Chapter 9

Biological and chemical analytical methods

This chapter describes methods of analysis that are appropriate for characterizing nutritional attributes of feeds. Two approaches are used: one is biological and the other is chemical. Biological methods have advantages where laboratory facilities are minimal since they require little more than a weigh scale and a drying oven and materials that can almost always be acquired in village and city markets.

The chemical methods that are described are those considered to be most relevant in the light of developing knowledge on the characterization of tropical feed resources. They relate closely to the criteria discussed in chapters 3 and 5 concerning the nutritional principles underlying utilization of tropical feed resources by monogastric and ruminant livestock.

The section on the in sacco *nylon bag method was contributed by E.R. Ørskov; that on the* in vitro *gas production by Kamal Khazaal; and the one on purine analysis by X.B. Chen, all of the Rowett Research Institute in Scotland. M. Rosales and Chris Wood, of the Natural Resources Institute, Chatham, UK described the modification to the gas production technique based on the original proposal by Theororou* et al. *1994. M. Rosales also contributed the section on tannins and described the quantitative methods for identification of a range of secondary plant compounds in plants.*

INTRODUCTION

It is not intended to provide a comprehensive description of all analytical methods used in animal nutrition research. The aim is to identify those procedures considered to be more applicable and critical to the characterization of feed resources for incorporation into livestock feeding systems in developing countries. Emphasis is given to methods which

are least demanding in terms of sophisticated facilities and equipment.

The measurements are the minimum needed to enable researchers to acquire the essential information for them to set up meaningful feeding trials. Observing and measuring animal response to dietary manipulation on the available feed resources are essential first steps in the development of feeding systems for application on farms. This is the correct order of priorities for allocation of resources aimed at development of animal feeding systems. Too often the research is "bogged down" in the laboratory without excursion into the field, which is a necessary prelude to any study of farmers' problems and finding possible solutions that might fit into existing farming systems (see Chapter 11).

The approach is aimed at resource persons working in national institutions and in non-governmental organizations (NGOs) but the methods are also applicable to international research centres. Obviously there is a special role for the latter and they require many more tools in their research. Their task must be to examine, in depth, the problems that arise in the field and which are generated by the pragmatic "local" approach that is advocated. Such centres should support national institutions, and NGOs, and be engaged in the more sophisticated basic studies that such research requires. The proposed research methods relate closely to the guidelines for utilization of feed resources set out in Chapters 4 and 6.

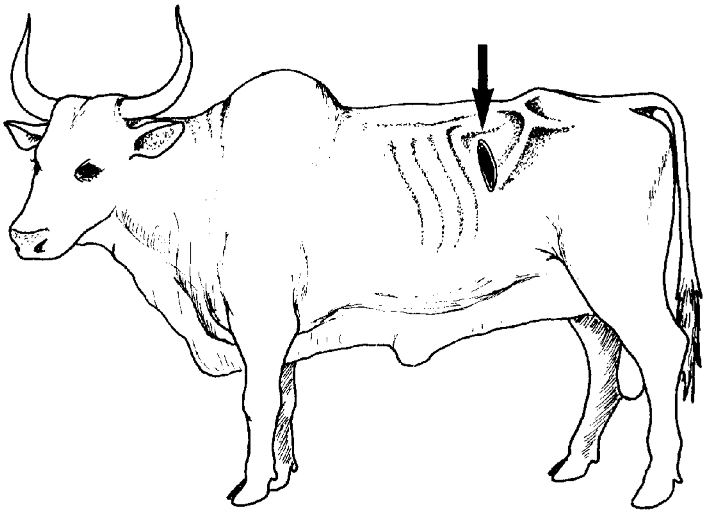
BIOLOGICAL METHODS

Rumen fistulation

Animals with rumen cannulae have proved to be one of the most useful tools for evaluation of feed resources and especially for determining the effect of a feed on the rumen environment, which has a major influence on the processes of digestion. The technique can be criticized on the grounds that it is an interference with the normal functioning of the animal and that if not carefully maintained the fistula and the cannula can be a source of stress. *In vitro* methods of gas production promise to be an appropriate alternative for measurement of the fermentation potential of a feed and of the relative effect of anti-nutritional compounds present in many tropical feed resources.

For the moment, animals with rumen cannulae will continue to be required as they represent one of the most useful tools available to researchers in developing countries. However, they are not indispensable, and alternatives approaches should be developed and used wherever possible.

Figure 9.1. Illustration of rumen fistula produced by the one-step method of Schalk and Amadon.



Two procedures for cannulation have been used by researchers. In 1928, Schalk and Amadon described a one-stage surgical technique. A two-step method was developed later by Jarrett (1948), mainly for use with sheep. Both methods have been used but, for unknown reasons, the Schalk and Amadon method seems to have been neglected except in Australia (Hecker, 1974). The surgery associated with establishment of rumen fistulas by the two-stage operation requires considerable skill, is laborious and can be stressful to the animal. The method is extremely difficult to carry out in laboratories that do not have the necessary facilities (e.g., an operating table). Furthermore, it was presumed that

such surgery was the domain of the trained veterinarian. This resulted in "a mental block" for many young scientists, particularly those in developing countries. One result of this has been an undue emphasis on feed analyses as a means of predicting nutritive value of feeds, to the neglect of studies on the live animal. The most appropriate method for establishing rumen fistulas, especially in laboratories with limited surgical facilities, is the one-step Schalk and Amadon procedure.

In the last 10 years in Australia, this technique has been considerably simplified allowing untrained but aware scientists to establish fistulas with a minimum of stress to the animal. For example, in a course at ILCA, in Ethiopia for young African scientists from many backgrounds (all with the mental block concerning surgery), each was able to establish (under guidance) a rumen fistula in either cattle or sheep. The animals that were surgically modified were in the preliminary phase of a feeding trial. Their feed intake was monitored both before and after surgery. The animal ate less on the day of the operation but quickly regained its appetite. With the two stage surgical method, animals go off their feed often for several days.

Principle of the method

A metal clamp is applied to a fold of the rumen wall exposed by an incision into the body cavity. The clamp holds the fold of rumen wall outside the body, occludes the blood supply and causes the damaged area below the fold to adhere to the body wall. In ten to fourteen days, the clamped piece of rumen 'sloughs off' leaving a fistula through which a cannula can be readily introduced and secured.

Facilities and equipment

Only minimum facilities are needed; a simple crush or some method of restraining the animal in a standing position (cattle only), a minimum of surgical equipment (scalpel, forceps, etc.), a tranquillizer and local anesthetic. The clamp consists of two brass rods 11 cm long and 0.6 cm in diameter (for sheep) and about twice this size for cattle (Figure 9.3). Each brass rod has two holes 2.5 cm from either end of the rods. The holes in one rod are threaded to take a brass screw which is fitted through a hole in the other rod so that, when the screws are turned, the two rods draw together forming a clamp.

Figure 9.2. (1) incision with rumen fold pulled through (2) placing wooden clamp on rumen fold (3) inserting sutures along clamped area, and (4) tying sutures (After Johnson, 1969).

