936), cited by Robinson *et al.* (1948), found no significant difference in the level of thiamine in four varieties of lucerne. Albonico and Fabris (1958) concluded that genetic factors should be considered as having only a weak influence on the vitamin content of forages.

In conclusion, plants that produce little leaf tend to contain low levels of β-carotene and α-tocopherol (Park *et al.*, 1983; McDowell, 1989). Other than the origin of the plant, the ratio of leaf to stem, which is the main factor influencing the levels of β-carotene and α-tocopherol in forage, is also influenced by both climatic conditions and the stage of maturity (Olsson *et al.*, 1955).

Climatic conditions

Bondi and Meyer (1946) measured the level of carotene in lucerne harvested at the budding and flowering stages at different times of the year in Palestine. The level of β-carotene was higher in April and May for the budding stage (266–273 mg kg^{-1} DM) and in November for the flowering stage (170 mg kg^{-1} DM) than in August (110 mg kg^{-1} DM for the budding stage and 72 mg kg^{-1} DM for the flowering stage). Albonico and Fabris (1958) demonstrated that the levels of β-carotene and α-tocopherol of 16 varieties of lucerne sampled in the south of Italy at the beginning of flowering were higher in March/April (349 and 282 mg kg^{-1} DM, respectively) than in July (251 and 110 mg kg^{-1} DM, respectively). Independently of the cycle of growth, the level of β-carotene of legumes and grasses was higher in the summer of 1942 – a fresh and humid summer – than in 1943 and 1944 – two summers that were hot and dry (Table 19.8; Olsson *et al.*, 1955). These results indicate that, for a given stage of growth and independently of the cycle of growth, a forage is richer in β-carotene and α-tocopherol when grown under mild, wet conditions. The beneficial effect of rainy conditions rests in the reduction in the quantity of sunlight received by the plant (Olsson *et al.*, 1955; Hjarde *et al.*, 1963; Park *et al.*, 1983). The ambient temperature and the light contribute more to the variability in the level of β-carotene than the supply of water (Olsson *et al.*, 1955; Repp and Walkins, 1958).

Beck and Redman (1940) showed that heat and intense light were detrimental to the production of carotene in the leaves of clover. However, the positive influence of the reduction in temperature and day length was also related to the increase in the leaf-to-stem ratio (Wilson, 1981). Thus the climatic conditions influence the levels of β-carotene and α-tocopherol of forages because they also influence the leaf-to-stem ratio.

The antirachitic value of forages is also dependent on levels of sunlight (Meissonier, 1981; McDowell, 1989). Green forages have low levels of vitamin D but, when the plant begins to die and the fading leaves are exposed to the ultraviolet light of the sun, high levels of vitamin D are synthesized (Thomas and Moore, 1948; McDowell, 1989).

Table 19.8. β-Carotene content (mg kg^{-1} DM) of grasses and legumes harvested at an early stage of growth during the summer, 1942–1944, in Sweden (from Olsson *et al.*, 1955).

	White clover		Red clover		Timothy		Perennial ryegrass	
Years	First cut	Second cut	First cut	Second cut	First cut	Second cut	First cut	Second cut
1942	538	395	462	362	369	222	214	147
1943	408	210	355	256	209	87	97	100
1944	387	225	270	298	133	63	95	68

The stage of maturity of the plant

The levels of β-carotene and α-tocopherol in the grasses and legumes are very high in the young stages and reduce as the plant matures (Smith and Wang, 1941; Brown, 1953; Clarke, 1953; Bondi and Sklan, 1984; Table 19.5). The levels of β-carotene and tocopherols of lucerne achieve maximum values between the end of the vegetative stage (Park *et al.*, 1983) and the budding stage (Burrows and King, 1968) and then decrease with maturity. The level of carotene drops at the end of the vegetative stage in clover (Olsson *et al.*, 1955) and cocksfoot (Clarke, 1953) and after the start of earing in timothy (Olsson *et al.*, 1955). According to Moon (1939) and Livingston *et al.* (1968c), the level of carotene in the leaves of grasses and legumes is maximal at the start of flowering and then diminishes with varying degrees of intensity (Thompson, 1949). Indeed, the green colour of their leaves is generally a good index of their carotene content (Maynard *et al.*, 1979). At maturity, plants may have 10% (in the case of grasses) to 40% (for legumes) of the value of carotene of immature plants.

As has already been indicated, the principal factor responsible for the variation in levels of β-carotene and α-tocopherol of forages in the course of their maturation is the change in the ratio of leaf to stem, because the leaves are considerably richer in these vitamins than the stems. The formation of stems is accompanied by an increase in the concentration of DM; there is thus a negative correlation between the DM content and the level of β-carotene (Olsson *et al.*, 1955).

Contrary to the situation when vitamin D is produced in fading leaves, the level of thiamine is positively correlated with the verdant nature of the forage leaf (Hunt *et al.*, 1935; Galgan *et al.*, 1950; McDowell, 1989). The vitamin D content of forages is also dependent on the stage of harvest. Indeed, vitamin D level increases even when the plant has reached maturity (Table 19.9; Keener, 1954), because of the increase in dead leaves (Thomas and Moore, 1951; McDowell, 1989). Thus, the levels of β-carotene and vitamin D are negatively correlated. Thomas and Moore (1948) demonstrated that the proportion of dead leaves in lucerne increases from 2.4% at budding to 6.5% at maturity. The dead leaves contain approximately 7064 IU vitamin D kg^{-1} DM, while the green leaves had levels close to zero. Thus, the quantity of dead leaves adhering to the plant during harvest can considerably modify the level of vitamin D, independently of the duration of exposure to sunlight during drying. During the same year, the level of vitamin D of forages at the same stage of development increases with the number of cuts (Keener, 1954; Table 19.9). It was suggested that the difference between a first and second cut was due to differences in the level of ergosterol and the relative importance of the sun's ultraviolet rays.

Methods of harvesting and conservation

The manner in which forage is treated between its harvest and being offered as feed to animals can influence its vitamin content.

Both β-carotene and α-tocopherol are destroyed by oxidation. This reaction is accelerated by ultraviolet light and heat (Seshan and Sen, 1942b; Bauernfeind,

Table 19.9. Influence of stage of maturity and number of cuts in vitamin D content of several forages (from Keener, 1954).

Cutting and date	Red clover (IU kg^{-1} DM)	Timothy (IU kg^{-1} DM)
First cutting		
25 June	31	40
2 August	750	571
15 September	1400	1100
Second cutting		
2 August	130	430
15 September	981	820
Third cutting		
15 September	500	540

1980). However, prolonged heating without oxygen has a minor effect (Seshan and Sen, 1942b; McDowell, 1989).

Before it becomes a photochemical process, the destruction of β-carotene in forages is first an enzymatic process and is due to a lipoxygenase system (Ferrando and Mainguy, 1970; Kalac and McDonald, 1981; Bondi and Sklan, 1984). The lipoxygenases, a group of isoenzymes, are present at varying levels in a wide number of species of plants (Waugh *et al.*, 1944; Larsen *et al.*, 1993). Lucerne is know to be an excellent source of lipoxygenases (Waugh *et al.*, 1944; McDowell, 1989). These enzymes are destroyed at around 80–100°C (Olsson *et al.*, 1955; Ferrando and Mainguy, 1970), wherein lies the interest of dehydration (Blaylock *et al.*, 1950; Bondi and Sklan, 1984).

According to Bauernfeind (1972), the enzymatic destruction of total pigments present in the leaves of lucerne is three to four times greater than photochemical destruction (Mitchell and Hauge, 1946). Enzymatic destruction of carotene begins when the forage is chopped and is more intense in the early stages of growth. Increasing the degree of chopping and grinding also accelerates enzymatic destruction, as does raising ambient temperature and humidity (Mitchell and Hauge, 1946; Ferrando and Mainguy, 1970; McDowell, 1989). During drying, the losses of β-carotene and α-tocopherol can equally be due to isomerization (Livingston *et al.*, 1966, 1970), but this isomerization represents only a small part of the total loss (Kalac and Kyzlink, 1979).

Vitamin D can be destroyed by excessive exposure to ultraviolet light (Ferrando and Mainguy, 1970; McDowell, 1989). When the crop is dry, thiamine is stable at 100°C for several hours, but the presence of humidity accelerates destruction (McDowell, 1989). Thus, thiamine is much less stable to heat in fresh material than in dried material. On the contrary, niacin is highly stable in air, to heat, to light and to the action of alkalis – hence its stability in feedstuffs (McDowell, 1989).

Haymaking

Drying crops either on the ground or in barns reduces the levels of β-carotene and α-tocopherol in forages (King *et al.*, 1967; Burrows and King, 1968; McDowell, 1989). Russell (1929) found that in excess of 80% of carotene from fresh lucerne was lost during the first 24 h of sun-drying. According to Akopyan (1958), cited by Kivimaë and Carpena (1973), the level of tocopherol in leaves of clover and maize was practically zero when the crop was dried for 4–5 days in the sun. With grasses, Ramanujan and Anantakrishnan (1958) obtained carotene and tocopherol losses in the region of 80% after 4 days of sun-drying. Park *et al.* (1983) showed that, as the exposure to the sun was increased, the level of destruction of β-carotene was increased (Table 19.10) and that this occurred irrespective of the stage of harvest of the lucerne. Forages exposed to rain and then dried in the sun have less β-carotene than sun-dried forage. Thus, if the forage rests exposed to the sun for an extended period of time and at the same time is exposed to several showers, the destruction of β-carotene is nearly complete.

According to Akopyan (1958) and Maynard *et al.* (1979), barn-drying has less of a destructive effect on carotene and tocopherol. Galgan *et al.* (1950) reported that barn-dried lucerne hay could contain three times more carotene than sun-dried lucerne hay. However, Ramanujan and Anantakrishnan (1958) reported that, when the drying time of grasses was considerably extended, irrespective of the method, the losses of carotene and tocopherol were practically the same.

During crop-drying on the ground, radiation from the sun can result in synthesis of vitamin D. However, again, it must be emphasized that the degree of radiation must not be excessive (Ferrando and Mainguy, 1970). Thomas and Moore (1948) showed that a sun-dried lucerne contained twice as much vitamin D (971 IU kg^{-1} DM) as a barn-dried lucerne (470 IU kg^{-1} DM).

The level of thiamine is much lower in sun-dried materials than in materials dried in an oven at 62°C (Galgan *et al.*, 1950). The lucerne dried in the sun had high losses of niacin when it was exposed to bad weather (Blaylock *et al.*, 1950; Scott, 1973).

Table 19.10. Effect of time of sun-drying (with or without rain) in β-carotene content of lucerne harvested at three stages of maturity during the second growth cycle (from Park *et al.,* 1983).

Duration of drying (h)	Vegetative (mg kg^{-1} DM)	Bud (mg kg^{-1} DM)	Flowering (mg kg^{-1} DM)
Sun-dry			
48 h	79.0	56.8	53.0
72 h	34.4	24.4	24.2
96 h	29.6	8.1	21.8
Rain damage, sun-dry			
48 h	22.2	22.0	22.6
72 h	13.9	12.0	23.6
96 h	12.7	5.3	10.8

Ensiling

In general, ensiling of grasses and legumes guarantees a better conservation of carotene and tocopherol than haymaking (Bauernfeind, 1980; Wolter, 1988; Hidiroglou *et al.*, 1994). As a result of a series of studies, Watson and Nash (1960) concluded that mean losses of carotene during ensilage were of the order of 30% (cited by Kalac and McDonald, 1981). It should be noted that the losses of β-carotene depend partly on the type of material ensiled, the quality of the ensiling process and the method of silage preparation. Kalac (1983) demonstrated that the losses of β-carotene during the aerobic phase (24 h) were of the order of 17 (cocksfoot) to 30% (lucerne and Italian ryegrass) (Table 19.11) and for maize approximately 19% after 48 h (Kalac and Kyzlink, 1980).

Either wilting prior to ensiling, which often precedes the ensiling of a forage, or the use of additives, such as organic acids (acetic, formic and propionic acids), tends to increase the loss of β-carotene during the preliminary aerobic phase (Kalac and Kyzlink, 1979, 1980; Kalac and McDonald, 1981). Acidification considerably increased the losses of β-carotene in lucerne, clover and maize, but did not have an effect on either Italian ryegrass or cocksfoot (Kalac and Kyzlink, 1980; Kalac, 1983). The destruction of β-carotene under acidic conditions is of enzymatic origin and results from an oxyreductase other than lipoxyreductases (Kalac and Kyzlink, 1980). The losses of β-carotene during fermentation differ according to the forage (Kalac, 1983), and are high for clover and lucerne (Patel *et al.*, 1966) and low for cocksfoot and Italian ryegrass (Table 19.11).

Ferrando and Mainguy (1970) and Kalac (1983) reported that there was no clear relationship between the quality of a silage and its carotene content.

Table 19.11. Changes in β-carotene content and pH of several types of silages (vegetative stage) (from Kalac, 1983).

Forages	Silage treatment	β-Carotene in fresh leaves (mg kg^{-1} fresh)	β-Carotene (% initial value), pH and quality of silage					
			Delayed sealing (h)			After fermentation and 180 days' storage		
			6 (%)	24 (%)	24 (pH)	(%)	(pH)	Quality
Cocksfoot	C	127	97.4	83.1	5.15	81.8	4.70	II
	F	–	92.7	80.8	4.30	76.3	4.40	II
Italian ryegrass	C	196	–	72.2	5.75	70.0	5.15	IV
	F	–	–	77.6	4.10	73.6	4.90	III
Lucerne	C	186	83.6	70.3	5.95	40.2	5.15	V
	F	–	47.0	11.3	4.35	8.4	5.25	IV
White clover	C	237	92.1	75.7	5.95	53.7	5.80	II
	F	–	69.7	48.6	5.10	28.6	5.30	III

C, control; F, formic acid.
Organoleptic assessment of quality: II, good; III, mean; IV, poor; V, very poor.

Bieber-Wlaschny (1988) indicated that grass silage could provide considerable levels of β-carotene (122 mg kg^{-1} DM), but that maize silage is a very poor source (11 mg kg^{-1} DM); lucerne silage (32 mg kg^{-1} DM) and clover silage (19 mg kg^{-1} DM) were intermediate.

Maize silage is practically devoid of vitamin E (Hidiroglou *et al.*, 1977; Bieber-Wlaschny, 1988). On the other hand, the ensiling of lucerne and brome preserves a major proportion of the vitamin E (King *et al.*, 1967).

The process of ensiling is therefore characterized by introducing great variability in the preservation of the β-carotene and α-tocopherol content of forages.

Dehydration

Rapid dehydration allows a major proportion of the β-carotene and α-tocopherol of forage plants to be conserved (Table 19.12). Dehydrated forages contain, on average, seven times the amount of carotene compared with when they are dried on the ground (Meissonier, 1974). The level of residual moisture of the treated material has a direct effect on the destruction of β-carotene and α-tocopherol (Ferrando and Mainguy, 1970; Burdick and Fletcher, 1985). Livingston *et al.* (1968a, b, 1970) demonstrated that the losses of β-carotene and α-tocopherol vary from 0 to 30% during the course of dehydration of lucerne. They observed that the highest losses occurred when the level of residual moisture was equal to or lower than 30 g kg^{-1}. The optimum moisture content appeared to be between 70 and 100 g kg^{-1} (Livingston *et al.*, 1970). Dehydration had no effect on losses of niacin (Blaylock *et al.*, 1950).

Storage conditions

The highest vitamin loss in absolute terms is observed in forages that are well preserved during their preparation, as in dehydrated materials (Park *et al.*, 1983). Bruhn and Oliver (1978) demonstrated that the losses of β-carotene and α-tocopherol in lucerne hay are significantly correlated with the duration of storage.

Table 19.12. β-Carotene and α-tocopherol losses of lucerne during dehydration.