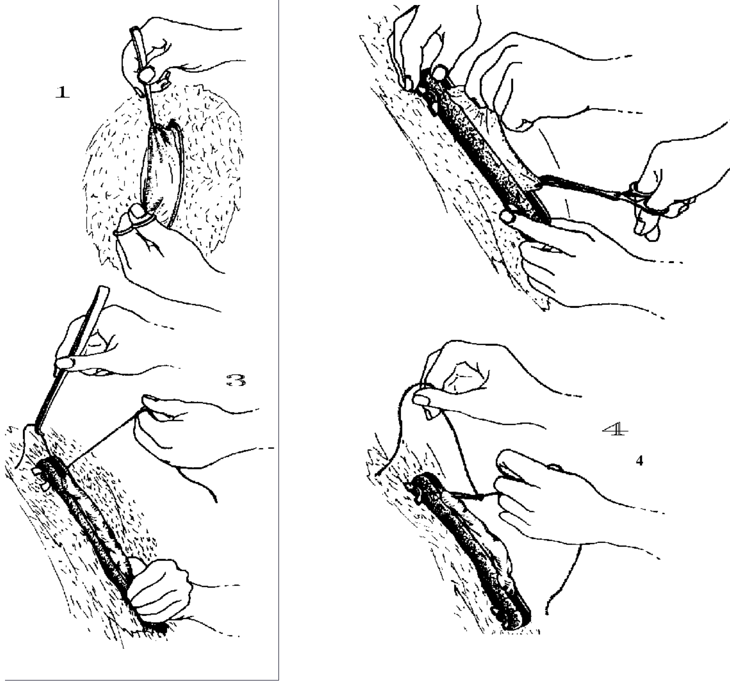
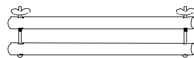


Figure 9.3. Illustration of the two bars that form the clamp used to hold the exteriorized rumen wall.



Preparation of the animal

It is not necessary to starve animals prior to surgery; in fact it is desirable to have the rumen relatively full. A tranquillizer given prior to the actual surgery is an advantage in the case of cattle.

The animals should be accustomed to handling by attendants and should be docile and easily led. The surgery is carried out with the fed animal standing in a crush or even restrained in the corner of a yard by a moveable gate. The animal is tranquilized by intramuscular injection (e.g., with Rompun) but this is not absolutely necessary. A 350 kg cow requires about 0.5 ml of Rompun to be sufficiently sedated.

Approximately 15 minutes after the injection of the tranquillizer, surgery may commence. The area of incision should be closely clipped or shaved and cleansed with a mixture of alcohol or alcohol and iodine. The incision should be made high on the left side in the anterior dorsal abdomen. The site of the incision is identified by marking a triangle from the point of connection of the last rib with the spine and moving the same distance along the spine from the last rib. The area between the last rib and where it connects with the spine should be sufficiently large to take the external flange of the cannula. In general the closer to the spine on the flank the incision is made the better; but only experience will allow accurate placing and estimation of size of the incision.

Before starting such operations cannulae of different sizes (5-12 cm internal diameter) should be on hand. Analgesia of the incision area can be produced by paravertebral anaesthesia; however, this requires experience and skill. A more practical approach is to inject a local anaesthetic in a series of subcutaneous and intramuscular injections immediately above and along the site of the incision. Approximately 25 ml of Zylocain is injected into a steer of 250 kg and 15 ml into a sheep.

The surgery

Once the site has been cleaned and disinfected, and the local anaesthetic injected, an incision about 5 cm for sheep and about 10 cm for cattle is made in the ventro-cordal direction through the skin, following the line identified previously.

In the original description of this method the underlying abdominal muscles and peritoneum are separated by blunt dissection to form an

opening in the abdominal wall. This requires considerable physical strength with large animals such as buffaloes and the bold use of the scalpel to cut to the peritoneum is less traumatic to the animal. On reaching the peritoneum, this is cut and the rumen wall which lies immediately below is drawn to the exterior to form a fold and held with two "Alice" forceps. The brass clamp is applied and the screws tightened (Figure 9.2).

Sutures should be placed through the skin and under the clamp and are tied to the clamp at both ends. These sutures hold the clamp to the skin and also prevent accidents which can occur if the rods catch on the sides of the pen. Stitching the skin is one of the most difficult aspects of the operation, particularly with buffaloes, and a sharp cutting needle is needed. In ten to fourteen days the rumen fold held by the clamps will slough off and can be removed quite easily. A flexible rubber cannula or rigid cannula prepared as described below is inserted and clamped into position.

Manufacture of rumen cannulas from locally available materials

The use of the one-step fistulation technique means that animals (cattle, buffalo, sheep or goats) can be prepared for use in almost any research laboratory. The lack of availability of manufactured cannulas has often been the reason for not preparing fistulated animals. It is relatively simple to devise rumen cannulae for both cattle and sheep. The method described below is taken, in part, from a paper by Rowe (1979).

Available materials

In most countries PVC conduit is available with diameters from 13 to 300 mm and with a wall thickness of 3-5 mm. In the Dominican Republic, PVC tubing has been used for cannulae which were placed in the fistula of cattle which were subsequently under experimentation for more than two years without apparent problems. Rubber tubing is also available in most countries (e.g., car radiator manifolds) and has been used to prepare cannulae. Car tyres or the protective band from inner tubes usually provide a suitable rigidity for retaining flanges for the cannula.

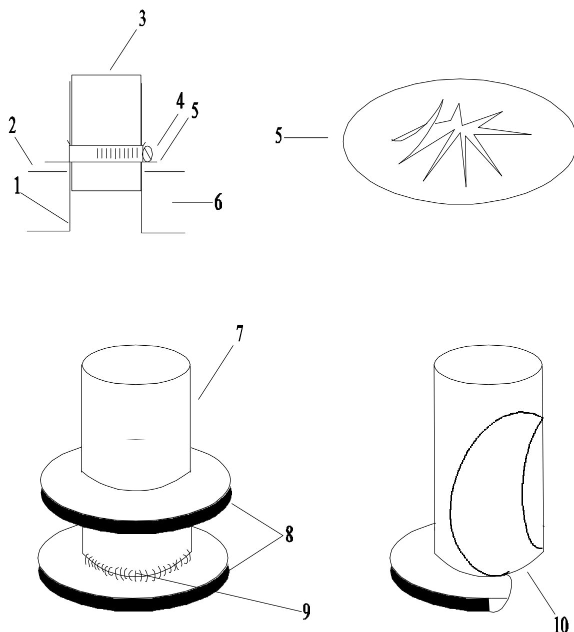
Construction of cannulae from radiator tubing

Flexible rubber cannulae are preferred since these can be easily

compressed to introduce them into the oval fistula that results from the method of cannulation.

For sheep, the components of the cannula can be constructed from a section of radiator hose and a round flat piece of rubber cut from a truck tyre. These two parts may be sewn together with nylon thread (as shown in Figure 9.4). Insertion of this cannula into sheep is facilitated by twisting a section of the retaining flange into the tube (see Figure 9.4). When the cannula is in position the retaining flange may be pushed out of the tube of the cannula to allow it to assume its normal shape but inside the rumen.

Figure 9.4: Rumen cannula for cattle made from rubber components (Source: Rowe, 1979). [1. Main body of cannula, tube and internal flange;2. External flange 3. Plastic bottle; 4. Hose clamp; 5. Clamped retainer flange made from inner tube 6. Rumen wall, muscle and skin; 7. Tube of cannula - radiator hose; 8. Two flanges - inner tube protector from truck tyre assembly; 9. Continuous stitching with nylon string].

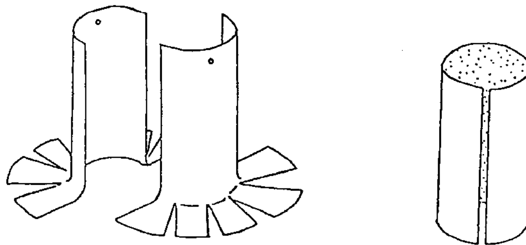


The cannula is held in position with a second rubber retaining flange and this is secured against the body of the sheep as shown. The retaining flange is kept in position by a radiator hose clamp. A suitable stopper for the cannula can be a plastic bottle inverted and inserted with the open end downwards into the tube of the cannula. This is extremely light and causes no problems to the animal.

PVC cannulae

The design of the PVC cannula is shown in Figures 9.5 and 9.6. The PVC tube is prepared with a flange by making cuts of up to 5 cm (for sheep) and 15 cm in length (for cattle) at four intervals around the circumference of the tube. When this is heated uniformly with a gas jet, the plastic becomes pliable and the flanges can be bent outwards at a right angle to the main tube. The flanges can be filed so that there are no rough edges and enclosed in rubber tubing.