Outlet temperature (°C)	Residual moisture of product (g kg^{-1})	Losses (%)		References
		Carotene	Tocopherol	
153	92	9	5	Knowles *et al.* (1968)
149	92	9	5	Livingston *et al.*
166	23	18	21	(1968a, b)
116–166	15–122	0–33	–	Livingston *et al.* (1970)
–	80	15	–	Ferrer (1972)

Kohler *et al.* (1955) demonstrated that losses in tocopherol during storage also depended on temperature; a ryegrass meal stored for 6 weeks at 3°C lost 8% of its tocopherol but the loss was of the order of 49% at 60°C (Maynard *et al.*, 1979). The conservation of dehydrated lucerne at ambient temperature (32°C) during 3 months results in considerable loss of carotene (42–72%) and tocopherol (35–73%) (Livingston *et al.*, 1968b, 1970). Thus, the storage at low temperature allows the maintenance of reasonable levels of β-carotene and α-tocopherol (Kivimäe and Carpena, 1973; Park *et al.*, 1983). The storage of dehydrated lucerne in a nitrogen atmosphere allows the duration of storage to be extended without any adverse effects on the quality of β-carotene (Melcion and Delort-Laval, 1973; Park *et al.*, 1983). Irrespective of the procedure of conservation, the carotene degrades slowly during storage; the loss is of the order of 50% in the first 6 months, but it can be greater (Fig. 19.2).

A second degradation of the carotene and tocopherol present after the period of conservation is known to occur during the time that forage is held in the feed bunker, prior to its distribution (McDowell, 1989).

Factors Affecting Vitamin Availability in Ruminants

The metabolic utilization of vitamins supplied by forages is not possible without transit through the digestive tract of the animal. The two most important factors limiting vitamin availability in ruminants are passage through the rumen and intestinal absorption. The act of digestion, principally at the level of the rumen,

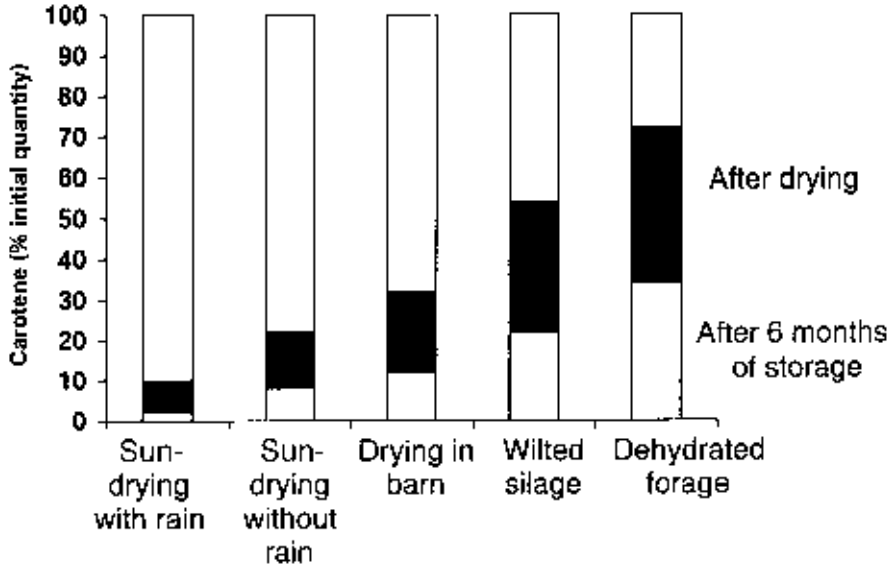

Fig. 19.2. Relative availability of carotene in comparison with its initial content in forages (from Wolter, 1988).

serves to release the vitamins from the combination in which they are found in plant tissue. Thus, the extent of degradation and liberation of vitamins from the forage in the rumen is an essential feature of the bioavailability of vitamins found in forage. According to Nadai (1968), the degree of liberation of carotene in the digestive tract varies according to the type of plant. This may explain why the carotene in green or dehydrated lucerne is more available than that from maize and, in particular, maize silage.

The variability of vitamin supply in forages identified in the foregoing section is one of the reasons why the ruminant-livestock industry has chosen the practice of meeting requirements from synthetic sources. In ration formulation, the supply of vitamins from raw materials is often ignored and the requirements for supplementation with synthetic vitamins are based on meeting 100% of the requirements. Most of the studies concerning the stability in the rumen and intestinal absorption of β-carotene and α-tocopherol have been carried out using synthetic sources. Certain comments on ruminal stability and intestinal absorption of vitamins A, E and D are therefore relevant to this discussion. Factors that need to be considered are the influence of rumen conditions on the availability of vitamins and also intestinal absorption. However, it is beyond the scope of this review to cover the ruminal stability and intestinal absorption of all vitamins and hence comments will be confined to vitamins A, E and D.

Vitamin A

Synthetic vitamin A is produced in the form of the ester, retinyl acetate, in the all-*trans*-isomer form, which has a biological activity of 100% (McDowell, 1989). Vitamin A is sensitive to the action of light, heat and oxidizing agents, as a result of the presence of the unsaturated side-chain. For this reason, synthetic vitamin A is found in encapsulated forms, in order to provide protection against the adverse effects of light and oxidation.

Degradation of vitamin A in the rumen and intestinal absorption

Many biochemical reactions that occur in the rumen are capable of destroying vitamin A, and a large number of studies have provided evidence of major losses of vitamin A in the rumen. These losses may be explained by different phenomena involving the rumen bacteria, including engulfment, oxidation and degradation. To date, no author has demonstrated absorption of vitamin A through the rumen wall, which would appear logical, given the vitamin's large molecular size.

Ruminal losses of β-carotene are, on average, 20%, with a range from 3 to 32% (Table 19.13). Potkanski *et al.* (1974) found that these losses were not affected by diet. Although Keating *et al.* (1964) showed that the addition of an antioxidant (ethoxyquin) or oxidizing agent (potassium nitrate) had no effect on the β-carotene disappearance, Cohen-Fernandez *et al.* (1976) suggested that the destruction of β-carotene could be due to partial oxidation. This contradicts the conclusions of Mitchell *et al.* (1967) and Lichtenwalner *et al.* (1973), who reported that degradation of vitamin A in the rumen is not significantly modified when oxidizing agents, including nitrates, were added to the rumen (Table 19.14). Given that the rumen is

Table 19.13. Ruminal disappearance of β-carotene.

Animal	Diet	Disappearance (%)	Method	References
–	–	31.9	*In vitro* 9 h	King *et al.* (1962)
Dairy heifer	High-hay	25.2 ± 5.6	*In vitro* 7 h	Davison and Seo (1963)
Steer	High-roughage	24.4	*In vitro* 16 h	Keating *et al.* (1964)
Mature wether	High-cellulose	23.1 ± 5.9	*In vivo*	Potkanski *et al.* (1974)
	High-starch	23.3 ± 6.9		
Cow	High-concentrate	4.7 ± 1.7	*In vitro* 24 h	Cohen-Fernandez w*et al.* (1976)*

* β-Carotene from different sources, pure β-carotene or commercial lucerne meal.

Table 19.14. Vitamin A recovery from abomasal fluid of beef cattle (%)* (from Mitchell *et al.,* 1967).

Steers no.	Observation no.	Supplement		Mean
		Control	Nitrate†	
1	1	43.1‡	62.2	
	2	32.4	46.2	
	3	44.6	55.8	
	4	69.1	32.2	48.2
3	1	41.8	45.1	
	2	35.2	39.5	
	3	42.1	66.4	
	4	35.5	55.7	45.7
Mean		43.0	50.4	

* Calculated from the ratio of vitamin A to chromic oxide in abomasal fluid compared with the ratio administered 24 h later.
† 90 mg of potassium nitrate steer^{-1} day^{-1}.
‡ Each observation is the average result of triplicate determinations.

a highly reducing environment, it is unlikely that oxidation plays a major role. It was therefore concluded that β-carotene disappearance in the rumen was due mainly to the action of bacterial hydrogenases (Ferrando, 1980) and it is probable that vitamin A undergoes reductive degradation. In *in vitro* studies, the highest rate of degradation of vitamin A incubated for 4 h in either rumen fluid or autoclaved rumen fluid was in the presence of an active rumen microflora (Table 19.15; Klatte *et al.*, 1964). It was concluded that the degradation of vitamin A was due to the action of the microflora, although it was accepted that autoclaving could have limited other biological functions. Rode *et al.* (1990) showed that nine different types

Table 19.15. Disappearance of vitamin A after 4 h in distilled water or ruminal fluid (from Klatte *et al.,* 1964).

Incubation fluid	Number of assays	Disappearance (%)
Distilled water	11	16
Rumen liquor	11	36
Autoclaved rumen liquor	2	13

of bacteria were capable of transforming retinol propionate without inducing an accumulation of retinol, which is the only form of vitamin A that can be absorbed (Table 19.16).

Rode *et al.* (1990) measured the disappearance of vitamin A from the rumen of steers given diets based on concentrates, hay or straw and found levels of degradation of 67, 16 and 19% respectively. Weiss *et al.* (1995) confirmed *in vitro* that degradation of retinyl acetate was higher (72%) with rumen fluid obtained from a concentrate-type fermentation, in comparison with fluid obtained when the diet was based on forage (20%).

Using measurements of rumen flow in steers (Mitchell *et al.*, 1967) and sheep (Brongniart, 1987), the degradation of vitamin A *in vivo* was shown to be of the order of 60%. From a review of the literature, a mean rate of degradation of vitamin A in the rumen of 66% was found (Table 19.17). Similar losses of vitamin A from the rumen after intraruminal administration of vitamin A were also reported by Granseigne and Robert (1985) (Fig. 19.3). The rate of degradation of the two esters (acetate and palmitate) was highest in the first 2 h and then tended towards a maximum of 60%. Retinyl palmitate appeared to be less degradable than the acetate form.

Intestinal absorption of fat-soluble vitamins is dependent on the digestion and absorption of fat (Ullrey, 1972; McDowell, 1989). Vitamin A is absorbed in the form of retinol via the same mechanism as lipids, requiring the activity of bile salts,

Table 19.16. Degradation of vitamin A by rumen bacteria (from Rode *et al.,* 1990).

	Retinyl propionate degradation (%)			Retinol accumulation (%)		
Strains	18 h	42 h	66 h	18 h	42 h	66 h
Ruminobacter amylophilus WP109	13	17	30	0	0	0
Ruminobacter amylophilus WP91	67	76	85	0	5	0
Eubacterium ruminantium G1–1(1)	37	45	37	2	3	5
Fusobacterium necrophorum 1A	18	50	69	0	0	0
Fibrobacter succinogenes D	9	26	27	0	0	0
Ruminococcus flavefaciens 4	0	69	63	0	10	5
Propionibacterium acnes ATC6919	48	48	73	0	0	0
Butyrivibrio fibrisolvens XBF	48	95	96	0	0	0
Megasphaera elsdenii AW106	46	59	46	0	0	0

Table 19.17. Measurements of ruminal degradation of vitamin A *in vivo.*

Animal	Diet	Degradation (%)	References
Mature steer	High-roughage	57 (31–67)	Mitchell *et al.* (1967)
Steer	High-hay	73.9 (70–80)	Mitchell *et al.* (1968)
Mature steer	20% concentrate	52	Warner *et al.* (1970)
	40% concentrate	56	
	60% concentrate	70	
	80% concentrate	62	
Sheep	High-concentrate	64 ± 9	Brongniart (1987)

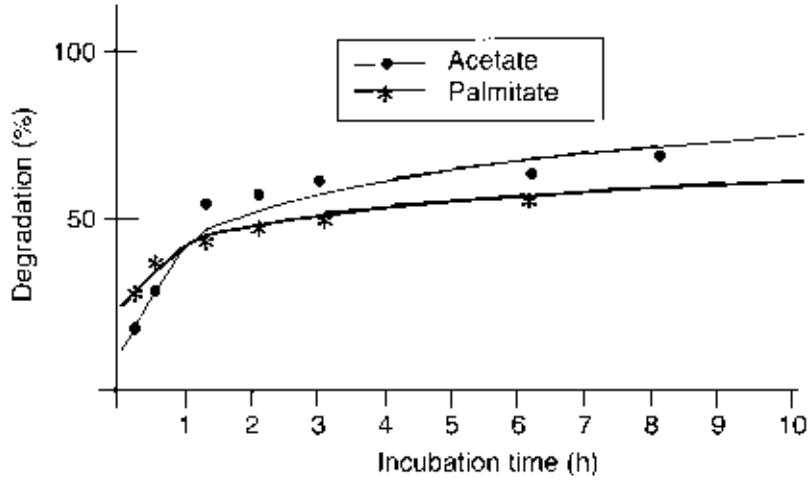

Fig. 19.3. Degradation curve of two esters of vitamin A in the rumen of sheep offered a concentrate ration (from Granseigne and Robert, 1985).

and thus any perturbation of lipid absorption results in a similar perturbation in vitamin A absorption (Bondi and Sklan, 1984; McDowell, 1989). Vitamin A transport across the brush border is not dependent on a membrane transport system but is dependent on the physicochemical characteristics of the surface contact of the molecule with the surface of the enterocyte. Absorption of vitamin A at the level of the intestine was found to be 30–50% of that ingested (Olson, 1984). This figure is in agreement with the level of degradation of 60% in the rumen, on condition that there is no further loss during intestinal absorption.

Ferrando (1980) reported that only 10% of β-carotene from forages would be used by ruminants. The low utilization of β-carotene from forages was due to its low solubilization and hence limited absorption (Bondi and Sklan, 1984), rather than its ruminal degradation. Indeed, Potkanski (1974), cited by Kalac and McDonald (1981), found that postabomasal absorption of carotene was only 5–13% of total carotene intake. Furthermore, intestinal absorption of carotene was reduced as the

level consumed increased (Olson, 1984). Following absorption, β-carotene is totally converted into vitamin A in the enterocytes of sheep and goats, but only partially converted in the enterocytes of the bovine. Eaton *et al.* (1959) found that differences existed between breeds in ability to convert carotene into vitamin A. The Holstein breed converted carotene into vitamin A 1.4 times more efficiently than the Guernsey breed.

A particular characteristic of the metabolism of vitamin A is that it is stored in the liver in the A1-all-*trans* esterified form by fatty acids, essentially palmitic acid. The liver may contain 90% of the total vitamin A in the organism and 30–60% of the quantity absorbed each day may be stored in the liver.

Studies in sheep on liver storage of vitamin A as a function of elevated dietary supply showed that the coefficient of liver storage, (vitamin A in the liver/vitamin A ingested) × 100, was of the order of 30% for supplementation with 20 IU g^{-1} of feed (Robert, 1987; Table 19.18). This value is relatively low in comparison with the coefficient of storage of 56% observed in monogastrics (Uzu, 1988), but is in accord with a substantial rumen degradation of vitamin A. In this study, it was also reported that coefficients of liver storage were reduced when levels of supplementation were increased. This could be explained by saturation of either the absorption pathway or the capacity of the liver to store vitamin A or a combination of the two.

During manufacture, synthetic vitamin A is normally encapsulated to guarantee stability against oxidation during fabrication of premixes and compound feed. It has been found that the type of encapsulation can play an important role in the level of ruminal degradation of the vitamin. Robert (1995a) measured the degradation of vitamin A in the rumen of lactating cows, using the nylon-bag technique. The results demonstrated that the encapsulation process could significantly reduce the rate of vitamin degradation in the rumen. It was further demonstrated that the solubilization of the encapsulation was significantly greater with a hay-based ration compared with a ration based on maize silage, probably as a result of the higher pH in the rumen with the hay-based diet compared with maize silage, producing conditions more favourable for the solubilization of the encapsulation material. The observations indicate that, dependent on the diet and the encapsulation process, different levels of vitamin A may bypass the rumen and be absorbed in the intestine.

Table 19.18. Measurements of hepatic storage of vitamin A (from Robert, 1987).