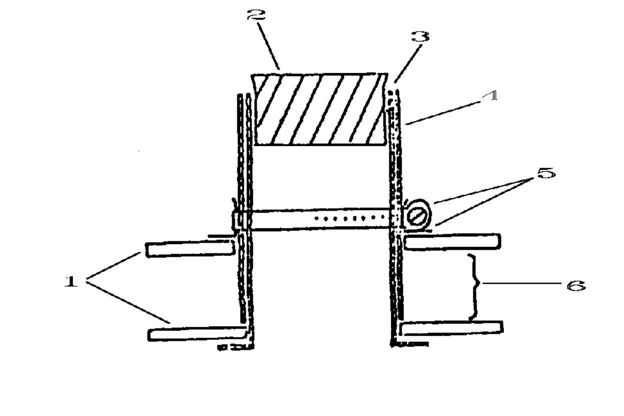
Figure 9.5. Diagram showing the construction of a rumen cannula from PVC tubes and rubber flanges (Rowe 1979).



The inner split-tube made from PVC showing the flange (bent out. The outer split-tube made from PVC after heating the material), and the two holes for securing the string tube of the same diameter as the internal tube.

To facilitate placing the cannula in the fistula, it is cut longitudinally in half and a small hole made in each half at the upper end of the flange to attach a length of string. The retaining flanges and the clamping arrangements are prepared as described for rubber cannulae.

Figure 9.6. Cross-sectional view of PVC cannula. 1. Internal and external flanges (see component 2 of Figure 9.5). 2. Rubber stopper. 3. Inner split-tube of cannula (see Figure 9.5). 4. Outer split-tube of cannula (see Figure 9.5). 5. Clamping assembly. 6. Rumen wall, muscle and skin.



To hold the two halves of the cannula together, an outer split tube is prepared from the same diameter PVC tube but with only a single cut. The cannula is inserted by first putting the two halves (attached to a length of string) into the rumen. The internal retaining flange is then passed around the string and into the rumen, before pulling the two halves together and positioning them in the fistula. The surface of the tube must be thoroughly dried before applying PVC cement and placing the outer split tube in position. The application of PVC cement is not necessary if the tube is clamped close to both the entry of the cannula into the rumen and at the top adjacent to the stopper. A lightweight plastic bottle makes the best seal for the entrance to the cannula. Any size cannula can be made in this way.

Collection of ruminal fluid by oesophageal tube

For sheep a plastic tube of 10 mm internal diameter and some 90 cm long is suitable. The rumen sampling tube should be moistened and the sheep's mouth opened by placing a thumb in the region without teeth. The tube is then passed over the back of the tongue and into the oesophagus. Test for its presence in the rumen by checking for the smell of rumen fluid, and the lack of respiratory air movements along the tube.

A vacuum pump is used to apply suction to draw the rumen liquid into the sampling bottle.

With cattle, a larger tube is required (15 mm internal diameter and 150 cm long). The rumen fluid can be obtained by lowering the animal's head until fluid runs from the tube. Move the tube in and out a few centimeters in taking the samples. Filter rumen fluid through gauze. Note that samples obtained in this way may be contaminated with variable amounts of saliva.

Rumen incubations with nylon bags

This method is given first priority for researchers in developing countries, as the most appropriate tool for providing information on:

- The nutritive value of a feed for ruminants;
- The efficiency of the rumen ecosystem.

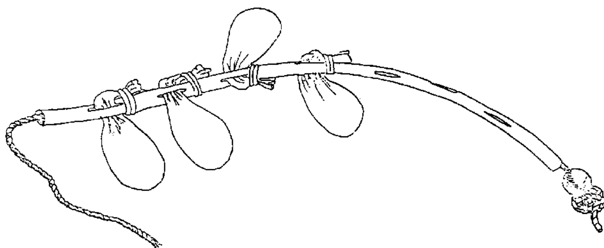
It generates useful information from the point of view of both the carbohydrate and protein status of a feed; and the degree to which it will be digested in the rumen or escape to the intestines. The method is described in detail for this reason.

Characteristics of the bag

The bags should be prepared from a nylon or other synthetic fibre material with a pore size of between 20 and 40 microns. The pore size is a compromise between minimal loss of small particles and making sure that microbes, including protozoa, can enter the bags uninhibited; and also that gas can escape from the bags. When gas does not escape the bags may float on top of the solid phase of the rumen and give very variable results.

The bags should be sewn with polyester or nylon thread with double seam and close stitching. Overall dimensions for cutting out should be 17 x 10 cm to give an effective length of about 12-14 cm. Smaller bags can be used if samples are smaller. It is not necessary to introduce a draw-string in the neck of the bag, as they can be closed with a separate length of nylon thread (e.g., fishing line), and/or attached to a long nylon string (e.g., baler twine) or a plastic rod (Figure 9.7). The bags can be reused as long as there are no holes in them; each time they should be checked for breakages.

Figure 9.7: Illustration of plastic tube and attachments of nylon bags for suspension in the rumen.



Sample size

The sample size has to be adapted to the size of the bag. With the size of bag suggested, samples of between 3 and 5 g of DM are appropriate. For smaller bags, the quantity should be less, but with a minimum of 2 g. To avoid forming micro-environments in the bag the material has to be able to move freely within the bags. If larger samples are needed for analysis, larger bags must be used.

Preparation of samples for incubation

The preparation of samples must, as far as possible, represent materials as they would appear in the rumen after they have been consumed naturally by the animal. It is recommended that the materials are processed through a hammer mill with a screen size of 2.5 mm; the same screen size can also be used for forages and cereals. For green and succulent materials and silage, a mincer with a 5 mm screen is more appropriate. If the apparatus for grinding materials is not available the sample can be broken down by pounding in the case of dry materials, or by chopping finely with a knife for succulent feeds. It is important to specify exactly what was done in the preparation process.

Position of bags in the rumen

If sheep are used, a 25 cm nylon cord is normally used to attach the bags to the cannula cap. The size of the animals might be considered. In many countries where the sheep and goats are small, it is probably better to use cattle. In cattle, depending on their size, the nylon cord should be about

40 cm. This allows the bag to move freely within the digesta, both in the liquid and solid phases. It is not usually necessary to anchor the string of bags with a weight. Inserting a glass marble or a brass weight in each bag sometimes helps to ensure that each bag is kept well within the digesta. Another method is to fix the bags to a nylon tube (Figure 9.7). This latter system simplifies withdrawal of the bags since bags with individual cords can become tangled and difficult to withdraw from the rumen.

Incubation times of bags in the rumen

Selection of the most appropriate times to withdraw bags from the rumen depends on the shape of the curve of degradation with time. It is not possible, therefore, to give absolute recommendations. Having tested one material, the test may have to be repeated with slightly different incubation times. It is important to describe the most sensitive part of the degradation curve and also the asymptote. For straw and other fibrous materials, incubation intervals of 12, 24, 48 and 72 hr are usually suitable. For protein meals shorter incubation times should be used (e.g., 2, 6, 12, 24 and 36 hr).

Replication of measurements

The important source of variation is between animals. There is little to be gained by repeating treatments within the rumens of the same animals. The number of animals needed will depend on the expected magnitude of the differences between treatments. To measure degradabilities of proteins, at least three animals are needed per treatment while, to test the effect of chemical treatment of straw, two replicates (animals) will probably suffice.