Diet	Number of animals	Planned vitamin A supplementation (IU g^{-1} feed)	Vitamin A level in feed (IU g^{-1} feed)	Total vitamin A hepatic storage (IU × 10^3)	Coefficient of hepatic storage (%)
1	15	0	2.4	11	–
2	15	20	19.8	523	34.1
3	15	40	45.6	961	25.0
4	15	60	61.3	928	18.9
5	15	80	83.1	898	12.1
6	15	100	109.2	989	10.1

Vitamin E

The biological role of tocopherols is related to the antioxidant activity of vitamin E, in particular as a means of protecting polyunsaturated fats in tissue membranes from peroxidation. Tocopherols have a low stability to heat, light and basic pH. Taking into account their antioxidant properties, in the presence of oxygen they themselves are oxidized, with the formation of quinones in the dimer and trimer forms. Acylation of the hydroxyl group present on the ring improves vitamin E stability and, for the most part, synthetic forms of vitamin E are present in the form of esters (acetate, succinate, …). These synthetic forms have no antioxidant properties *per se* but this property is recovered at the metabolic level, given the fact that absorption at the intestinal level occurs in the form of the alcohol.

Degradation of vitamin E in the rumen and intestinal absorption

Effective supplementation with vitamin E requires that the dietary vitamin supplement arrives intact at the site of absorption in the small intestine, where it can be absorbed and participate in metabolism.

The question of apparent low plasma appearance has been addressed by several authors. Using a preparation in which mature ewes had the pylorus ligatured, Alderson *et al.* (1971) were unable to obtain increased blood levels of vitamin E when 5000 IU of α-tocopherol (1 mg = 1.36 IU) was introduced into the rumen. It was concluded that vitamin E is not absorbed through the rumen wall. The addition of either an oxidizing agent (sodium nitrate) or an antioxidant (ethoxyquin) into the rumen of steers had no influence on the rate of disappearance of supplementary vitamin E added to the rumen at the level of 4190 IU (Tucker *et al.*, 1971; Table 19.19). It is notable that, in these two experiments, quite disparate and unexplainable rates of degradation of vitamin E were reported (27% and 62%). Hidiroglou *et al.* (1970) reported that there was a higher retention of radiolabelled tocopherol in sheep when it was administered intramuscularly compared with oral administration, which could be explained by pre-intestinal destruction of vitamin E. The fact that the rumen microflora contains vitamin E strengthens the hypothesis of a direct action of the rumen bacteria (Hidiroglou and Jenkins, 1974).

The destruction of vitamin E by the rumen microorganisms was studied in rumen-fistulated steers, each supplemented with 20,000 IU of vitamin E day^{-1} and given four diets containing different levels of lucerne hay and maize (Alderson *et al.*, 1971; Table 19.20). Degradation of vitamin E ranged from 8 to 42% as the level of maize incorporation in the ration increased. These results tend to suggest that

Table 19.19. Influence of oxidant or antioxidant agents on the degradation of vitamin E *in vivo* (from Tucker *et al.,* 1971).

	Trial 1		Trial 2	
	Control	Ethoxyquin	Control	Sodium nitrate
% of vitamin E degraded	26.8	24.4	61.5	64.3

Table 19.20. Disappearance of α-tocopherol in the rumen.

Animal	Diet	Disappearance (%)	Method	References
Steer	20% concentrate 40% concentrate 60% concentrate 80% concentrate	8.4 ± 1.2 22.2 ± 3.5 25.0 ± 5.3 42.4 ± 7.7	*In vivo*	Alderson *et al.* (1971)
Adult steer	High-concentrate	39–52	*In vivo*	Shin and Owens (1990)
Sheep	Forage/concentrate 50/50	6 ± 4	*In vitro* 24 h	Astrup *et al.* (1974a)
Beef steer	High-concentrate	4	*In vitro* 24 h	Leedle *et al.* (1993)
Jersey steer	High-forage	7.7 ± 3.5	*In vitro* 24 h	McDiarmid *et al.* (1994)
Dairy cow	High-silage	18	*In vivo*	Robert (1995b)
Dry cow Dairy cow lactating	High-forage Forage/concentrate 50/50	0 0	*In vitro* 24 h	Weiss *et al.* (1995)

vitamin E was partly degraded by the rumen microflora and particularly the amylolytic bacteria. Shin and Owens (1990) also demonstrated that between 39 and 52% of dietary supplemental vitamin E disappeared before reaching the duodenum of steers.

These results are contrary to those obtained by Leedle *et al.* (1993), who, based on assumptions made by Alderson *et al.* (1971), examined the stability of vitamin E in an *in vitro* system, using rumen liquor obtained from steers which had been offered a diet rich in concentrates. After periods of incubation of up to 24 h at 39°C, there was no loss of 1000 IU of DL-α-tocopherol acetate (1 mg = 1 IU). These authors concluded that there was no destruction of vitamin E by the rumen microorganisms and they suggested that the contradictory results obtained by other authors were due to the methods of vitamin E extraction and analysis. Using a diet based on 50% oats and 50% hay and with an *in vitro* rumen system, Astrup *et al.* (1974a) observed only slight degradation of vitamin E (Table 19.20). Similarly, with diets rich in either forage or concentrates, McDiarmid *et al.* (1994) and Weiss *et al.* (1995) observed only slight degradation of DL-α-tocopherol acetate.

Leedle *et al.* (1993) considered that structural differences between vitamins E and A were responsible for the relative differences in stability. Vitamin A possesses four double bonds in the side-chain, which are susceptible to reduction in the anaerobic rumen environment (biohydrogenation), whereas vitamin E possesses only one double bond on the inside of the aromatic ring, conferring greater stability.

It is quite possible that values obtained using *in vitro* fermentation systems may underestimate ruminal degradation, compared with values measured *in vivo*. In an *in vivo* trial with cows cannulated at the duodenum and given diets based on maize silage (ratio of forage to concentrate 80/20), Robert (1995b) reported only moder-

ate degradation of vitamin E (approximately 20%) (Table 19.20). The level of supplementation was 1500 IU animal^{-1} day^{-1}, which was an exceptionally high level but which was used for reasons of experimental methodology. Indeed, it is possible that this very high dose caused a saturation of the microbial degradation of vitamin E.

In ruminants, absorption of vitamin E is slower than in other species, the peak level in the plasma occurring approximately 24–48 h after oral administration. Intestinal absorption is linked to the absorption of lipids and requires the presence of bile salts and pancreatic lipases. Only the alcohol form is directly absorbed from the jejunum. This absorption process can be saturated. Using radiolabelled L-α-tocopherol, Tikriti (1969) obtained levels of absorption of the order of 40–50% of that escaping degradation in the rumen of dairy cows and, in goats, Astrup *et al.* (1974b) obtained levels of absorption of approximately 58% of the vitamin E that was introduced into the abomasum. Similarly to the case with β-carotene, intestinal absorption reduces as the level consumed increases (Machlin, 1984).

Vitamin E is stored both in adipose tissue and in the liver, but all tissues contain detectable amounts. Adipose tissue would appear to be capable of storing an unlimited quantity, whereas storage of vitamin E in the liver occurs for only a limited period. Furthermore, high-level supplementation of vitamin E for a short period does not increase the level of reserves in a similar manner to that following similar supplementation with vitamin A.

A low level of degradation of vitamin E in the rumen, which as yet remains to be confirmed, would be in accordance with responses in terms of animal health and performance obtained in practice. It is also in accordance with the more recent practice of using high-level supplementation with vitamin E to improve carcass quality. Bozzolo *et al.* (1993) (Table 19.21) were able to improve the quality of external carcass fat in sheep by supplementing with 4 g of DL-α-tocopherol acetate for 4 days prior to slaughter. The improvement resulted from the antioxidant properties of tocopherols. It is important to note that these authors obtained a significant increase in the plasma concentration of vitamin E, which was similar to that reported by Hidiroglou and Karpinski (1988). However, the high variability recorded in the concentrations of vitamin E in the treated sheep demonstrates a lack of homogeneity in the response. The authors considered that the variability was a result of the oral administration.

For improvement in meat quality, different authors have recommended supplementation with vitamin E at elevated dose levels (500–1000 IU animal^{-1} day^{-1}) during a period of 90–100 days before slaughter of cattle. Such levels of supplementation have proved beneficial in increasing tissue levels of α-tocopherol and reducing the percentage of metmyoglobin in muscle and the concentration of free radicals in the meat. Mitsumoto *et al.* (1993) orally supplemented steers with 1500 IU vitamin E animal^{-1} day^{-1} for 250 days before slaughter. Proportions of metmyoglobin, which is a brown oxidation product, were, in muscle of the control and treated animals, 19 and 7% on the day of slaughter and 87 and 40% after 9 days of storage, respectively. This reduced oxidation has the potential to dramatically improve the quality and storage life of meat obtained from beef animals and available on supermarket shelves (Faustman *et al.*, 1989a, b; Arnold *et al.*, 1992).

Table 19.21. Vitamin E and external tissue colour of sheep (from Bozzolo *et al.,* 1993).

	Control	Supplemented
Number of animals	120	120
Supplementary supply of DL-α-tocopherol acetate	0	4 g per animal (4 days before slaughter)
Colour of fat (%)		
Creamy white	19	38
Creamy brown	46	27
Yellow	34	34
Plasma level of vitamin E (μg ml^{-1})	0.7 ± 0.18	1.93 ± 0.83

Vitamin D

In high-producing, lactating, dairy cows at the start of lactation and under normal conditions, the presence of vitamin D prevents hypocalcaemia and the occurrence of milk fever. The presence of 1,25-dihydroxycholecalciferol also stimulates mineral uptake and particularly calcium uptake by bone, which explains the occurrence of rickets and bone dystrophy in the absence of vitamin D (rickets found in young animals and osteomalacia in lactating females).

Theoretically, the requirements for supplementary vitamin D in ruminants are low, because animals exposed to sunlight are capable of synthesizing sufficient vitamin D and, as indicated earlier, a supply can be obtained from sun-dried forages. Regular supplementation with vitamin D allows establishment of adequate reserves, which cover requirements during periods when the climate is unfavourable and when diets based on ensiled materials are offered. It is for these reasons that synthetic vitamin D has been developed, which confers the additional advantage of providing a controlled supply.

Studies of degradation of vitamin D incubated for 24 h in rumen liquor obtained from steers fed a diet based on forage showed a loss of approximately 75% of vitamin D (Sommerfeldt *et al.*, 1979b). However, incubation of vitamin D in sterilized rumen fluid resulted in no degradation, indicating that degradation is due to the activity of the rumen microorganisms (Sommerfeldt *et al.*, 1979b). Such activity produces three metabolites of vitamin D (stereoisomers of 10-ceto-19-norvitamin D_3) (Horst and Reinhardt, 1983), which have anti-vitamin D activity but which have the advantage of protecting ruminants from vitamin D toxicity in the event of consumption of high quantities. Although D_2 is comparable to D_3 in antirachitic function (McDowell, 1989), Sommerfeldt *et al.* (1979a, 1983) showed that the two forms of vitamin D (vitamin D_2 and D_3) do not pass through identical metabolic pathways in the rumen, resulting in a reduction of vitamin D_2 activity in comparison with vitamin D_3. Vitamin D is absorbed from the intestine in association with lipids and thus requires the presence of bile salts. Only approximately 50% of the vitamin D ingested is absorbed (Miller and Norman, 1984; McDowell, 1989).

It is probable that a proportion of supplementary vitamin D in the diet is destroyed by the rumen and does not reach the small intestine. This is justification for retaining the use of supplementary vitamin D in the diet as a means of covering requirements during periods of need. However, account must be taken of the ease with which hypervitaminosis and vitamin D toxicity can occur. Severe bone demineralization (osteofibrosis) in conjunction with hypercalcaemia and the occurrence of urinary calculi plus calcification of soft tissues has been observed following chronic doses of between 50 and 100 times requirement. In Europe, legislation limits dietary supplementation for ruminants to a maximum of 4000 IU vitamin D kg^{-1} of diet.

Conclusion

This review has demonstrated that, whilst forages may be considered as a potential source of vitamins, there is great variability in the quantity of key vitamins that remain bioactive in the feed and, perhaps of more importance, it is almost impossible, without costly feed evaluation, to accurately predict the quantity that will be supplied to ruminants. The review has also identified the major influence that rumen fermentation has on the quantity of vitamins reaching the small intestine. The value of correct vitamin supply to ruminants cannot be overstated and there is increasing evidence of the benefits of hypersupplementation with certain vitamins.

In view of the tremendous importance of correct vitamin supply, plus the uncertainty of supply from feed components, it is totally realistic to rely on the precision and reliability that is achieved by supply from synthetic vitamins. Accuracy of vitamin supply to ruminants is assisted by the use of formulation technology, which protects vitamins during feed processing and also helps ensure the delivery of the target dose to the absorption site in the intestine. In this manner, optimum benefit can be gained from these highly valued but relatively inexpensive feed additives. Although forage has the potential to contribute to the vitamin supply of ruminants, it is very difficult to envision how this supply can be reliably incorporated into ration formulation systems.

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20 Secondary Plant Compounds and Forage Evaluation

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Introduction

Secondary plant compounds (SPC) are a diverse group of molecules that are involved in the adaptation of plants to their environment but are not part of the primary biochemical pathways of cell growth and reproduction. There are over 24,000 structures, including many compounds that have antinutritional and toxic effects on mammals (Harborne, 1993). The SPC that occur in forages include alkaloids, non-protein amino acids, cyanogenic glycosides, volatile terpenoids, saponins, phenolic acids, hydrolysable tannins (HT) and flavonoids, including proanthocyanidins (PA) and oestrogenic isoflavones. Secondary plant compounds are involved in defence against herbivores and pathogens, regulation of symbiosis, control of seed germination and chemical inhibition of competing plant species (allelopathy). Therefore, SPC are an integral part of the interactions of species in plant and animal communities (Harborne, 1993). Research on the SPC in forages has concentrated on their toxic and antinutritional effects on livestock. These effects can be classified into two groups (Reed 1998):

1. Toxic compounds that are present in plants at low concentrations (generally less than 20 g kg^{-1} of the dry matter) and have negative physiological effects when absorbed, such as neurological problems, reproductive failure, goitre, gangrene and death. Examples are alkaloids, cyanogenic glycosides, toxic amino acids, saponins, isoflavonoids and many others.

2. Non-toxic compounds that lower the digestibility and palatability of plants. Higher concentrations (> 20g kg^{-1} of dry matter) of these compounds are required for negative effects and the primary site of activity is in the digestive tract or through sensory organs associated with feeding behaviour. This class includes lignin, tannin, cutin, biogenic silica and volatile terpenoids. Compounds that have a structural role in the plant (i.e. lignin, silica and cutin) lower the extent of microbial degradability in cell-wall polysaccharides. The primary role of tannins and terpenoids may be in plant defence against predators.

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The division between these groups of SPC is not well defined. For instance, HT are potentially toxic to ruminants, because microbial 'tannases', which hydrolyse galloyl esters, are present in the rumen (Skene and Brooker, 1995). The gallic acid released is further metabolized to potentially toxic phenols, which are absorbed from the rumen (Murdiati *et al.*, 1992). The major lesions are haemorrhage, gastroenteritis, necrosis of the liver and kidney damage, with proximal-tubular necrosis (Dollahite *et al.*, 1962; Holliman, 1985; Filippich *et al.*, 1991). Excessive consumption of oaks and other tree species that contain more than 200 g kg^{-1} HT results in high mortality and morbidity in cattle and sheep.

However, SPC in forages are also associated with improved nutritive value and may have beneficial effects on animal health. PA, more commonly called condensed tannins in the animal nutrition literature, in forage legumes, such as sainfoin (*Onobrychis viciaefolia*), bird's-foot trefoil (*Lotus corniculatus*) and Lotus *pedunculatus*, are associated with improved protein digestion and metabolism in ruminants and in protecting ruminants against legume bloat (see reviews by Reed, 1995; Waghorn *et al.*, 1998). PA may also protect ruminants against helminthiasis. Undrenched lambs grazing sulla (*Hedysarum coronarium*), a forage that contains PA, had lower faecal egg counts and *Trichostrongylus colubriformis* worm burdens and higher average daily live-weight gains than undrenched lambs grazing lucerne (alfalfa) (*Medicago sativa*), which does not contain PA (Niezen *et al.*, 1995). Growing interest in the potential health-promoting effects of SPC in human foods has prompted research on their potential to prevent or treat cancer, circulatory disease and viral infection. The mechanisms by which SPC have beneficial effects on health are likely to be the same as their toxic effects, and the difference between toxicity and beneficial effects is probably dose-dependent (Shahidi, 1995).