Use of sheep or cattle

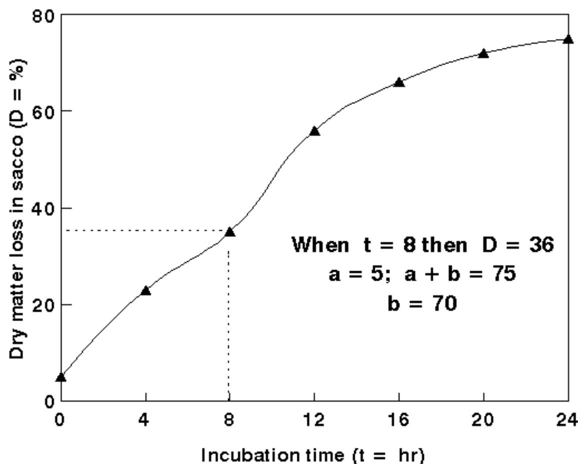
If the available sheep weigh less than 40 or 50 kg, it is probably better to use cattle. Cattle are much easier to work with than very small sheep and goats; moreover, it is often convenient to be able to insert a hand directly through the cannula into the rumen so as to introduce, and later extract, the bags more easily.

Interpretation
Irrespective of whether the results are going to be used for estimation of degradability of protein or dry matter, the most appropriate method of describing the results is in the form of an equation (Ørskov and McDonald, 1979). The expression: $p = a + b(1 - e^{-ct})$ is the most appropriate equation.

In this equation, p is the degradation which has taken place; a is the intercept; b is the amount which in time t will be degraded c is the degradation rate constant and e is the natural logarithm. If computers or scientific calculators are not available, the equation can be derived by eye.

The procedure is to fit the curve to the measurements obtained in Figure 9.8. It can be seen that the intercept a is 5; the asymptote is 75 (i.e., a + b = 75) which means b is 70 (i.e., 75-5). Taking a value on the curve where degradation is occurring most rapidly (e.g., t=8) the D = 36.

Figure 9.8. Estimating degradabilities of feeds by the nylon bag (*in sacco*) method: calculation of degradation rate (c) from disappearance curve fitted by eye.



It is now possible to describe the equation as:

$$e^{-ct} = (a+b-D)/b$$

which means that

$$e^{-c\,t} = (5+70-36)/70$$
$$e^{-ct} = 0.557$$

By taking the natural logarithm on both sides of the equation it is found that c = 0.086. All the constants in the equation are now known and they will be found to agree closely with those obtained more accurately with the computer. A computer program on diskette (NEWAY) produced at the Rowett Research Institute is available on request.

Effect of outflow rate

Protein-rich meals, derived from oilseed cakes and by-products from cereal processing and animal slaughter, contain quite a high proportion of small particles which can escape easily from the rumen. The effective rate of degradation of the protein will then depend on the solubility (a), the rate at which the b fraction is degraded (c) and the outflow rate from which k which can be measured by mordanting the protein supplement with chromium. The expression which combines these three factors is:

$$P = a + bc/(c + k)$$

where a, b and c are from the equation describing degradability and k is the outflow rate per hr.

Characterizing the rumen ecosystem

The second major use for the nylon bag technique is to measure the adequacy of a diet for a particular purpose. Under these conditions a standard material is put in the bags and the rumen ecosystem varied by supplementation or other means. For example, if the objective is to assess the adequacy of the rumen ecosystem to digest cell wall carbohydrate, then a fibrous substrate with a relatively high potential fermentability (e.g., soya bean hulls) is put in the bags (see Chapter 5 for the application of this method). The effect of supplementing the basal diet (e.g., with urea or highly digestible forage) can be investigated by measuring the relative loss of the soya bean hulls during a 48 hr incubation period.

The other important feature of the rumen ecosystem is the extent to which it permits dietary protein to escape to the intestines. To assess this effect the test protein meals are incubated in nylon bags in the rumen of animals subjected to manipulation of the basal diet by, for example, adding urea or molasses.

Evaluation of roughages

In the case of roughages there is often a lag phase, that is a period in which there is no net disappearance of substrate. This is in part due to the microbial invasion of new substrate (e.g., the cell walls). The consequence for the equation:

$$p = a+b(1-e^{-ct})$$

is that by extrapolation the a value can be negative or very small and does not indicate solubility as for instance with protein supplements. In this case, it is useful to determine the water solubility in the laboratory by standard procedures or, more simply, the dry matter loss incurred when the substrate is washed in the nylon bag without rumen incubation using the same procedures as those adopted for washing the nylon bags after incubation. This can be done by two methods: either rinsing under a tap until the water is clear or in a washing machine with a 15 minute rinsing cycle.

In this case, the roughages are best described by: (i) the determined solubility denoted as A; (ii) the insoluble but fermentable fraction denoted as B which is now (a+b)-A; and (iii) the rate constant C. These three parameters have been shown to describe better the degradability character-istics. They can also be used in multiple regression analyses to predict feed intake, or at least the relative feed intake that can be achieved by similar animals. This index of feed "potential" (Ørskov and Ryle ,1992) is only applicable however to feeds that are relatively well balanced for other nutrients (especially fermentable nitrogen). For example, it gives erroneous results with tropical feed resources such as banana pseudo stems and sugar cane. For both these feeds, the stem is more degradable than the leaf but intakes are higher with the leaves, which are better balanced with other nutrients (Montpellier and Preston, 1977; Ffoulkes and Preston, 1978).

Limitations to the method

One of the great advantages of the *in sacco* method is that, unlike *in vitro* methods, it is not dependent on a constant supply of electricity. If need be, the samples can even be sun-dried. There are however feeds which are not suitable to be evaluated by this procedure. For instance, highly soluble feeds such as molasses cannot be assessed in this way.

Some feeds have a very small particle size, such as blood-meal and single cell proteins. The particles of these feeds will pass through the pores of the nylon bags and invalidate the estimate.

It is also appropriate for measuring the presence and effects of antinutritive factors in the feeds which affect rumen microbes. This is because of the vast amount of rumen inoculum in the rumen relative to the small amount of substrate in the nylon bags. The presence of any antimicrobial factors in the feed are swamped by the large inoculum. In this case the *in vitro* gas production test (see later) is much more suitable as the inoculum relative to substrate is far greater. The effect of anti-microbial substances in feeds can be measured by using such feeds as the basal diet or supplement of the host animal and putting in the bags a standard substrate (see Chapter 9).

The *in sacco* method is also unsuitable for measurements of protein degradability in roughages as the microbial N adhering to the particles often exceeds the N in the feed sample.

The use of rumen ammonia concentration to determine when urea supplementation is necessary

The level of rumen ammonia is critical for efficient microbial fermentation of feed (Chapter 5). Rumen ammonia concentration can therefore be used to diagnose a deficiency of fermentable N in a diet. This will indicate when urea supplements are required. The critical ammonia level in the rumen for efficient microbial growth on different substrates is likely to vary according to the fermentability of the substrate.

As a rule of thumb, rumen ammonia nitrogen should be at least 15-20 mg/100ml rumen liquor (see Chapter 5). Where rumen ammonia is to be used as a diagnostic tool then the times of sampling of rumen fluid are critical. It is necessary to synchronize the availability of ammonia with the fermentation of the carbohydrate. The ammonia level at 4 to 6 hours post feeding or following the commencement of grazing is the recommended time for sampling. Ammonia concentrations in rumen fluid must be above the critical level for prolonged periods on fibrous diets which are only slowly digested in the rumen. For this reason the concentration immediately before feeding may also be an index of the need to supplement.

Assay for by-pass protein in a supplement

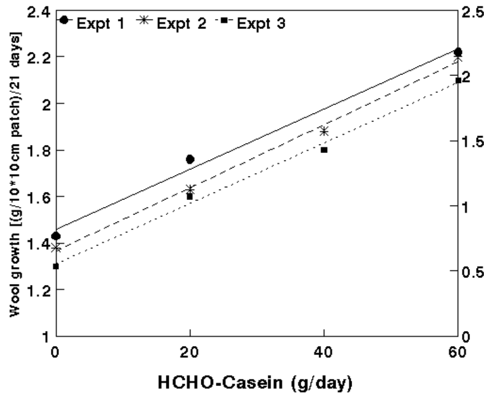
Wool growth in sheep is highly dependent on the quality of amino acids absorbed from the intestines, in particular the sulphur amino acids. However, these amino acids are not absorbed other than from the protein (dietary and microbial origin) digested in the intestines. Increased wool growth rate in response to ingestion of a protein supplement is directly related with the content of by-pass protein in the supplement.

Procedure

Mixed sex, cross-bred wool sheep (the original work was done with 1-year-old Merino x Border Leicester sheep) are housed in individual pens and given *ad libitum* a basal ration of low-nitrogen roughage (e.g., rice straw) plus a mineral mixture and 1% urea (to ensure adequate fermentable-N in the rumen). The sheep are randomized to the treatments (protein-containing supplements) with 10 animals per treatment. In the validation of the method, these were 60g/d untreated casein and 0, 20, 40, and 60 g/d of formaldehyde-treated casein (HCHO-casein) prepared as described later (formaldehyde treatment leads to almost complete protection of the casein from degradation in the rumen). Wool growth was estimated by clipping and weighing the wool from a 10 cm square patch on the flank of the sheep every three weeks (Leng *et al.*, 1984).

Preliminary studies indicated that the carryover effects of diet on wool growth were reduced to insignificant levels after 3 weeks. Trials are therefore carried out for a six week period and only the wool growth in the final three weeks is measured and related to the amount of protein in the supplement. In subsequent studies, the sheep are re-randomized before being allocated to treatments. The data in Figure 9.9 show the results obtained with different levels of formaldehyde-treated casein.The amounts of wool clipped from the patch were linearly related with the level of HCHO-casein added to the basal diet. When soluble casein was added to the diet, wool growth rate was only slightly increased over the control animals indicating that this protein had no by-pass characteristics.

Figure 9.9. Wool growth in sheep given a standard basal diet (oat hay and urea) and supplemented with different amounts of casein protected with form-aldehyde. The three experiments were run consecutively. Wool growth was measured during the last 21 days on a 10*10cm patch (Source: Leng *et al.,* 1984).



In subsequent experiments, wool weight from the clipped patch in sheep fed 100 g/d protein meal (containing say 40 g protein) was related to the wool grown when HCHO-casein was fed.

Selected results from the use of this assay to evaluate a number of plant protein sources are given in Table 9.1. The wool growth represents the level of by-pass protein relative to formaldehyde-casein. Meals that had received most heat treatment gave the highest wool growth and were therefore the best sources of by-pass protein. Sunflower seed meal was a poor source of by-pass protein, especially when the oil had been extracted by the expeller system. The better by-pass characteristics of the protein in meals produced by the solvent extraction process are because these meals are usually "toasted" at 120°C after the oil is extracted. Similar temperatures may be reached in the expeller process but the results are more variable, as the temperature is produced by friction in the press and this varies with the kind of oilseed being processed.

Feeding trials ranked the protein meals in the same order as indicated by the wool growth assay (Leng R A, unpublished data). In this case the criteria were feed intake and liveweight gain, both of which are good

indicators of the by-pass protein status of a supplement when added to a low-protein diet.

Table 9.1. Wool growth in sheep given a basal diet of oat hay supplemented with different sources of protein. The feeding trial lasted 42 days and wool growth (on a 10cm square patch) was measured over the last 21 days. Results are expressed as wool growth (g/100g N) (Leng *et al.* 1984).

	Relative wool growth
Casein	0.3
Sunflower meal	3.3
Rapeseed meal	3.9
Extracted soya bean meal	4.5
Fish meal	7.5
Cottonseed meal	7.2
HCHO casein	10.0
Linseed meal	10.6

Preparation of formaldehyde-treated casein as a standard for the wool growth assay

- Place 5.08 kg of casein into a food mixer (a cement mixer is normally used).
- Put 140 ml of formalin (47% formaldehyde) and 240 ml water into a beaker using a measuring cylinder and transfer to a pump fitted with a fine spray.
- Cover with a sheet of plastic the opening in the mixer containing solid casein. The plastic cover has a hole to take the nozzle and add the formaldehyde whilst mixing.
- Put the formaldehyde-casein in plastic bags for a week prior to feeding.

Biological test of protein quality in non-conventional sources of protein (leaves multi-purpose trees and water plants)

It has been shown in preliminary experiments (Vargas, J.E. and Sarria, P., 1994, unpublished data) that it is feasible to detect differences in growth rate in male chicks over the period 7 to 13 days of age caused by addition of leaf meals (20% level) to a commercial concentrate diet. As the concentrate contains cereal grain (unbalanced protein) and a varied (and unknown) array of nutrients from unknown sources, the test has been modified to use a protein-free energy source (raw sugar) and a

known source of protein (soya bean meal). In this way, the relationship between chick growth and the protein in the test foliages should be strengthened.

In the test, soya bean meal is used as a standard and the experimental treatments are graded rates of replacement of the soya bean meal with the test foliage. Fibre levels are kept constant by using soya bean hulls. The experiment is done at medium and low overall protein levels so as to identify "additive" effects (or otherwise) of the test foliage. An example of the approach is given below:

Constant protein level (20%); foliage replacing soybean

Raw sugar	34	34	34	34	34
Soya bean hulls	22	16.5	11	5.5	0
Palm oil	2	2	2	2	2
Foliage (20% protein) (leaf meal)	0	10	20	30	40
Soya bean meal	40	36	32	28	24
Mineral/vitamin mix	2	1.5	1	0.5	0

Varying protein level with soya bean levels as above

Raw sugar	34	34	34	34	34
Soya bean hulls	22	26	30	34	38
Palm oil	2	2	2	2	2
Soya bean meal	40	36	32	28	24
Mineral/vitamin mix	2	2	2	2	2

Groups of 3 chicks (male rejects of dual purpose laying strains) are allocated to each of three replicates of each dietary treatment. The trial lasts 14 days, starting at 7 days after hatching: the first seven for adaptation to the experimental diets and the last seven for measurements of weight gain and feed intake. Feed intake is recorded daily. Liveweights are taken at the beginning and end of the 7-day experimental period. The effect of treatment is assessed by regressing body weight gain on level of test foliage (or of soya bean meal in the case of the standard diets without the foliage). The aim of running the standard diets (without test foliage) at the same time is to separate the effects due to protein level and to secondary compounds in the foliages.

Biological test of soil fertility

Feed resources are generated from the land and specifically from soil and water. To maintain the fertility of soil and, even better, to enrich this basic natural resource, is one of the most important indicators of sustainability. This parameter is obviously a complex one and will depend on many factors both chemical and physical. To analyze the individual components is costly and time-consuming and is not technically nor economically feasible in the majority of tropical developing countries. There are therefore good reasons for developing a simple method which can be performed with minimum inputs and which gives a broad assessment of the capacity of the soil from a given cropping system to support the subsequent growth of another crop.

This issue was addressed in an experiment carried out by CIPAV in Colombia in April-May 1994 (Gomez, M.E., 1994).

About 10 kg of soil (cores taken to a depth of 30cm) taken from the test plot are air-dried and placed in 0.5 litre plastic bags arranged in blocks in the open. Samples are replicated three times in each of four blocks in a random design.

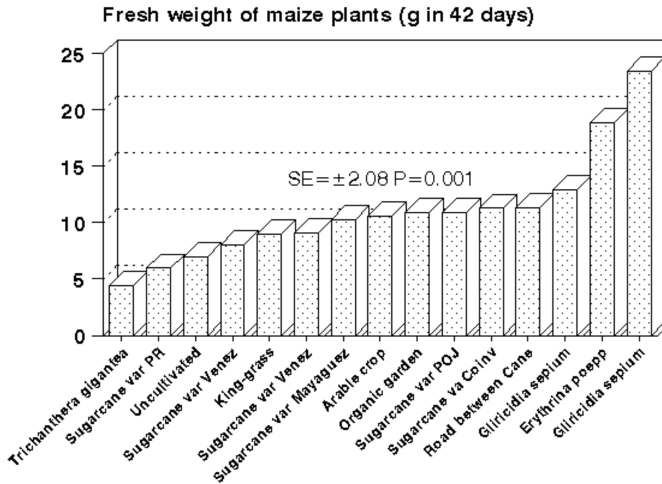
One maize seed was placed in each bag and the biomass harvested 42 days later for fresh and dry weight determination. Adequate moisture was ensured by frequent irrigation. No plant nutrients were used.