This chapter will not attempt to review the extensive literature on the toxicity and nutritional effects of SPC in forages. Recent reviews are available (D'Mello, 1997; Cheeke, 1998; Reed, 1998) and readers are also referred to a review on mammalian metabolism of SPC by Scheline (1991). In this chapter, we shall discuss results from our research on two forages, *Sesbania seban* and *Trifolium pratense* (red clover), as a way of illustrating the effects of SPC on forage evaluation.

Plant Polyphenols and Protein Digestion in Ruminants

Our research on *Sesbania* and red clover is directed at the manipulation of the plant phenolic chemistry in order to improve protein digestion and metabolism in ruminants. Rapid rates of proteolysis and deamination of amino acids in the rumen are nutritional problems of productive forage legumes, such as lucerne (Broderick, 1985; Broderick and Buxton, 1991). Problems associated with extensive proteolysis and deamination of amino acids limit production in modern feeding systems (Beever *et al.*, 1989). Over 75% of the protein in lucerne is degraded in the rumen (Broderick and Buxton, 1991). Excessive proteolysis and deamination create high levels of rumen ammonia, which is not used by rumen microbes for synthesis of amino acids. This ammonia is absorbed, metabolized to urea in the liver and excreted in the urine and represents a net loss of both energy and protein to ruminants. Lucerne has a higher rate of proteolysis than forage legumes that contain

PA, such as sainfoin and sericea lespedeza (*Lespedeza cuneata* (Dum-Cours) G. Don) (Broderick and Albrecht, 1997). Feeding experiments with forage legumes that contain PA indicate that, at high levels of protein intake, PA have a positive effect on nitrogen (N) retention and increase the flow of essential amino acids to the duodenum (Thomson *et al.*, 1971; Harrison *et al.*, 1973; Egan and Ulyatt, 1980; John and Lancashire, 1981; Barry and Manley, 1984; Beever and Siddons, 1985; Waghorn *et al.*, 1987). Although apparent and true digestibility of protein is depressed, urinary loss of N is sufficiently reduced to compensate for the greater faecal loss. These results suggest that it may be possible to manipulate the PA chemistry of forage legumes in order to improve protein utilization.

The phenolic chemistry of forage legumes also has a large effect on the proteolysis that occurs in silages. Extensive proteolysis during ensiling also creates high levels of soluble non-protein N (SNPN) (Albrecht and Muck, 1991). Plant proteases are responsible for most of the proteolysis that occurs during wilting and the initial phases of the silage fermentation (Muck, 1988). The SNPN in legume and grass silages also contributes to high levels of rumen ammonia and inefficient use of protein. Albrecht and Muck (1991) demonstrated that proteolysis is lower in silage produced from bird's-foot trefoil, sainfoin and red clover in comparison with lucerne. SNPN was negatively correlated with tannin content in silage made from sericea lespedeza, sainfoin, bird's-foot trefoil and lucerne ($r^2 = 0.93$).

However, forage legumes seldom contain a single class of SPC. In addition to PA and related phenolic compounds, forage legumes may contain toxic compounds that interfere with the normal metabolic function of ruminants. As illustrated in the following discussion, the effects of toxic SPC in *Sesbania* and red clover need to be considered in research on the manipulation of phenolic chemistry to improve protein digestion and metabolism.

Secondary Plant Compounds in the Evaluation of Red Clover

Red clover contains a soluble polyphenol oxidase (PPO) and a high level of phenolic substrates (Jones *et al.*, 1995a, b). The action of PPO on phenolic substrates produces quinones and leads to a rapid browning reaction when red clover is harvested and allowed to wilt. The quinones react with amino, sulph-hydryl, thioester, phenolic, indole and imidazole groups of proteins (Matheis and Whitaker, 1984). Subsequent cross-linking of proteins by quinones reduces solubility and enzymatic proteolysis during field wilting and the ensiling process. Although red clover does not contain PA, this legume has lower rates of proteolysis and ammonia production in the rumen in comparison with lucerne (Jones *et al.*, 1995a, b; Yocum, 1995). The effect of the PPO and phenolic substrates on proteolysis in red clover are similar to the effects of condensed tannins in forage legumes that contain PA (Yocum and Reed, 1994). The content of soluble phenolic compounds in red clover is more than twice the content in lucerne and other forage legumes that do not contain PA (Fig. 20.1). The higher levels of soluble phenolics are associated with high amounts of neutral-detergent-insoluble N (NDIN). In a study of six forage legumes, red clover had the highest content of NDIN and this was higher than many species that contain PA (Table 20.1). The NDIN and acid-detergent-insoluble N (ADIN)

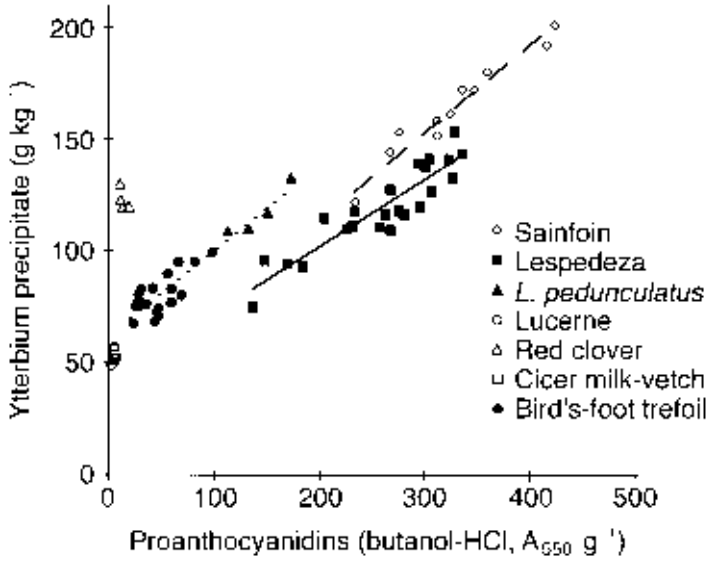

Fig. 20.1. The relationship between soluble phenolic compounds, as measured by ytterbium precipitation, and proanthocyanidins (PA), as measured by butanol-HCl, in seven species of forage legumes. Red clover (*Trifolium repens*) has a similar content of soluble phenolics to sainfoin (*Onobrychis viciaefolia*) and *Lotus pedunculatus* but does not contain PA. Lucerne (*Medicago sativa*) and cicer milk-vetch (*Astragalus cicer*) also do not contain PA, but their concentration of soluble phenolics is less than 40% of the concentration in red clover. The relationship between ytterbium precipitate and PA is highly linear in sainfoin (dashed line, $r^2 = 0.95$), lespedeza (*Lespedeza cuneata*, solid line, $r^2 = 0.84$) and combined *L. pedunculatus* and bird's-foot trefoil (*L. corniculatus*, dotted line, $r^2 = 0.88$). HCl, hydrochloric acid.

in red-clover silage was significantly higher than in lucerne silage and similar to those in sainfoin silage when expressed as a percentage of the total N (Table 20.2) (Yocum, 1995).

Two phenylpropenoid compounds, clovamide and phaselic acid, were isolated and identified in leaves of red clover. Both phenolic compounds are substrates for the PPO in red clover, as indicated by their rapid disappearance during the oxidation process (Fig. 20.2). Red-clover PPO that was isolated by using ammonium sulphate fractionation and chromatography on *Sephadex* G-150 had a pH optimum of 6.5 with catechin and 10.5 with 4-terbutylcatechol. These results indicate that red-clover PPO catalyses the conversion of *ortho*-dihydroxyphenols to quinones (Fig. 20.3). Our laboratory is currently purifying the enzyme, using different chromatographic techniques, in order to determine molecular weight and study enzyme kinetics.

Silages from lucerne, red clover and sainfoin were fed to wethers with rumen cannulae and placed in metabolism crates with feeding mechanisms for continuous feeding in a 3 × 3 Latin-square design (Yocum, 1995). Rumen ammonia and blood urea N was highest and N in the rumen particulate dry matter was lowest in sheep

Table 20.1. Nitrogen, neutral-detergent fibre (NDF) and neutral-detergent-insoluble N (NDIN) in six species of forage legumes. Red clover (*Trifolium pratense*) does not contain proanthocyanidins (PA) but had higher amounts of NDIN than *Lotus corniculatus*, *Lotus pedunculatus*, *Onobrychis viciaefolia* and *Lespedeza cuneata*, which do contain PA. The effect of the polyphenol oxidase and phenolic substrates may decrease the solubility of proteins in detergent.

	N		NDF		NDIN		
Forage	Mean (g kg^{-1} DM)	SD	Mean (g kg^{-1} DM)	SD	Mean (g kg^{-1} DM)	SD	% of N
Medicago sativa	36.0	2.7	406	16	10.7	0.9	29.7
Trifolium repens	28.6	0.8	385	15	18.4	1.9	64.3
Lotus corniculatus:							
var. Norceen	35.4	3.0	350	8	8.7	1.3	24.6
var. Viking	33.6	0.7	349	20	7.9	0.7	23.5
Lotus pedunculatus:							
var. Red Maku	39.2	1.8	345	43	18.4	2.1	46.9
Onobrychis viciaefolia							
var. Remont	35.3	5.6	357	8	15.2	1.6	43.0
var. Eski	39.5	1.8	307	22	16.3	1.7	41.3
Lespedeza cuneata							
var. Au Lotan	28.4	1.6	524	31	14.5	0.9	51.0
var. Serala	27.1	1.2	496	26	12.8	0.3	47.2

DM, dry matter; SD, standard deviation.

Table 20.2. Nitrogen, neutral-detergent fibre (NDF), neutral-detergent-insoluble N (NDIN), acid-detergent fibre (ADF) and acid-detergent-insoluble N (ADIN) in lucerne, red-clover and sainfoin silages. Red-clover silage does not contain proanthocyanidins but has a similar concentration of NDIN and ADIN to sainfoin silage.

Component (g kg^{-1} DM)	Lucerne	Red clover	Sainfoin	LSD
N	26.6^{ab}	25.3^{a}	27.8^{b}	2.3
NDF	478^{ab}	446^{a}	499^{b}	43.1
NDIN	3.2^{a}	7.9^{b}	9.9^{c}	0.04
ADF	358	359	357	21.8
ADIN	1.2^{a}	1.7^{b}	1.8^{b}	0.3
NDIN (% N)	11.80^{a}	31.25^{b}	35.70^{b}	4.65
ADIN (% N)	4.60^{a}	6.70^{b}	6.40^{b}	1.68

Within rows, means with different superscripts differ ($P < 0.05$).
LSD, least significant difference; DM, dry matter.

fed lucerne silage (Table 20.3). Wethers fed sainfoin silage had the highest excretion of total faecal N, faecal NDIN and ADIN, and had the lowest excretion of urinary N (Table 20.3). Wethers fed red-clover and sainfoin silage had higher N retention than wethers fed lucerne silage. The polyphenols in sainfoin and red clover have a large effect on parameters of protein digestion and metabolism, such

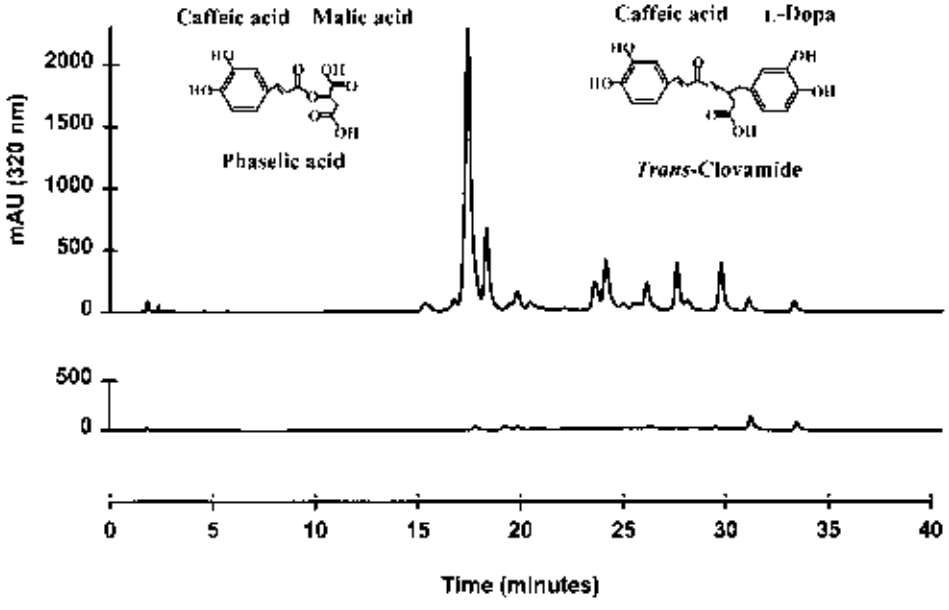

Fig. 20.2. Reverse-phase high-performance liquid chromatography of aqueous methanol extracts of red-clover leaves. Peaks corresponding to phaselic acid and *trans*-clovamide disappear after leaves are allowed to wilt and turn brown, in approximately 30 min.

as lower rumen ammonia and blood urea N and a shift from urinary N excretion to faecal N excretion in comparison with lucerne silages. These effects are accompanied by an increase in the faecal excretion of fibre-bound N and, in the case of sainfoin, a significant depression in the true digestibility of N, but the net result may be a positive effect on N balance. Red-clover silage, on the other hand, had a true N digestibility that was similar to that of lucerne silage, because the NDIN of red clover has a high total-tract digestibility. The NDIN fraction of red-clover silage has an *in vitro* degradability of 74% and digests at a greater rate than the neutral-detergent fibre (NDF) (Yocum, 1995; Fig. 20.4).

Red clover also contains the oestrogenic isoflavones formononetin, daidzein, biochanin-A and genestein (Farnsworth *et al.*, 1975). Formononetin in red clover and subterranean clover (*Trifolium subterraneum*) is the isoflavone that causes reproductive disorders in sheep (Millington *et al.*, 1964; Davies, 1987; Lundh, 1990). Although formononetin is less oestrogenic than the other isoflavones, formononetin is metabolized by rumen microbes to equol, a potent oestrogen (Nilsson *et al.*, 1967; Shutt *et al.*, 1970; Davies and Hill, 1989; Fig. 20.5). Biochanin-A and genestein are degraded to non-oestrogenic compounds by rumen microorganisms (Batterharn *et al.*, 1965; Nilsson *et al.*, 1967). The method of preservation of red clover has an effect on the oestrogenic activity of the forage (Kallela, 1975, 1980). Ensiling may preserve the oestrogenic activity (Kallela, 1980), whereas drying may decrease it.

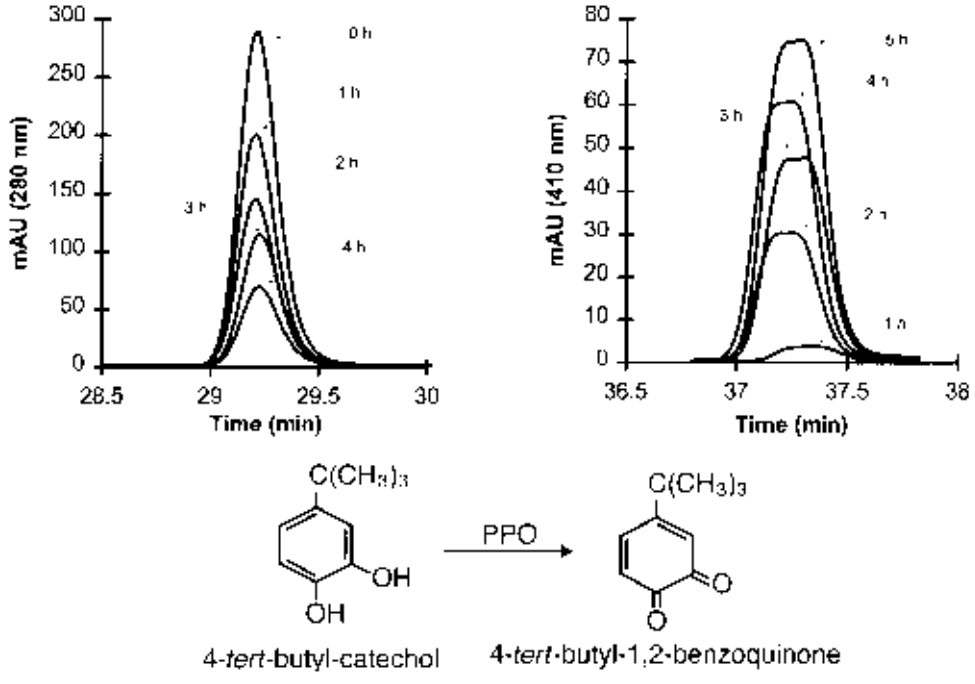

Fig. 20.3. The conversion of dihydroxyphenols to quinones by red-clover polyphenol oxidase (PPO). Reverse-phase high-performance liquid chromatography shows the disappearance of 4-*tert*-butyl-catechol and subsequent appearance of 4-*tert*-butyl-1,2-benzoquinone.