The results obtained from a comparison of different cropping systems being evaluated in the Instituto Mayor Campesino, Buga, Valle, Colombia, are shown in Figure 9.10. Details of the cropping systems (from left to right in the figure) are given in the footnote.

Two points can be made. The first is the relatively good repeatability of the test (SE of mean = ±2.06). The second is the positive effects on soil fertility of the legume trees, *Gliricidia sepium* and *Erythrina poeppigiana*.

It would be useful to include a "standard" soil type in future tests so as to have a reference point that would facilitate comparisons among sites within and across countries. This could be based on sand with three levels of, say, excreta from laying hens or from cattle.

Figure 9.10. Relative growth rates of maize plants grown in soils taken from a variety of cropping systems producing livestock feed (Source: Gomez, M.E., 1994).



CHEMICAL ANALYSES

Estimation of rumen ammonia concentration - field method

The concentration of ammonia in the rumen is one of the most important factors that determine the rate and efficiency of digestion of fibrous feeds (Chapter 5). There are also recent reports that rumen protozoa populations are reduced when rumen ammonia levels are high (Leng R.A., 1994, personal communication).

There are two methods of measuring rumen ammonia which are relatively simple, one of which can be used under field conditions (i.e., the use of an indophenol-dye to produce a colour reaction with ammonia). This method is used by extension officers in Queensland, Australia to predict when urea supplementation through drinking water is likely to be effective in increasing productivity of grazing ruminants (McMeniman, 1981). The method, as modified by Leng (Leng R.A., 1985, unpublished data), is as follows:

Rumen ammonia kit
Collection tube
Beaker
1 litre 0.2 hydrochloric acid
Muslin
200 ml sodium salicylate reagent
200 ml dichloroisocyanuric acid reagent (DIC)
Ammonia standards 0, 2.5, 5.0, 7.5, 10.0 mg NH_3-N/100 ml (These will have already been diluted with HCl).
Test tubes
Test tube rack
Syringes

Reagents
- Solution A (Salicylate): Dissolve 85 g of Na and 100 mg of Na nitroprusside in a litre of distilled water.
- Solution B (DIC): Dissolve 5 g of sodium dichloroisocyanurate in a litre of 0.3M NaOH containing 5% commercial bleach (50 ml commercial bleach and 12 g NaOH in one litre of water).
- Solution C (Stock solution): Weigh 3.28 g NH_4Cl (equivalent to 100 mg NH_3-N/100 mg) and dissolve in one litre 0.1M HCl (in distilled water).

Chemical principles
Ammonia reacts with free chlorine to form chloramine which then condenses with two phenol molecules to form an indophenol dye (strongly reducing compounds in rumen fluid are oxidized by the hypochlorite). Since excessive amounts of both salicylate and hypochlorate are present, the amount of dye produced depends on the amount of ammonia present. The relatively high concentration of salicylate was chosen to "swamp" the effect of any phenolic compounds which may be present in the rumen fluid.

Obtaining a sample
- Using an oesophageal tube (see above), obtain 20-30 ml of liquor from the sheep.
- Strain the liquor through four layers of muslin into the beaker.

Analyzing the sample

- Using a syringe or pipette, measure 0.2 ml of the range of standards into labelled tubes, starting with the lowest concentrations and taking care to blow out any fluid left in the syringe between standards.
- Using a plastic syringe for rumen fluid, measure 0.2 ml of strained rumen fluid into a second row of tubes already containing 0.2 ml of 0.1M HCl; blow out the syringe between samples.
- Add 1 ml of the salicylate solution to each tube using a 5 ml syringe.
- Add 1 ml of DIC solution to each tube using a 5 ml syringe; cap and shake to mix.
- Allow 5 minutes for colour development, then add 0.2 ml rumen fluid to all standards; compare samples with standards. Do not allow samples to stand longer than 10 minutes as colour will over-develop.

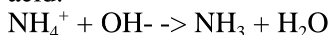
Points to note in analysis

- The reagents are relatively stable but they are unlikely to keep indefinitely. If the zero standard is highly coloured then the standards have been contaminated and the salicylate needs renewing.
- If the colour developed in the range of standards is the same, the DIC reagent needs renewing. Add 5-10 drops of commercial bleach to 100 ml of the DIC and repeat the test, before remaking the solution. The standards are in an acid solution and will absorb ammonia from the air, therefore the standards must be sealed at all times and, where analysis appears unreliable, this is the most likely site for investigation. Sampling from 6-8 sheep in a grazing group gives a reliable estimate of the mean rumen ammonia concentration of the group of animals.

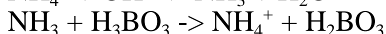
Laboratory techniques for estimation of rumen ammonia

Principle

The ammonia is separated from rumen fluid by steam distillation, collected in boric acid solution and determined by titration with standard acid.



$NH_4^+ + OH^- \rightarrow NH_3 + H_2O$



$NH_3 + H_3BO_3 \rightarrow NH_4^+ + H_2BO_3$

Equipment

Standard steam distillation apparatus is used to isolate ammonia from rumen fluid.

Reagents

- Ammonium sulphate - a standard solution is prepared from analytical reagent (AR) material and diluted for use.

Working acid solutions

- 0.05N H_2SO_4
  50 ml N H_2SO_4 standard diluted to 1 litre with distilled H_20.
- 0.0075 N H_2SO_4; 7.5 ml standard 1N H_2SO_4 is diluted to 1 litre with distilled water
- Boric acid solution (20%):
  Dissolve 20 g AR boric acid in approximately 700 ml hot distilled water, cool and make 1 litre in a volumetric flask.
- Sodium tetraborate solution (5%) saturated:
  Dissolve 50 g AR sodium tetraborate (powder or crystals) in 1 litre distilled H_2O
- Universal indicator solution (BDH Chemicals Ltd.)
  Range pH 4 - pH 11 product No. C21049.
- Silicone antifoaming agent (BDH Chemicals Ltd):
  An aqueous emulsion containing 30% Silicon product no. 33151 - use discretely and in extremely small amounts to counter "greasiness" of glass ware.

Mixed indicator solution
0.1% ethyl red in 85-95% ethanol
0.1% Bromocresol green
40 ml 0.1% methyl red, made up to 2 litres
8 ml 0.1% bromocresol green with 2% boric acid solution

Preparation and distillation of samples

Strained rumen fluid is centrifuged for 15 min (3000 rpm) and the supernatant frozen after being acidified with 2-3 drops of concentrated sulphuric acid.

Pipette 5 or 10 ml rumen fluid into a distillation flask, add a few drops of universal indicator solution; followed by a small drop of defoaming agent (if necessary) and 10 ml sodium tetraborate solution - distill immediately. Distill until 30-40 ml is collected (about 4 min), remove conical flask and titrate the distillate using 0.1 M HCl. A standard ammonium sulphate should also be titrated.

Gas liquid chromatography of volatile fatty acids in ruminal fluid

The total concentrations and individual proportions of the volatile fatty acids in the rumen are an indication of the animal status for glucogenic compounds. Fermentations giving high proportions of propionic acid (25-35%) are desirable since these fermentation patterns are most efficient energetically (less heat is lost as methane). The level of total VFA is also indicative of total fermentation rate. For these reasons a method for VFA analysis by gas-liquid chromatography (GLC) is recommended.

Column packing

Inert support: chromosorb 'W', acid washed 50-80 mesh
Liquid phase: (a) phosphoric acid (1.5% by weight of the inert support)
(b) polypropylene glycol sebacate (PPGS) (18% of the inert support)

Method

- 12 g of the chromosorb W are placed into an evaporating basin (12 cm diameter).
- 0.2 g of 88% phosphoric acid (H_2PO_4) in 70 ml of distilled water is then added and the mixture gently stirred until the chromosorb W is uniformly wetted.

The mixture is then oven dried at 80°C.

- 2.1 g of PPGS is dissolved in 70 ml of methylene chloride and added to the chromosorb W - phosphoric acid mixture in the evaporating basin and then dried in an oven at 80°C.
- The column is packed using slight suction from a vacuum pump while vibrating the column. Acid washed glass wool is packed into the end of the column then attached to vacuum pump and suction applied. The free running packing is held in a funnel attached to the other end of the column. Vibration or tapping causes the mixture to run into the column.

Operating procedures

Operating conditions using FID (Flame Ionization Detector):

Column temperature - 135°C
Detector temperature - 180°C
Injection port temperature - 200°C
Nitrogen carrier gas flow - 60 ml/min
Hydrogen flow to FID - 49 ml/min
Air flow to FID - 400 ml/min

Preparation of rumen fluid for GLC with an internal-standard

- Make a stock solution of the internal standard - 1.6% isocaproic acid in formic acid. Store at 4°C.
- Composition of standard VFA (standard A) solution for GLC (mM/litre): Acetic acid 56, Propionic acid 18, Isobutyric acid 3, Butyric acid 9, Isovaleric acid 3, Valeric acid 3.

The concentration of each acid must be known accurately and can vary slightly from that indicated. The mixtures are prepared from AR grade acids and stored neutralized (because a variable loss of individual acids from frozen samples occurs). The formic acid/isocaproic acid, internal standard acidifies the samples prior to injection onto the column. A solution of the internal standard for use (Standard B) over a few days is prepared weekly.

- 10 ml of stock internal standard solution (see Standard A) + 10 ml of 15% metaphosphoric acid (freshly prepared) + 30 ml of formic acid. Store at 4°C.

Sample preparation

- Ruminal fluid is centrifuged at 3000 rpm for 10 min
- 0.4 ml of the internal standard solution B is placed into a small (1.5 ml) disposable centrifuge tube followed by 0.7 ml of ruminal fluid. This is mixed and if necessary the sample is again centrifuged at 3,000 rpm for 5 min. These samples can be stored for 1-2 days at 4°C.
- Standards: 0.4 ml of internal standard solution B plus 0.7 ml of mixed VFA standard. 1 to 4 microlitres of this mixture is injected into the column.

Calculation of total VFA concentration and VFA proportions using the internal standard method

- The relative response factor (representing the area under the peak of that acid) for each volatile fatty acid is calculated using the standard VFA mixture which is chromatographed in every group of 50 samples, e.g., fAc = CAc x Std A (ic)/Std.A (Ac) where:
  fAc = the relative response to acetate
  CAc = concentration (μM/ml) of acetic acid in the VFA standard
  Std.A(ic) = area under the iso-caproic acid peak in the standard.

Std.A(Ac) = area of the acetic acid peak in the standard.
Factors are similarly calculated for the other VFAs.

- These factors are used to calculate the individual VFA concentration for each sample.
  e.g., Acetic acid = fAc x sample A(Ac) x sample A(ic)
  where:
  sample A(ic) = area of iso-caproic acid peak in the sample, sample A(ic) = area of iso-caproic acid peak in the sample.
- The sum of all the individual VFA concentrations for each sample is the total VFA concentration (in μM/ml of ruminal fluid).
- By taking the sum of all the individual VFA concentrations as 100% the molar percentage of each acid is calculated.

Acetate clearance as an indicator of the balance of absorbed nutrients
Hypothesis
It is proposed that in, cattle and sheep, acetate clearance rates reflect the balance of nutrients available for metabolism for a given productive state and that acetate clearance from blood will be directly related with feed intake (Weston, 1966). Accumulation of acetate in blood indicates that there is a shortage of co-factors (NADPH) that are required to incorporate the acetate into lipids at sites of deposition. These co-factors are derived from glucose or its precursors, or from amino acids. If the clearance rate of acetate from the basal diet is slow, this indicates that supplementation is necessary with "by-pass" protein or with glucose precursors.

Method
Cattle (about 150 kg) are injected with about 2.5 mM sodium acetate per kg liveweight. The injection can be done *via* an in-dwelling cannula in the jugular vein or injected directly into the vein. The injection should be done slowly over about 4 minutes. Blood samples (10 ml) are taken at intervals post injection for analysis of acetate or total VFA.

Injection solution
Dissolve 30 g of sodium acetate in 300 ml sterile double-distilled water. Inject directly into the jugular vein.

Blood samples

When an in-dwelling cannula is used, this is normally filled with dilute heparin solution (100 units/ml of 0.9% saline) to prevent it being blocked with coagulated blood, and then sealed with a nail. Prior to taking samples, remove the heparin solution and 5 ml of blood from the cannula and discard. Take a further 20 ml of blood into a bottle containing two drops of heparin (3,000 units/ml). Flush the cannula with the dilute heparin solution and seal it. Take samples at 10, 20, 30 and 40 minutes post injection.

Chemical analysis

- Centrifuge blood at 3,500 rpm for 10 minutes.
- Take off the plasma; store 5 ml; use 5 ml for analysis.
- Put into 50 ml centrifuge tubes the following:
    - 20 ml 0.2N H_2SO_4
    - 0.4 ml of isobutyric acid (1.75g/litre)
    - 5 ml 10% sodium tungstate
- Leave for 10 min at room temperature.
- Centrifuge at 3,000 rpm for 10 min.
- Put supernatant in a conical flask and add 1 drop of phenolphthalein.
- Neutralize with about 0.5 ml of 3M NaOH (add drop at a time until a pink colour persists).
- Evaporate to about 1 ml by boiling the solution on a hot plate (add glass beads to prevent bumping).
- Add 0.5-1.0 ml of metaphosphoric acid (36% HPO_3).
- Inject 1-2ul into GLC.
  (See above)
    - H_2 gas flow 30 ml/min
    - Air flow 350 ml/min
    - N_2 carrier gas flow 40 ml/min
- On the GLC set:
  Range 10 Injector temperature 210°C Attenuator 128 Detector temperature 180°C 10mv recorder Column temperature 135°C
- Column: 1.5 m X 4mm ID glass column
- Inert support: Chromosorb W acid washed
- Column coating: 17.5% polypropylene glycol

Calculations
Divide the area of the acetate peak by the area of the iso-butyrate peak to obtain the relative concentration of acetate in blood. Regress the relative concentration of acetate against time from injection and calculate the time for the concentration of acetate to fall to half the value following injection.

Chemical analysis of feed and faeces

Preparation of samples
Samples of material to be analyzed must be oven-dried at 65°C and then ground to pass through a 1 mm screen. Further drying to constant weight may be necessary to remove residual moisture. Dried material may be stored at room temperature in sealed vessels or plastic bags, preferably under nitrogen gas.

Moisture
A sample containing the equivalent of about 2 g dry matter is dried to constant weight at 95-100°C for 24 hr. Use an aluminium dish or porcelain crucible. Calculate percentage moisture from the loss in weight (to first decimal place).

Ash
Weigh a 2 g sample into a weighed porcelain crucible and place in a temperature-controlled furnace preheated to 600°C. Take care to avoid loss of material by convection currents. Hold at this temperature for 4 hr. Transfer crucible directly to desiccator, cool and weigh immediately. Calculate percentage ash (to first decimal place).

Kjeldahl Nitrogen determination

The Kjeldahl technique can be divided into three basic steps:

- Digestion of the sample in concentrated sulphuric acid during which all organic compounds are broken down, and organic N is converted to ammonia.
- Over-neutralization of the solution with a caustic soda solution and distillation and collection of the ammonia.
- Titration of the ammonia.