Table 20.3. Parameters of nitrogen metabolism in wethers fed lucerne, red-clover and sainfoin silages. Red clover and sainfoin differ from lucerne because of the effects proanthocyanidins in sainfoin and the effects of polyphenol oxidase and phenolic substrates in red clover on proteins during wilting and ensiling (Yocum, 1995).

	Lucerne	Red clover	Sainfoin
Rumen parameters			
Ammonia N (mg kg^{-1})	415^{a}	166^{b}	24.9^{ab}
Particulate N (mg kg^{-1})	27^{b}	34^{a}	35^{a}
Blood urea N (mg dl^{-1})	18.5^{a}	15.2^{b}	14.1^{b}
Nitrogen excretion (g day^{-1})			
Urinary N (g day^{-1})	14.8^{a}	11.1^{ab}	10.3^{b}
Faecal N (g day^{-1})	6.6^{c}	9.4^{b}	11.1^{a}
Faecal NDIN (g day^{-1})	1.4^{b}	1.7^{b}	6.7^{a}
Digestibility (%)			
True N	93.5^{a}	92.1^{b}	71.7^{c}
Acid-detergent-insoluble N	2.9^{a}	-26.3^{a}	-170.2^{b}
Lignin	1.4^{a}	-19.1^{a}	-73.1^{b}

Within rows, differences between means with different superscripts are significant ($P < 0.05$).

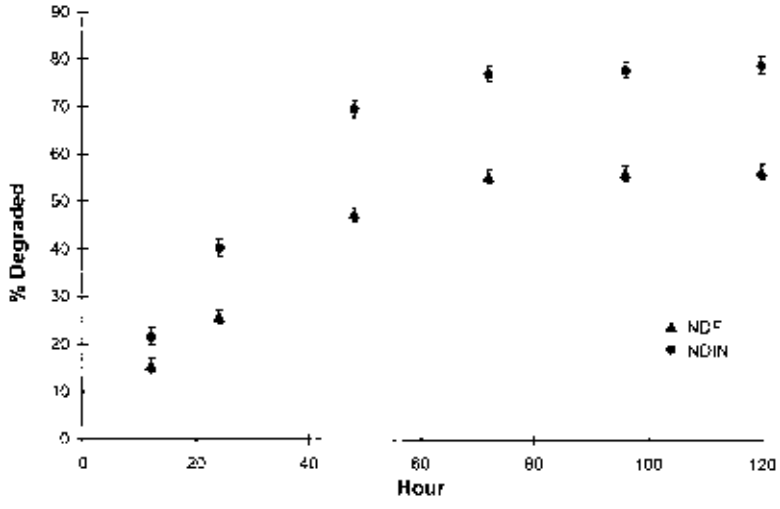

Fig. 20.4. *In vitro* degradability of neutral-detergent fibre (NDF) and neutral-detergent-insoluble N (NDIN) from red-clover silage. The rate of degradation for NDF and NDIN was 0.033 h^{-1} and 0.041 h^{-1}, respectively. The *in vitro* extent of degradation was 74%, a value that is close to the total-tract digestibility obtained in feeding trials with sheep (Yocum, 1995).

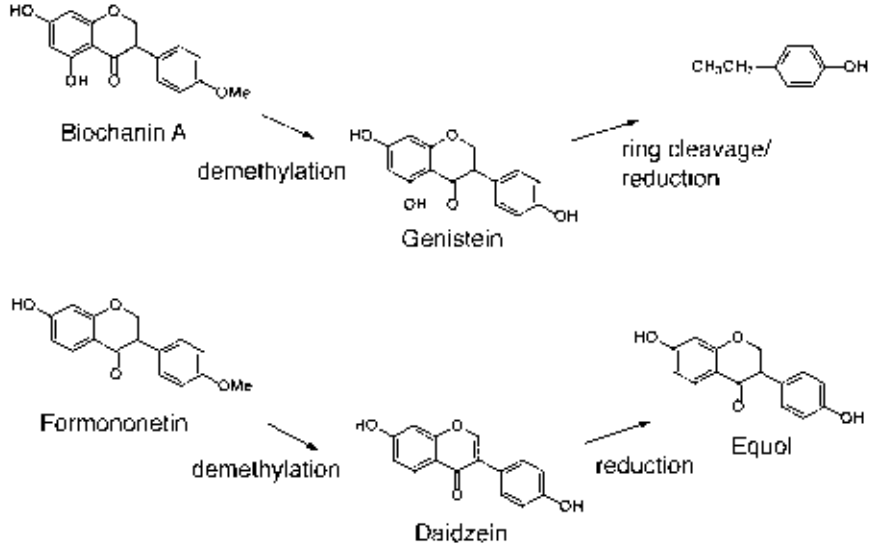

Fig. 20.5. Degradative pathway of red clover isoflavones by ruminal microbes in sheep (from Nilsson *et al.*, 1967).

We studied the effect of feeding ensiled red clover, lucerne and sainfoin to lactating ewes nursing twin lambs during January, February and March in order to determine if differences among silages in protein digestion and metabolism would have an effect on ewe milk yield, as indicated by lamb growth. Ewes were assigned to three silages: 20 ewes to lucerne, 17 ewes to red clover and 15 ewes to sainfoin. During the first 26 days, the average daily gains for lambs from ewes fed lucerne were significantly higher ($P < 0.05$) than for lambs from ewes fed red clover or sainfoin. Average daily gain from birth to 40 days and from birth to weaning were significantly higher for lambs from ewes fed sainfoin ($P < 0.05$) than for lambs from ewes fed lucerne. The higher average daily gains for the first 26 days for lambs from lucerne treatment indicates that milk yield during this period was higher for the ewes fed lucerne than for ewes fed red clover and sainfoin. When lambs consumed solid feed after 26 days, lambs from the sainfoin and red-clover treatments had a higher average daily gain than lambs from the lucerne treatment. These results suggest that the lower protein degradability of red-clover and sainfoin silages may have had positive effects on the average daily gains of lambs but not on ewe milk production. However, none of the 17 ewes from the red-clover treatment lambed when they were bred during the March breeding period. Forty-five per cent of the ewes fed lucerne and 69% of the ewes fed sainfoin lambed when they were bred during this period. The lambing percentages for ewes that were fed lucerne and sainfoin were normal for the breed type for the March breeding (Berger, 1993). These results indicate that formononetin in red clover had a negative effect on conception during the early spring breeding for accelerated lambing. The variety of red clover used in this trial is 'Marathon' and this has a high level of formononetin in comparison with varieties that have been selected for a low content (Fig. 20.6).

Although formononetin is linked to reproductive problems in sheep, there is much interest in the health-promoting effects of the isoflavones in soybeans and soya products in human diets. Soybeans contain the isoflavones genistein and daidzein, which have 0.002–0.001 of the oestrogenic activity of oestradiol (Cassidy *et al.*, 1995). High consumption of soybean products by Asians is linked to decreased incidence of breast cancer compared with Western women and decreased mortality due to prostate cancer compared with Western men (Barnes, 1995; Peterson, 1995; Stephens, 1997). Genistein inhibits the *in vitro* proliferation of tumour cells (Fostis *et al.*, 1995) and the growth of human prostate cancer cells (Peterson and Barnes, 1993).

Secondary Plant Compounds and the Evaluation of *Sesbania*

Sesbania is a large genus of over 50 species from Africa and Asia (Gillet *et al.*, 1971). *Sesbania sesban, S. goetzei* and *S. keniensis* from Africa are fast-growing and have potential for cut-and-carry forage production on resource-poor farms. *Sesbania sesban* is the most widely distributed, performs well in tropical highlands and has high potential for development as a forage (Gutteridge and Shelton, 1995). The International Livestock Research Institute (ILRI) maintains the world's largest germplasm collection of this genus and has over 100 accessions of *S. sesban* collected from East and Southern Africa.

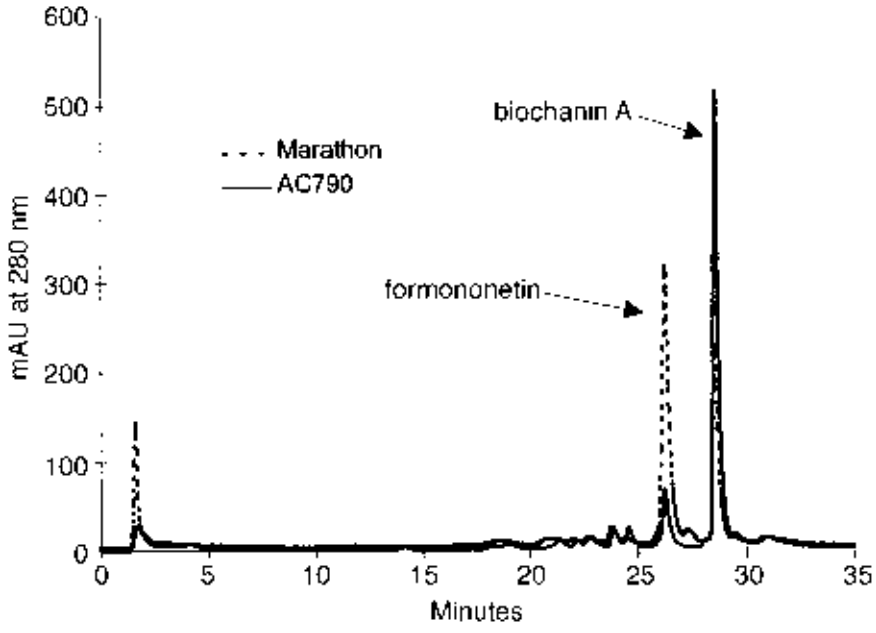

Fig. 20.6. High-performance liquid chromatography of two selections of red clover. 'Marathon' is grown in Wisconsin; AC790 is from New Zealand and has been selected for low formononetin.

However, variation among accessions of *Sesbania* in the quantity and types of secondary compounds affects its nutritive value and utilization as a forage (Tothill *et al.*, 1990; Wiegand *et al.*, 1995). In a sheep metabolism trial at ILRI, accession 15036 (cv. Mount Cotton) had the highest content of PA and the lowest protein digestibility of the three *S. sesban* accessions tested (Wiegand *et al.*, 1995). The presence of other SPC has also raised concerns about the potential toxicity of some *S. sesban* accessions (Brown *et al.*, 1987; Shqueir *et al.*, 1989a, b). The detection and analysis of SPC in the ILRI germplasm collection are required to avoid extensive agronomic evaluation of accessions that are potentially toxic.

Research on the feeding value has concentrated on both the chemical characterization of the entire collection and more detailed nutritional studies, including feeding trials, on some accessions that were identified as promising in agronomic trials. A study of SPC indicated a large amount of variation among accessions (Heering, 1995). High-performance liquid chromatography (HPLC) was used to analyse the flavonoids. A range of distinct fingerprints was found, which showed considerable variation in the content of PA and other types of flavonoids (Heering *et al.*, 1996). Sheep fed accessions that had a high content of PA had the lowest intake of cereal-crop residue and the lowest digestibility of protein, N retention and growth rate (Wiegand *et al.*, 1995). Nevertheless, sheep fed an accession with a moderate content of PA had a higher growth rate and N retention than the sheep fed on the accession with the lowest content. These results suggest that the variation in PA in *Sesbania* could be used to select accessions with a level of PA that

does not have a detrimental effect on protein digestion but does improve N metabolism and growth.

However, the results of *Sesbania* toxicity trials with day-old chicks indicate that some accessions contain a toxic SPC (Brown *et al.*, 1987; Shqueir *et al.*, 1989a, b; Reed and Aleemudin, 1995). Researchers in Kenya and Malawi have also reported sporadic toxicity in ruminants (Semenye *et al.*, 1987).

Our research indicated that accessions with a high content of saponin were the most toxic to day-old chicks (Reed and Aleemudin, 1995; Table 20.4). The major saponins in *S. sesban* are glycosides of oleanolic acid (Dorsaz *et al.*, 1988; Fig. 20.7). The biological activity and toxicity of these saponins depend on the pattern of glycosylation (Hostettmann and Marston, 1995). Saponins in *Sesbania* accessions were assayed by thin-layer chromatography (TLC). Six saponins were detected. The total number of spots and their relative areas were larger in *S. sesban* in comparison with *S. goetzii* (Table 20.5). Accessions 10865 and 15019 had the highest relative amounts of saponins and the lowest content of PA.

These results indicate that accessions with a low content of PA are the most toxic to day-old chicks. The toxicity study ranks the accessions in the inverse order to their ranking by sheep growth rate and content of PA (Table 20.6). The chick toxicity contradicts the results from feeding trials, where sheep fed acessions that were high in saponins but low in PA had the highest growth rate. There appears to be an inverse relationship between saponins and PA in *Sesbania*. Toxicity may be an effect of the saponins, which affect chicks more than growing sheep, whereas depressed growth rate in sheep is caused by poor protein digestion in accessions that contain a high level of PA. There is also the possibility that interactions between PA and saponins in accessions with higher levels of PA reduce the toxicity of saponin (Freeland *et al.*, 1985).

Although *Sesbania* saponins may be toxic to day-old chicks, there may be beneficial effects of saponins in the diet of sheep. The molluscicidal activity of the

Table 20.4. Toxicity of *S. sesban* and *S. goetzii* leaves to day-old chicks when included at 30% of the diet. *Sesbania* accessions 10865 and 15019 had the higest content of saponins and the lowest content of proanthocyanidins and were the most toxic to chicks, as indicated by the high mortality rates compared with the lucerne and maize/soybean control diets.

	Per cent mortality	
	Mean	SD
Maize/soybean	0	0
Lucerne	0	0
S. sesban		
10865	37	9.9
15019	35	7.1
15036	5	3.5
S. goetzii		
15007	0	0

SD, standard deviation.

Compound	R^1	R^2
1	β-D-glucopyranosyl	H
2	β-D-glucopyranosyl	β-D-glucopyranosyl
3	β-D-glucopyranosyl	β-D-xylopyronsyl

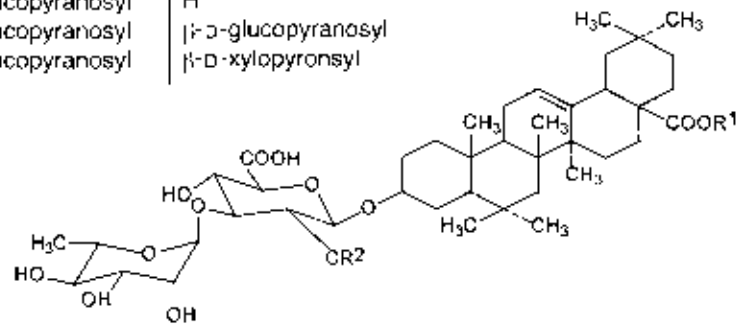

Fig. 20. 7. The oleanane saponins from *Sesbania sesban*. Haemolytic and spermatocidal activity depends on pattern of glycosylation (Dorsaz *et al.,* 1988). Monodesmosidic saponins are not glycosylated at R^1 and have greater haemolytic and molluscicidal activity and may be generated by hydrolytic or enzymatic cleavage of bidesmosidic saponins (Hostettmann and Marston, 1995).

Table 20.5. Thin-layer chromatography of saponins from *Sesbania sesban* and *S. goetzii*. Accessions 10865 and 15019 had the highest total area and area of individual saponins and were the most toxic to chicks.

	Area (mm^2)			
	S. sesban			*S. goetzii*
	10865	15019	15036	15007
TLC spot				
1	22	20	23	23
2	55	40	35	35
3	50	50	38	18
4	52	40	33	ND
5	37	55	33	ND
6	30	30	25	ND
Total area	245	235	186	76

ND, not detected.

oleanane saponins is potentially useful for treating water against the snail vector of schistosomiasis and may have anthelmintic effects. *Sesbania sesban* is used in traditional medicine for protection against mosquito bites, as a remedy for guinea-worms and as a remedy for scorpion stings (Dorsaz *et al.*, 1988). Other plants that contain glycosides of oleanolic acid have anti-inflammatory and antiviral activity (De Tommasi *et al.*, 1991; Liu, 1995).

Table 20.6. Rank, from highest to lowest, of three accessions of *Sesbania sesban* (10865, 15019 and 15036) and one accession of *S. goetzii* (15007) in parameters of nutritive value.

Accession	Sheep growth	Chick mortality	Condensed tannins	Saponins
10865	2	1	4	1
15019	1	2	3	2
15036	3	3	2	3
15007	4	4	1	4

Conclusions

The factors that determine the nutritional value of forages are complex. Laboratory analyses of forages are carried out to determine the relationship of intake and digestibility to content of nutrients (energy, protein, vitamins and minerals). However, many laboratory methods for estimating nutrient content and availability are inaccurate when applied to forages that contain SPC. There are large differences in nutritive value among genotypes of forage species, which may be related to variation in SPC. These differences in nutritive value are not easily predicted by content of nutrients (Wilson, 1977; Wilson and Harrington, 1980) or the analysis of individual SPC, such as phenolics, because plant species seldom contain a single type of SPC. This problem is often greatest in the nutritional evaluation of tropical legumes that have been selected as new potential forage species, as in the case of *S. sesban*. Some species of tropical herbaceous and woody legumes may be agronomically successful but unsuitable for feeding livestock because of their content of SPC. However, it is also apparent that SPC interfere with the nutritional evaluation and utilization of well-known temperate legumes, such as red clover. The variation in the amount and types of SPC within a forage species needs to be investigated from the standpoint of both potential toxic and antinutritional effects and potential beneficial effects.

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21 Current Procedures, Future Requirements and the Need for Standardization

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How Well Do Current Evaluation Techniques Assess Nutrients Available from Forage?

Most past and many current approaches to forage characterization are designed to meet the needs of nutrition/feeding models that essentially ask: how much energy and protein does the animal need to meet the requirements of maintenance and a given output of milk, meat, etc.? Thus, the energy value of forages is commonly estimated from the apparent digestibility of dry matter (DM) or organic matter through the entire digestive tract. This approach has led to the development of *in vitro* and near-infrared reflectance spectroscopy (NIRS) approaches, which predict *in vivo* digestibility, as described in Chapters 6, 7, 8 and 15.

Whilst these approaches have provided a valuable insight into forage quality, ruminant-animal production is facing new challenges, including the need to design diets that not only offer the maximum differential between feed costs and product sales but also reduce negative environmental impact. In addition, for high-genetic-merit dairy cows, at least, it seems essential that the dietary influence on the partition of dietary energy between body-tissue deposition and milk secretion is predicted (Agnew *et al.*, 1998). There is also an increasing need to predict the output of milk constituents (fat, protein, lactose), rather than simply energy and protein. A major consequence of these developments is the industry's desire for approaches to nutrition based on multiple responses of the animal to the nutrients supplied by the diet. Implicit in this is the need to predict voluntary feed intake, microbial transformations in the rumen and the end-products of digestion. This is dealt with in detail in Chapter 4.

For example, one problem that current approaches to herbage evaluation have failed to explain is the poorer productivity of autumn-grown compared with spring-grown grass. Early research (e.g. Corbett *et al.*, 1966) showed that this was likely to be the result of increased partition of metabolizable energy (ME) into heat production,

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although the reasons for this were unclear. More recently, MacRae *et al.* (1985) showed that, per unit of ME intake, spring grass led to increased incremental intestinal absorption of α-amino nitrogen compared with autumn grass. Recent data (van Vuuren *et al.*, 1992) suggest that most of the difference is likely to be due to increased microbial protein synthesis from the spring grass. MacRae *et al.* (1985) suggested that the additional glucogenic amino acids absorbed would provide additional nicotinamide adenine dinucleotide phosphate (NADPH) and glycerol phosphate necessary for conversion of acetate into long-chain fatty acids and thus improve the flow of energy into animal production. The development of a nutrient-based description of forages, along with suitable models of metabolism, would allow such situations to be revealed and allow corrective action to be taken. However, it is vital not only to know what the nutrient-supply potential of any forage is, but also to understand what factors are responsible for the value of the forage (e.g. molecular structure) and how it could be favourably altered (e.g. by plant breeding).

Volatile fatty acids

In most ruminant-production systems, rumen volatile fatty acids (VFA) represent some 0.6–0.7 of the carbon available for metabolism. It is clear that the various VFA produced during rumen fermentation have different metabolic properties and, for example, do not elicit the same insulin response (Trenkle, 1970). Most rationing systems have not explicitly represented VFA production, although there have been a number of attempts to use stoichiometry-based rumen mechanistic models to predict VFA production and profile (for review, see Sauvant *et al.*, 1995; see also Chapter 4). Many of these approaches have appeared to be unsatisfactory and, whilst improvements to these models are ongoing, the role of *in vitro* systems needs examination.

Early work with a simple gas-production apparatus (O'Hara and Ohki, 1973) showed a strong correlation between gas volume and total VFA *in vitro*. More recently, Blümmel and Bullerdieck (1997) proposed that gas production is, indeed, only a reflection of VFA production, while Rymer and Givens (1999) found that some of the gas-production parameters estimated from the *in vitro* fermentation of various diets based on hay plus micronized maize were closely correlated with at least some of the rumen VFA and pH values estimated *in vivo*. These findings raise the possibility that VFA measurements within *in vitro* gas-production systems may provide a reflection of the amounts and proportions of VFA *in vivo*, although more data are needed. However, this approach may only give the broad picture, since the VFA profile *in vivo* is dynamic, can change substantially after a meal and is influenced by factors such as meal frequency and rate of eating (Sutton *et al.*, 1986).

It is particularly noteworthy that, in the case of grass silage-based diets, there is considerable evidence that the proportions of fermentation acids in the silage can greatly influence the proportions of rumen VFA. In particular, there is a strong relationship between the lactic acid content of the silage and the proportion of propionate in rumen fluid in both cattle (van Vuuren *et al.*, 1995) and sheep (Martin *et al.*, 1994) and there is an effect on the lipogenic : glucogenic VFA ratio (Fig. 21.1). These relationships also appear to hold good for diets containing up to at least

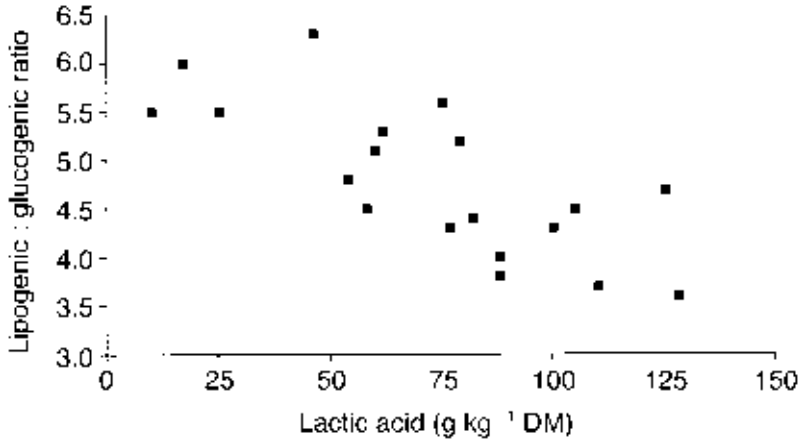

Fig. 21.1. Effect of silage lactic acid on rumen VFA lipogenic : glucogenic ratio (from van Vuuren *et al.,* 1995).

400 g kg^{-1} of concentrates, and Martin *et al.* (1994) suggested that the relationship between the lactic acid content of silage and nutrient supply is likely to be an important factor in the regulation of nutrient utilization for milk production from high-silage diets. This, in part at least, may be a result of its conversion to propionate in the rumen and the impact that the latter may have on gluconeogenesis and the insulin response.

Therefore, it seems vital that, if nutrient supply from silage-based diets is to be predicted, knowledge about the fermentation acids in the silage is required. Whilst this can readily be achieved by gas chromatography, it is slow and expensive. Recent developments in NIRS (Gordon *et al.*, 1998) which allow the obtaining and interpretation of spectral data from fresh (undried) silage have much to offer in this regard. As described in Chapter 15, NIRS can be used to predict lactic acid and VFA.

Microbial protein supply

With most forages, the rumen is the main site of digestion, with some 0.9 of total organic matter being degraded therein (Beever *et al.*, 1986; see also Chapters 2 and 4). A major function of the rumen is the degradation and subsequent fermentation of both soluble and cell-wall carbohydrates to provide energy in the form of adenosine triphosphate (ATP) for microbial maintenance and the synthesis of microbial protein. Microbial protein often provides up to about 0.7 of the absorbed α-amino nitrogen (Sauvant *et al.*, 1995) and, in the case of diets based on grazed herbage, this figure may exceed 0.9. It is now recognized that differences in the amount and proportion of amino acids and other nutrients absorbed can substantially determine the yield and composition of milk (Thomas and Martin, 1988).

It is also noteworthy that microbial growth has a specific demand for hexose in

the synthesis of amino acids and nucleic acids from ammonia (Baldwin *et al.*, 1970) and the partition of hexose between synthesis and fermentation can significantly affect the efficiency of microbial protein synthesis (Beever, 1993). Thus, probably the major priority in the development of an improved characterization of forages is a means of better assessing the quality of its complex carbohydrate make-up, how this is likely to be utilized in the rumen and hence the potential for microbial protein synthesis.

There has been considerable interest in the use of the gas-production technique (see Chapter 10) for assessing microbial protein production, both directly and indirectly. Blümmel *et al.* (1994) reported, for cereal straws, an inverse relationship (r = 0.64–0.77; $P < 0.001$) between *in vitro* microbial biomass yield, estimated as the difference between the apparent and true degraded residue, and gas production per unit of substrate truly fermented. Although Blümmel *et al.* (1994) proposed a relationship between gas production and *in vitro* microbial biomass yield, it seems unlikely that such a relationship would hold true for microbial protein yield under all rumen conditions. It may be that the combination of estimates of hexose available (e.g. by the *in situ* approach, as described in Chapter 9) and ATP production (i.e. by gas production), together with a suitable model of rumen function, would provide substantially improved estimates of microbial protein production, compared with current relationships based solely on organic matter apparently degraded in the rumen. Further research in this area is needed.

Outflow rates

All current *in situ* and *in vitro* techniques for estimating dynamic aspects of forage degradation and fermentation in the rumen use models to calculate the effective amount of a substrate degraded or fermented, which include assumptions about fractional outflow rates of small particles (see Chapter 9). In many cases, the outcome is very sensitive to the assumptions made, which are still largely those presented by the Agricultural Research Council (ARC, 1984) based on studies with chromium-mordanted protein particles. The ARC (1984) recommended that further data on the outflow of larger forage particles were required and that is still the case today. Of note is the recent finding of Romney and Gill (1998) that the short-term intake rate (STIR) of poor-quality forages at least has considerable potential to predict passage rate. If STIR relates to rumen outflow, it may provide a relatively rapid means of obtaining new data.

Impact of physical structure and sample preparation

Most *in vitro* techniques for forages rely on the use of feeds that have been previously dried and milled. This is derived from the need to use small samples that are homogeneous and representative. However, sample preparation needs considerable care, since, under some circumstances, the destruction of the physical structure can alter the kinetics of the response *in vitro* in a manner different from what occurs in the animal. For example, Sanderson *et al.* (1997) compared the gas-production data

from grass silages incubated either as freeze-dried and milled, chopped fresh or unchopped fresh and observed that the freeze-dried and milled material produced substantially higher gas production than the other treatments (Table 21.1). In the same experiment, the authors concluded that wilted silage fermented more rapidly than unwilted silage. This is an important finding which requires further research, since the rate of release of nutrients *in vivo* may also be modified by the likelihood of increased rumination time on the wetter silage.

On the other hand, Deaville and Givens (1998) argued that there may be some merit in pre-drying silages before assessment with the gas-production technique, since the drying largely removes the fermentation acids. If these remain, interpretation of the gas-production data may be flawed, since they lead to gas release from the buffer, which is not associated with fermentation and hence ATP production.

Therefore, it appears vital that *in vitro* techniques are used in a way that prevent destruction of important aspects of physical structure, since failure to account for this may give very misleading results relative to the response *in vivo*.

How Well Do Current Evaluation Techniques Predict Voluntary Intake?

Impact of physical/cellular structure

There is much literature to show that reducing the particle size of forages by a sufficient amount, e.g. by milling (not chopping), increases intake and that the effect is greater in sheep than cattle and is greater the lower the digestibility of the forage (Owen, 1979; Walker, 1984; Fahey *et al.*, 1993). Predicting the response in intake due to physical treatment *per se* has received little recent research. Interest in milling and pelleting high-temperature dried forages declined from the mid-1970s, because of the high cost of fossil fuel. However, as the increase in intake with

Table 21.1. Gas-production data from grass silages incubated either as freeze-dried and milled, chopped fresh or unchopped fresh (after Sanderson *et al.*, 1997).

Silage preparation	Gas asymptote (ml g^{-1} DM)	Lag phase (h)	Fractional rate at 50% of total gas (h^{-1})
Silage 1			
Fresh, unchopped	254[a]	1.44	0.0600[a]
Fresh, chopped to 3 mm	261[a]	1.71	0.0693[a]
Freeze-dried, milled 1 mm screen	284[b]	1.30	0.0995[b]
Silage 2			
Fresh, unchopped	285[c]	0.56	0.0571[a]
Fresh, chopped to 3 mm	303[d]	0.77	0.0670[a]
Freeze-dried, milled 1 mm screen	297[d]	0.87	0.1071[b]

Within columns, different superscripts denote significant differences ($P > 0.05$).

milling is considered to be largely due to increased rate of passage, being able to predict the latter would presumably indicate the response in intake. As noted earlier (see 'Outflow rates' above) and in Chapter 3, STIR (Romney and Gill, 1998) appears to hold promise as a measure of the physical structure of forage and its impact on intake. Future research may well show that intake of forage is better predicted by a combined measure of STIR and rate of digestion in the rumen, estimated by *in vitro* gas production (e.g. Mauricio *et al.*, 1999a). It is likely that the regression relationships between intake and a combination of rate of digestion and STIR will need to be established for forages of similar type (e.g. grasses or legumes) and animals of a given species and level of productivity. Sileshi *et al.* (1996), working in Ethiopia, showed that the correlation between parameters of *in situ* degradation and *in vitro* gas production varied with forage type.

Selectivity

Prediction of intake of non-grazed forage is normally based on *ad libitum* intake, measured indoors, with the amounts offered being restricted to 15% more than the animal will consume, in order to minimize selectivity. However, indoor studies with tropical forages (Zemmelink, 1980) and crop residues, such as barley straw (Wahed *et al.*, 1990) and stovers of sorghum (Osafo *et al.*, 1997) and maize (Methu *et al.*, 1997), show that intake increases with increasing levels offered in excess of *ad libitum*. The intake response is due to animals selecting for the more nutritious leaf and leaf-sheath fractions. Similar selection occurs with grazed forage, provided the allowance rate is sufficient (Gibb and Treacher, 1976). NIRS appears to hold promise as a method of predicting the leaf content, at least in samples of straw (Islam, 1997). A recent workshop on intake in grazing animals (Gibb, 1998) concluded that use of *n*-alkanes as markers was useful for estimating both intake and composition of grazed herbage. On the other hand, Minson and Wilson (1994) concluded that it was probably simpler and more accurate to estimate intake under grazing from equations relating intake of DM and live-weight and production level than to use an equation based on feed composition.

Interactions between forages when fed together

When different forages are fed together, the nutrients they supply often interact and modify the ensuing metabolic processes, to the extent that the digestibility and intake of the combination often differs from the average values for the component forages. Such associative effects reflect the lack of additivity between the nutrients in the forages (Sauvant and Giger, 1989), a process largely ignored by current forage evaluation systems. Interactions may be positive, negative or absent, depending on the nature, physical form and substitution rate of the components, their influence on the rumen ecosystem, the level of feeding and outflow rates and the adequacy of essential nutrients. For instance, synergism was only observed between grass and legume components of a mixed ration when dietary crude protein and mineral levels were deficient in one of the forages and adequate in the other

(Minson, 1990). Likewise, when straw or poor-to-medium-quality hay is supplemented with high-protein feeds, positive effects on intake and digestion are often observed (Butterworth and Mosi, 1986; Reid *et al.*, 1987; Bowman and Asplund, 1988). This is because the additional nitrogen enhances the utilization of the straw.

Associative effects often lead to a higher intake of mixed-forage diets than of the components fed in isolation (Phipps *et al.*, 1995). The reasons for this are unclear, though an increase in palatability upon mixing forages may be involved. Positive associative effects also result from the enhancement of rumen digestion, as seen by decreases in methane production, rumination and heat production (Van Soest, 1994). Simultaneously offered cereal and legume forages, which, respectively, have high readily fermentable energy and protein contents, may synchronously release energy and protein within the rumen and thereby enhance microbial protein production and hence animal performance. Vogel *et al.* (1989) reported that stocking densities could be doubled without adverse effects on live-weight gain in steers fed wheat or Bermuda-grass pastures supplemented with silage, because of positive associative effects between the forages.

Negative associative effects also occur frequently. A common example is when a forage with a high fermentable energy content is fed with one with a high cell-wall content. This is because the fermentation of the carbohydrates in the former produces acidic conditions, which depress the digestibility of the cell walls in the latter. This type of negative associative effect was thought to partly explain the low intraruminal digestion of whole-crop wheat in dairy cows, since the forage has high cell-wall and starch contents (Adesogan *et al.*, 1998). However, there are several accounts of mixed-forage feeding without associative effects (Milne and Bagley, 1976; Eldridge and Kat, 1980; Odoi, 1985), and more work is needed to elucidate the underlying mechanisms and digestive interactions between forages fed together.

As a result of associative effects, the nutritive value of mixed-forage diets is often over- or underestimated when estimated conventionally, because the ingredients are over- or undervalued (Van Soest, 1994). McDonald *et al.* (1995) noted that associative effects pose a serious objection to the determination of the digestibility of components of a mixed ration by difference. They also cited experiments that showed that, for certain ingredients, the efficiency of ME utilization for fattening depends on the nature of the basal ration to which it was added and concluded that values for the efficiency of ME utilization for individual foods are of limited significance. Similarly, Van Soest (1994) noted the continuing problem of oversimplification in relation to prevailing concepts of net energy (NE) values in feed tables and emphasized that it is the composition and character of the total diet that determines the animal response and therefore the NE.

Associative effects between feeds are often ignored when the nutritional potential of diets is assessed. Such interactions can be tested by feeding supplements at different ratios to a basal diet (Van Soest, 1994). Continuous analysis can be applied to evaluate the associative effects of two-component diets, provided the components are, at most, complementary (Franci and Acciaioli, 1998). For more complex diets with several components, the response surface methodology, associated with multiple regression analysis, can be employed (Franci and Acciaioli, 1998). This model was shown by Franci *et al.* (1997) to be a useful and precise means for studying the associative effects of complete diets.

Problems Specific to Silages

Volatile fermentation products in silages

One main difference between silages and the forages from which they are made is the presence of volatile constituents in the silages, which are formed from protein and carbohydrate fermentation during ensiling. Since the volatile compounds typically have higher energy contents than their precursor compounds, silages are usually higher in gross energy (GE) than the forages from which they are formed. Under ideal conditions, the GE of silages would be determined on fresh, unprocessed silage samples. However, the need for uniform samples that are representative of the silage fed necessitates some form of sample processing. Many of the processing methods currently used result in an underestimation of GE content, because they increase the losses of the energy-dense volatiles. The best method appears to be to chop the forage samples in liquid nitrogen. Porter (1992) showed that this method gave more accurate GE results than freezing and cutting with a band saw, shredding, freezing and mincing or chopping in a commercial food processor.

Losses of volatile fermentation products also occur during oven-drying of silages, leading to an underestimation of the DM content. Such underestimations are higher in wetter silages with higher volatile constituents and DM losses of up to 240 g kg^{-1} have been reported (Aerts *et al.*, 1974). If the losses are not corrected, the energy value and chemical constituents in the silage will be overestimated, while the digestibility, DM intake and feed-conversion efficiency (kg feed kg^{-1} gain) will be underestimated (Kaiser *et al.*, 1995). This has resulted in the advancement of several methods for determining the true DM content of silage. One of the most widely used is the toluene distillation technique (Dewar and McDonald, 1961), which quantifies the volatile compounds lost and then uses this information to correct the apparent DM value. However, the method does not always account for all the volatiles lost, especially in silages with high alcohol contents (Kaiser *et al.*, 1995; Porter and Barton, 1996). It is labour-intensive and hazardous and therefore not routinely practicable.

An alternative approach involves quantifying the fermentation products lost during oven-drying and using correction equations, such as that of Porter *et al.* (1984), to account for such losses. However, such equations are forage species-specific and only exist for the more common silages. Many of the equations are also flawed, because they assume that the volatility of the fermentation products is constant across several silage types. Although the development of different equations for grass silage, maize silage and lucerne by Dulphy and Demarquilly (1981) overcomes the interaction between volatility and silage type, the approach does not cover all silage types and requires separate quantification of fermentation products. Such determinations are not required in the method advocated by Givens (1986). This entails using a mean correction factor of 1.9 percentage units, derived from the analyses of 300 silage samples, to account for volatiles lost during oven-drying. While this approach involves no further analysis, it is not sufficiently accurate for some purposes.

Other methods that have been used include low-temperature (70–85°C) oven-

drying, freeze-drying, microwave-drying, saponification (Hood *et al.*, 1971), Karl Fischer titration (Galleti and Piccaglia, 1988), gas chromatography and NIRS. Kaiser *et al.* (1995) compared all but the last two of these methods for analysing the DM content of a diverse set of grass silages. They found that only the saponification and Karl Fischer methods appeared to account for all of the volatiles present. They also found that the saponification technique required more time and gave higher standard errors and therefore concluded that the Karl Fischer approach was best for routinely analysing the DM of silage, because it was rapid, accurate and simple. Porter and Barton (1996) also found more accurate DM determinations with the Porter *et al.* (1984) correction equation, gas chromatography or Karl Fischer titration than with the alcohol-corrected toluene distillation and low-temperature oven-drying techniques. However, gas chromatography requires specialist equipment, which may not be available in many laboratories. The availability of odourless, low-health-risk, stable end-point, long shelf-life, pyridine-free reagents overcomes some of the traditional problems of the Karl Fischer titration. However, anhydrous methanol and exclusion of atmospheric moisture are still required and the Dulphy and Demarquilly (1981) correction equation gives comparable results (Galleti and Piccaglia, 1988).

NIRS scanning of undried forages can also provide accurate determinations of silage DM content. Although the cost of purchasing and calibrating the NIRS machine are substantial, once these are done, the technique can be conveniently used for routine DM determinations. Kaiser *et al.* (1995) suggested that NIRS machines should be calibrated with data derived from Karl Fischer titrations.

In conclusion, it is essential to account for volatiles lost during oven-drying of silage. The widely used toluene distillation technique does not account for all the volatiles lost during oven-drying, even when corrected for volatile losses. Therefore, the Karl Fischer titration, gas chromatography, NIRS or corrected oven DM method should be used when accurate DM determinations are paramount. Future work should develop NIRS calibration equations for grass silages and oven DM correction equations for the less common silages.

Volatile compounds can also be rapidly lost from exposed silo faces, leading to differences between the nutritive value of the silage sampled at silo opening and that fed later. Such losses are minimized when good silo management is practised. Nevertheless, future work should examine the extent to which such losses reduce the nutritive value of silages.

High concentration of soluble nitrogen

Soluble nitrogenous compounds in silages include short-chain peptides, ammonia, non-volatile amines and amino acids. In addition to nitrate, these compounds make up the non-protein nitrogen (NPN) fraction, which is about 200–300 g kg^{-1} total nitrogen (N) in fresh forages and 600–700 g kg^{-1} total N in silages (Van Soest, 1994). This difference arises from the proteolytic degradation, which increases NPN at the expense of true protein during ensiling. Deamination of the resulting amino acids leads to increases in concentrations of ammonia, diamines and isoacids (Van Soest, 1994).

Soluble nitrogen is potentially available for ruminal degradation, but its nutritive value is not usually realized, because it is readily converted into ammonia, absorbed and excreted as urea. Some of the soluble N escapes ruminal degradation and is available for peptic digestion in the abomasum. The value of such escape soluble N depends on its amino acid profile, while the extent of rumen escape is affected by the enzyme-to-substrate ratio, the outflow rate and the presence of a readily fermentable energy supply. Brookes and McGuire (1973) found that, in spite of being completely digestible, soluble N was poorly utilized in sheep diets unless the energy-to-protein ratio was improved.

Several authors have found that animal performance improved as nitrogen solubility in the diet decreased. Table 21.2 summarizes some of these findings and reveals the importance of measuring N solubility, so as to regulate its inclusion in ruminant diets. However, there is no standard method for measuring N solubility in silages. Some of the traditional methods include solubility in detergents, cold or hot water, saliva-simulating buffers and salt solutions. The water and buffer solubility methods do not always account for all the soluble true protein and do not differentiate adequately between NPN and true protein in the soluble fraction (Van Soest, 1994). Trichloroacetic acid and tungstic acid methods are superior in these respects.

The UK metabolizable-protein system (AFRC, 1993) determines the quickly degradable protein (QDP) fraction in feeds from the immediately soluble N frac-

Table 21.2. Effect of N solubility (N sol) level in the diet on animal performance.

Author	Feed	Species	Observation
Majdoub *et al.* (1978)	Sorghum silage and concentrates	Dairy cows	Low N sol improved milk yields
Dingley *et al.* (1975)	Maize silage and concentrates	Dairy cows	Low N sol decreased essential and total amino acid supply to the udder
Zook (1982)	Maize silage, lucerne hay and concentrates	Dairy cows	Low N sol improved milk yield and milk fat and protein contents
Beever *et al.* (1976)	Grass	Sheep	Low N sol diets improved efficiency of ruminal VFA synthesis
Mader and Britton (1986)	Lucerne	Steers	Better utilization of lucerne hay than lucerne silage
Knowlton *et al.* (1992)	Lucerne silage and concentrates	Dairy cows	Low N sol diets improved the yield of bacteria per unit silage DM and the metabolizable protein per unit of silage crude protein

tion, which is determined as the N lost when feeds in polyester bags are washed with cold water. This method overestimates true N solubility, due to particulate losses through the pores of the bags, which may include degraded, insoluble N. Such losses can be accounted for by using correction equations (Dhanoa, 1988; Lopez *et al.*, 1994), providing the rate of degradation of the particulate matter lost is similar to that of the material remaining in the bag. Although it is more accurate to determine N solubility by cold-water extraction of feeds suspended in filter-paper (Cockburn *et al.*, 1993), such extractions do not completely account for the solubilization of true protein (Pichard and Van Soest, 1977).

The Agricultural and Food Research Council (AFRC, 1993) assumes that the QDP fraction is utilized in the rumen with an efficiency of 0.8 and recommends a maximum QDP inclusion level of 0.4 of the effective rumen-degradable protein to prevent ammonia toxicity. Although this is a useful guide for preventing excess intake of soluble N, the model is premised on an assumed efficiency of utilization and overestimates the soluble N content of rations, because of the insoluble, readily degradable proteins in the QDP fraction.

The model of Pichard and Van Soest (1977) overcomes some of the shortcomings of the UK metabolizable-protein system by using different solvents to distinguish the water-soluble NPN fraction, containing primarily nitrate, ammonia (NH_3) and free amino acids, from three water-insoluble true-protein fractions that differ in their degradability. This model can improve the accuracy of ration formulation and enhance the efficiency of protein utilization. However, in order to use this model, further work is required to define optimum levels of inclusion of soluble true protein and soluble NPN in livestock diets. Additionally, there is a need for formulae that can be used for estimating the economic and nutritional value of protein supplements from solubility estimates.

Since silage forms the basal diet of most ruminants in the UK, improving the utilization of silage N by reducing silage intake is not always feasible. Alternative methods that have been examined include thermal treatment (Murphy and McNiven, 1994), dehydration (Beever *et al.*, 1976), pelleting (Alloui *et al.*, 1994), wilting (Kowalski *et al.*, 1993) and treatment with iron alum (Antoniewicz, 1993), formic acid, formalin (Beever *et al.*, 1976) and condensed tannins (Ulyatt *et al.*, 1980). Effective methods of minimizing the soluble N content of silages include reducing N fertilization at pasture and employing management practices that ensure good-quality fermentation.

Prediction of silage intake by using near-infrared reflectance spectroscopy

As discussed in Chapter 3 and earlier in this chapter, many approaches have been examined for predicting the voluntary intake of forages. Prediction equations have poor performance when applied to ensiled forages. It was proposed some time ago (AFRC, 1991) that NIRS could be one of the most promising techniques for predicting the voluntary intake of grass silage, since the spectra contain information on the entire chemistry of the feed. In fact, NIRS has been successfully used to predict the intake of mainly non-fermented forages. This is shown by the work of Norris *et al.* (1976) and the more recent studies of Ward *et al.* (1982) and Flinn *et al.* (1992).

However, it has also been shown recently (Steen *et al.*, 1995) that NIRS can accurately predict the voluntary intake of grass silage by cattle. Steen *et al.* (1995) measured the voluntary intakes of 136 grass silages, using 192 individually fed beef cattle, and compared NIRS with some 30 other laboratory measurements on the silage for their ability to predict voluntary intake. The best overall relationship was between NIRS spectra and intake, with R^2 values of 0.86 and 0.71 for calibration and blind validation, respectively, and a standard error of prediction of 5.5 g DM kg^{-1} live-weight $(LW)^{0.75}$ daily. Of the other measurements, the next best relationships with intake were obtained with electrometric titration (R^2 0.53), digestibility *in vivo* (R^2 0.30) and DM degradability (R^2 0.28).

The fact that NIRS seems to offer considerable opportunities for predicting intake suggests that such studies should be extended to include the impact of feeding concentrates and interactions between forages fed together.

Applicability of Techniques

Appropriateness and cost

The discussion in previous chapters has centred on reviewing the literature on given techniques. Whilst there is a need to standardize techniques (see below), there is also a need to consider the appropriateness of techniques for given circumstances. Choice of technique will be a function of many factors. For example, in developing-country laboratories, often the only technique available for a given analysis is one donated by a developed country or development agency. Unfortunately, such donations are often inappropriate, because they do not sufficiently consider local needs and circumstances. Techniques often require chemicals and backup services not available locally, which have to be imported at large expense.

Increasingly, in developed countries, there is concern to devise non-invasive techniques, because of public aversion to vivisection. Thus, for evaluating the kinetics of organic-matter degradation in the rumen, the *in situ* technique (Chapter 9) is likely to be superseded by *in vitro* gas-production methods (Chapter 10). The *in situ* technique has been much promoted and used in developing-country laboratories, because of its low cost, robustness and lack of dependence on a reliable electricity supply. However, animal-welfare issues apart, the *in situ* technique is only able to accommodate a few samples at a given time and is therefore inappropriate when large numbers of samples have to be screened, e.g. as in forage breeding.

The question of cost and numbers also applies to gas-production methods. Automated systems (Chapter 11) are attractive, in that they obviate the need to use labour for night-time recording, but are expensive to purchase and are only able to evaluate a few samples at a given time (e.g. a 12-unit Cone *et al.* (1996) system costs around £10,000). On the other hand, Mauricio *et al.* (1999a) have recently developed a semi-automated version (Reading pressure technique (RPT)) of the manual technique of Theodorou *et al.* (1994). RPT has a capacity to evaluate concurrently 70 forages in triplicate, and its capital cost is low (less than £1000). Importantly, Mauricio *et al.* (1999b) have also shown faeces to have potential for

replacing rumen liquor as an inoculum in RPT. Use of faeces in the Tilley and Terry (1963) *in vitro* digestion technique (e.g. Akhter *et al.*, 1999) was discussed in Chapter 7. Use of faeces instead of rumen liquor has much potential application, particularly in developing countries, but its use requires much more research and development.

Increasingly in the future, choice of analysis and technique will be influenced by the use to be made of the information generated. Thus issues such as accuracy, speed and cost of analysis will receive different emphases, depending on whether the customer is a researcher, commercial operator (e.g. feedstuff manufacturer), extensionist or farmer. As mentioned previously, the location will also affect the choice. In developing countries, extensionists are being encouraged to make greater use of simple assessments of nutritive value such as leaf-to-stem ratio, colour and smell (Thorne, 1998). NIRS combines many advantages of accuracy, speed and low recurrent costs and will be used increasingly in developed countries. However, use of NIRS in developing countries will increase only when there is a major reduction in capital cost.

Need for standardization of techniques

Although forage characterization is most accurate when conducted *in vivo*, public concerns and high costs associated with such procedures have necessitated the development of *in vitro* techniques. Such methods are constantly being modified, adapted and ultimately replaced by faster, cheaper or more accurate methods by the commercial firms, consulting analytical chemists and official advisory services that use them. Thus a plethora of methods is currently available for analysing the chemical constituents in forages. Nevertheless, outdated, inaccurate methods are still being used to predict nutritive value. For instance, modified acid-detergent fibre (MADF) is currently used in some laboratories for predicting the ME value of grass silages. Yet the error associated with the prediction is much higher (standard error of prediction = 1.30; r^2 = 0.14 (Barber *et al.*, 1990)) than that recommended as the maximum permissible (0.5 MJ kg^{-1} corrected DM) to prevent inaccurate ME predictions and attendant wastes in feed outlay or lost production (Offer, 1993). Also, equations that are accurate for predicting nutritive value in certain feeds are often applied, without validation, to other feeds that were not represented in the population for which the equation was developed. The inaccuracy of this approach has been widely demonstrated in temperate (McLeod and Minson, 1972; Barber *et al.*, 1989; Givens *et al.*, 1995; Adesogan *et al.*, 1998) and tropical (Acamovic *et al.*, 1997) forages.

An added complication is the intra- and interlaboratory variation in expertise and analytical procedures used, such as sample-preparation methods, reagents, equipment, computation of results and technique. This can lead to considerably different results for the same sample. Givens *et al.* (1990) found substantial differences between the organic-matter digestibility of straws measured at different laboratories. After sending maize silage samples to nine different laboratories, Beever *et al.* (1996) concluded that the interlaboratory variation, especially in starch, crude-protein and NH_3-N content, was too great to be acceptable for use in

farming practice. Considerable interlaboratory or between-method differences have also been shown in the estimation of ME (Van der Meer, 1983; AFRC, 1993), *in situ* rumen degradability (Huntington and Givens, 1995), gas-production kinetics (Rymer and Givens, 1997), *in vitro* digestibility (Aufrère and Michalet-Doreau, 1988; Narasimhalu, 1985), GE (Porter, 1992), neutral-detergent fibre and ether extract (Lanari *et al.*, 1991). In addition, it is disconcerting that some techniques said to have the least predictive ability in some reports were found to have the highest in others. For instance, De Boever *et al.* (1994) reported that rumen-fluid digestibility techniques were more accurate than cellulase-based methods for estimating *in vivo* digestibility, whereas contrary results were shown by Aufrère and Michalet-Doreau (1988).

These factors emphasize the need for standardization of forage characterization procedures. Within laboratories, there is an ongoing need to ensure that experiments are precise, reproducible and repeatable. This may be effected by internal and external calibration (Minson, 1998). Internal calibration involves using results of standard samples analysed alongside test samples, to correct for week-to-week deviations in accuracy, while external calibration involves comparing test results with those determined *in vivo* (Minson, 1998).

In addition to the intralaboratory standardization, it is imperative that interlaboratory standardization procedures are developed to facilitate comparison of results from different centres. National and European Union (EU)-wide ring tests have already shown that, when the same procedures are employed, similar estimates of starch (McClear *et al.*, 1994) and mycotoxins (Schuhmacher *et al.*, 1997) can be achieved in different laboratories. There is a need to involve more laboratories in such ring tests and ultimately to ensure that accurate, standardized procedures are used throughout the feed industry. This may be achieved by the introduction of an accreditation scheme, whereby results from laboratories will only be considered acceptable if the laboratory is accredited for the test in question. Unless such a scheme is introduced, laboratories using the less accurate techniques may continue generating misleading results, and the validity of comparing analytical results from laboratories employing different techniques will remain questionable.

Health and safety issues related to techniques

The formal consideration of health and safety aspects related to laboratory techniques is relatively new. However, it is now a matter given increasing attention by laboratory managers and institutions in developed countries, if only because of fear of litigation. In the UK, in 1989, the government introduced legislation to protect health and safety of people at work, known as the Control of Substances Hazardous to Health (COSHH) (Simpson and Simpson, 1991). The COSHH requirements cover prevention or control of exposure to hazardous substances, use of control measures, maintenance of control measures, monitoring of control measures, health surveys and information and training. Employers have a duty under the law to ensure, so far as is reasonably practicable, the health, safety and welfare of their employees at work. The regulations require a COSHH risk assessment to be undertaken on each substance used in a given technique. The assessment includes the

occupational exposure limits of the substance, whether it is corrosive, an irritant, a carcinogen, a mutagen, a teratogen, a neurotoxin, an allergen or sensitizer and whether it affects the central nervous system. Furthermore, the assessment identifies precautions required for handling the substance, control measures to be used (special ones or 'good laboratory practices'), first-aid procedures, spillage response and action in case of fire. There is an increasing volume of literature on health and safety issues in laboratories (Bretherick, 1986; Web addresses). Health and safety aspects of forage evaluation techniques will need to be considered increasingly in the future, particularly in developing-country laboratories, where this aspect has tended to be neglected to date.

Role of Forages in Animal and Human Health

The effect of toxigenic fungi in forages on human and animal health and performance

There is little published information on the role of forage moulds and their associated mycotoxins in animal and human health. This is partly because the delay between mould contamination and disease incidence makes it difficult to inculpate mycotoxins in the aetiology of animal or human diseases. Although some forage mycotoxins have been implicated in the development of livestock diseases, toxigenic fungi are not perceived as a major threat to animal production in the developed world. Yet they adversely affect the efficiency and profitability of livestock production through subclinical toxicoses, such as immunosuppression, reduced feed intake and decreased feed-conversion efficiency. Moulds also reduce the nutritional value of forages by degrading water-soluble carbohydrates, lactic acid, cellulose and other cell-wall fractions (Oldenburg, 1991). In addition to the threat from mycotoxin contamination, the inhalation of fungal propagules can lead to kidney damage, respiratory-tract problems and irritation of the eye and skin (Scudamore and Livesey, 1998).

Mycotoxin-synthesizing moulds have been isolated from grass, grass silage, maize silage, whole-crop wheat straw and hay samples. The major toxigenic fungi in forages belong to the *Fusarium*, *Penicillium* or *Aspergillus* species. *Fusarium* species are field moulds that produce toxins such as trichothecenes, fumonisins and zearalenone. The moulds are commonly found in grazed or harvested forages and could also be endemic in inadequately dried (> 150 g moisture kg^{-1}) hay and straw bales, where the aerobic conditions enhance their stability. *Fusarium* species pose a lower threat in silages, because the fungi and most of their associated mycotoxins are degraded during anaerobic ensiling (Scudamore and Livesey, 1998). A notable exception is zearalenone, which has been shown to persist in maize silage after 12 weeks of ensilage (Oldenburg, 1991). Although zearalenone toxicity has been linked to infertility, reduced milk production and hyperoestrogenism in ruminants and pigs (Cheeke, 1995; D'Mello and MacDonald, 1996), it is not a major problem in the UK (Scudamore and Livesey, 1998).

Penicillium and *Aspergillus* species are storage moulds that produce ochratoxin and aflatoxin, respectively. Ochratoxin A causes kidney damage in pigs, but ruminants are

less susceptible. Ochratoxin A also has carcinogenic, teratogenic, nephrotoxic and immunosuppressive properties (Rosenthaler *et al.*, 1984; Oldenburg, 1991) and is transmissible from grains to edible animal products (D'Mello and MacDonald, 1996).

Aflatoxin is produced from *Aspergillus* species, which thrive between 25 and 30°C (D'Mello and MacDonald, 1996). Hence aflatoxin contamination is mainly associated with tropical feed ingredients, such as groundnut cake and cottonseed cake, which are banned for use as livestock feed ingredients in the UK. Previous surveys have not found aflatoxin to be a major contaminant of forage in the UK (Scudamore and Livesey, 1998). However, formic acid treatment of forage increases the risk of *Aspergillus* infestation and aflatoxin production. In Sweden, an increased incidence of aflatoxin M1 (the metabolic derivative of aflatoxin B1) in milk produced on farms using formic acid culminated in a ban on formic acid treatment of moist grain (Peterson *et al.*, 1989). Nevertheless, formic acid-treated silages may be safe for animals, since aflatoxin B1 degrades during ensiling (Scudamore and Livesey, 1998). Ruminants are less susceptible to aflatoxicosis than pigs, but clinical signs of the acute condition have been observed in cattle (Diekman and Green, 1992). Chronic exposure to aflatoxin lowers feed intake and milk production. This may be due to the fact that, in the rumen, aflatoxin decreases cellulose digestion, VFA production, proteolysis and motility (Scudamore and Livesey, 1998).

Mycotoxin production and mould growth in forages are inhibited by ensuring that the following conditions are not exceeded during storage: temperature 2.2°C, relative humidity 70%, moisture content of grains 150 g moisture kg^{-1} and oxygen 5g kg^{-1} (Diekman and Green, 1992). Mycotoxin activity in grains has also been inhibited by treatment with NH_3 and aluminosilicates (Diekman and Green, 1992). Previous work has also shown that increasing the protein or amino acid content of the diet beyond normal levels could alleviate poor performance in pigs that were fed on mycotoxin-contaminated grain. However, the foregoing relates to inhibition of grain mycotoxins. Further work is required to ascertain the effectiveness of these techniques in forage mycotoxins.

Although the disease risk from toxigenic fungi is relatively small, their role in animal and human health requires further study, because of the reduction in animal performance due to subclinical toxicoses and the possibility of transfer of carcinogenic metabolites from animal tissues to humans. Future studies are required to identify forage mycotoxins that produce residues of toxic metabolites in edible animal products. This requires the development of rapid detection techniques, as the methods currently used for grain mycotoxins (mass spectrometry and thin-layer, gas and high-performance chromatography) have not been fully tested and validated on forage mycotoxins (Scudamore and Livesey, 1998). More studies like those being undertaken at Aberystwyth (A.T. Adesogan, 1999, unpublished) are required to ascertain the effect of mycotoxins on digestive processes in livestock and the extent to which the mycotoxins are degraded during the digestion process. Coker (1999), in reviewing mycotoxins, calls for much further research, particularly to develop rapid and inexpensive mycotoxin analysis methods, which can be used in unsophisticated laboratories, e.g. in developing countries.

In conclusion, although toxigenic fungi have not posed a major threat to livestock production in the UK in the recent past, more attention should be focused on them because of the increasing use of alternatives to grass silage for winter feeding

of livestock. Forages such as maize silage, whole-crop wheat, baled-grass silages and legume silages have higher DM contents than clamped-grass silage. They are therefore more susceptible to microfloral contamination and aerobic spoilage.

Forages as sources of drugs

The possible beneficial role of secondary plant compounds was alluded to in Chapter 20. Butter *et al.* (1998), at Nottingham University, reported dietary quebracho tannin and/or high protein being effective in reducing parasite eggs in lambs infected with *Trichostrongylus colubriformis*. The same group is currently investigating the effectiveness of tanniniferous forages in Tanzania for reducing parasitic infection in goats. In view of the complexity of tannin chemistry and the large variation within and between species, there will be a need to develop methods for screening forages for their beneficial or antinutritional secondary plant properties.

Because of the high cost of veterinary drugs in developing countries, there is growing interest in the use of ethnoveterinary medicine based on local plants (ITDG and IIRR, 1996). Current research at the Institute of Grassland and Environmental Research, Aberystwyth, in partnership with Xenova Discovery Limited, is characterizing natural products from temperate forages and plants which may have beneficial drug properties (Nash, 1998). In the same programme, the use of modern analytical techniques, such as gas chromatography and high-performance liquid chromatography coupled to mass spectrometry (GC-MS and HPLC-MS), is enabling the active ingredients in plants used in folk medicines and toxic plants to be identified.

Forages as sources of n-*3 fatty acids and antioxidants*

Whilst, traditionally, forages have been regarded primarily as sources of energy and protein, the data reviewed in Chapter 19 clearly show that, under certain circumstances, forages can be an important source of antioxidants, which may ultimately make a useful contribution to the human diet. Also in relation to the human diet, there is increasing evidence that an increased intake of *n*-3 fatty acids is desirable in relation to cardiovascular health (see review by Givens *et al.*, 2000). The most important *n*-3 fatty acids are the long-chain polyunsaturates eicosapentaenoic acid (EPA, C20:5 *n*-3) and docosahexaenoic acid (DHA, C22:6 *n*-3), but there is evidence (Scollan *et al.*, 1997) that increased amounts of these acids can occur in the meat and milk of ruminants fed diets enhanced with the shorter-chain linolenic acid (C18:3 *n*-3). Although the total lipid concentration in the DM of most forages is relatively low, because they are often consumed in large quantities they can make a sizeable contribution to total lipid consumed.

In this context, Dewhurst (1997) confirmed earlier reports that lipids in grasses are a rich source of linolenic acid. However, Dewhurst (1997) also showed that this was influenced markedly by grass genotype (Table 21.3), plant maturity and methods of conservation. These results highlight the very important role grass-based forages could play in introducing increased amounts of *n*-3 fatty acids into the human

Table 21.3. The major fatty acids in various species of grasses (after Dewhurst, 1997).

Fatty acid* (g kg^{-1} DM)	*Lolium perenne*	*Lolium multiflorum*	*Lolium* hybrids	*Lolium*–fescue hybrids	Fescue	Cocksfoot	Timothy	Effect of species
Total fatty acids	22.5	20.8	23.2	23.0	21.9	19.1	21.8	$P < 0.001$
C16:0	4.7	4.5	4.9	4.6	4.4	4.0	4.1	$P < 0.001$
C18:2 (*n*-6)	3.0	2.7	3.2	3.1	2.6	2.9	3.6	$P < 0.001$
C18:3 (*n*-3)	11.6	10.5	11.9	12.3	12.1	10.3	11.3	$P < 0.05$

* Mean of three sampling dates.

diet. It also highlights the future need to regard forages as more than simply sources of energy and protein for the animal. Much more work in this area is needed.

Environmental factors

The need to minimize environmental pollution from livestock production systems will mean that future evaluation of forages must consider aspects such as methane emission (Moss and Givens, 1997) and efficiency of nitrogen (e.g. Castillo *et al.*, 1999) and phosphorus (e.g. Shah, 1999) utilization when ingested by ruminants.

Conclusions

The results presented and reviewed in all chapters of this book highlight not only the immense importance of forages worldwide as part of the diet of ruminants but also that forages can no longer be regarded as simply sources of energy and protein. They must be characterized in terms of the nutrients they supply to the animal and, more particularly, to the rumen microbes. Their wider potential in relation to their important role as indirect suppliers of important biologically active compounds to the human diet needs considerable further exploitation and development. All of these factors highlight the challenge that faces those developing means of characterizing forages. It also seriously calls into question the value of traditional books and databases on the nutritive value of forages, not only because of the inherent variability of forages but also because the data they contain will rapidly become less relevant to the needs of the industry.

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