Reagents

50% Sodium Hydroxide:

Dissolve 600 g of NaOH in distilled water and make up to a volume of 1 litre. When the pellets of sodium hydroxide are added to water, stir with a glass rod. This is necessary to prevent NaOH from fusing to the bottom of the beaker. Keep in a rubber- or plastic-stoppered bottle.

Digestion mixture:

Mix 8 g selenium with 400 g potassium sulphate, add the mixture into 2 litres concentrated sulphuric acid and heat until all reagents are dissolved. Note: When the chemicals are mixed the Se and potassium sulphate set solid so it is easier to put the chemicals into the digestion flask and then add the acid. Alternatively, Se catalyst tablets can be purchased and concentrated sulphuric acid is used as the digestion mixture.

Sample size

Determination of sample size assumes some prior knowledge of the material under investigation. For maximum accuracy, a sample size should be taken which will require 10-20 ml of the standard acid. The amount of titrant can also be varied by changing the normality of the standard acid. Some feeds may be low in protein, and it may be difficult with small samples to obtain truly representative samples. Consequently, a considerable amount of dry material must be digested. Using 0.1 M acid as titrant for the ammonia that had been distilled, it is recommended that the following sample sizes are used:

- Dry feed samples 300 mg
- Milk, except colostrum 1 ml (or 1 g)
- Colostrum 300 mg
- Plasma and serum 0.5 ml
- Urine 0.2 ml

Very dilute samples (e.g., rumen fluid) may require use of a 0.01M standard acid for titration. Because of the sensitivity of the analysis, high accuracy cannot be obtained without thorough mixing of the material to be analyzed prior to sampling. This is especially true with materials which have been frozen and allowed to thaw.

Digestion

To the 100 ml Kjeldahl digestion flask, add:

- Sample (approximately 150 mg DM)
- One glass bead to prevent bumping
- 5 ml conc. H_2SO_4
- 1 Se catalyst tablet

Heat the mixture on the digestion rack in an area with air extraction. If foaming occurs, the early part of the digestion can be carried out at a lower temperature. Silicone antifoam agents should never be used (contrary to several current texts). The silicone spray coats the sides of the digestion tube producing a non-wetting surface. Large water droplets collect, and when sufficiently large, drop into the superheated anhydrous digestion mixtures, with violent consequences.

Following removal of all the water, white sulphur dioxide fumes will be evolved. These fumes are irritating and toxic and must be exhausted in a hood with sufficient capacity to prevent transfer into the laboratory. During the digestion, charred material can be washed down into the digestion mixture by swirling the digestion flask. If swirling does not flush all charred material into the digestion mixture, let the mixture cool completely and wash the charred material down with a fine stream of water. Then redigest until the mixture clears. After white fumes are no longer evolved and the boiling mixture is clear, allow the digestion to proceed for a further 30 minutes. Then allow the flasks to cool. Add about 20 ml of deionized water and mix immediately to prevent crystallization of the sodium sulphate.

Distillation

This is the same as for ammonia estimation. Turn on the heater under the steam generator and increase the heat to boil the water steadily (not violently) and turn on water to condenser. Put the empty digestion flasks on the collector tubes and, with the alkali stopcock closed and steam directed into the apparatus, run steam through the assembly and collect the condensates in 100 ml beakers for several minutes. This serves to warm up the apparatus, and flush out any residual alkali.

When the apparatus is preheated, open the alkali stopcock and direct the steam into a water drain. Place samples in the distillation apparatus and place 100 ml flasks containing 20 ml 2% (w/v) boric acid (containing indicator) under the condenser stem. Be sure the tip of the condenser stems are below the surface of the boric acid solution. Admit alkali solution through the alkali stopcock (about 5 ml alkali for 1 ml of H_2SO_4 used in the original digestion) and close the alkali stopcock. Then turn steam on through the apparatus and allow steam distillation to proceed for 6 min. Near the end of this period, lower the receiving beaker so that the distillate washes any remaining ammonia solution from the tip of the condensing units.

When the distillation is completed, turn steam stopcock into the position which diverts the steam to sink waste and another opens the distillation flasks to atmospheric pressure. Remove distillation flasks and turn steam stopcock to the off position.

Quantification of the ammonia

Titrate the ammonia-boric acid solution to the pink end-point with standardized acid (0.1N HCl or 0.05N H_2SO_4). Appropriate blanks must be run and their values subtracted from the sample titration values.

Calculations

There is a direct mole-per-mole relationship between ammonia released, the acid needed to titrate that ammonia, and the total N originally present. The number of ml of acid multiplied by its molarity gives the millimoles of ammonia. Since the average protein is 16% N, multiplication of per cent N by the factor 6.25 gives per cent crude protein (some factor other than 6.25 may be used for particular proteins).

Precautions

Care must be taken when working with hot concentrated acid and alkali. Take normal precautions: safety goggles must be worn when starting distillations. In each step where water is added to acid and alkali to acid, the solutions must be cool, otherwise the reactions can be quite violent.

The *in vitro* gas production techniques

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Background

In most laboratory techniques used for feed evaluation, the disappearance or solubilization of substrate is measured. On the other hand, the gas production technique, which was originally developed by Menke and Steingass (1988), measures the evolution of gases (methane and CO_2) which are produced as end products of fermentation by rumen microbes.

Production of CO_2 is partly from the fermentation and partly as result of formation of volatile fatty acids which expels CO_2 from the carbonate buffer solution.

The gas technique provides a great advantage in that the fermentation takes place in a glass syringe which allows for several measurements to be made on the same sample by measuring the gas volume at different intervals of time. This means that not only the possible extent of fermentation but also the rate of fermentation can be measured. In this respect, the technique is similar to the nylon bag method and the same exponential equation can be used. Thus the gas technique complements the nylon bag technique by measuring end product formation and not substrate disappearance. Results from studies using this approach to predict animal performance (digestibility and intake) showed that the gas technique was slightly inferior to the nylon bag but a much better predictor than other *in vitro* techniques or chemical components of feeds (Blummel and Ørskov, 1993; Khazaal *et al.,* 1993; Dentinho *et al.,* 1994).

Recently, the fact that the gas technique differs from other *in vitro* techniques by measuring evolution of gas as a result of fermentation has been used to adapt it as a biological assay to estimate the level of phenolics-related anti-nutritional factors in feed (Khazaal and Ørskov, 1994; Khazaal *et al.,* 1994). This is achieved by adding phenolic binding agents such as polyvinylpyrrolidone (PVP) or polyethylene glycol (PEG) to the substrate. As a result, the phenolics-related anti-nutritional compounds bind to the phenolic binding agent and their negative effects on fermentation are alleviated.

Menke' gas production method

Apparatus

The apparatus used in the gas production technique may vary slightly from one laboratory to another. At the International Feed Resources Unit (IFRU), the apparatus used is simple. It consists of glass syringes of 100ml capacity which are incubated in a water bath where the temperature is accurately controlled with a water stirring heater. The following procedure is based on the apparatus used in our laboratory.

Syringes

Good quality syringes are essential (syringes of Hliberle Labortechnik, 7901 Lonsee Ettlenschieu, Oberer Seesteig 7, Germany are recomm-ended). The syringes and their pistons should be numbered with a permanent (water-proof) dye starting for example with number 1. A few extra syringes are left as replacement for broken ones.

Buffer Solution

Stocks of the main elements solution (pH 6.8), the buffer solution (pH 8.1), the resazurin solution and the trace element solution can be prepared and stored in dark bottles. The reduction solution must be freshly prepared. The preparation of all solutions is as described on page 9 of the paper by Menke and Steingass (1988). The pH of the buffer mixture solution (i.e. main elements + buffer solution + resazurin solution + trace element + reduction solution) should be about 7.1 ± 0.15.

Preparation of Sample

The samples are milled through a 1.0 mm screen and their DM content determined. Before weighing the samples, the position of the syringes in each run should be planned. Ideally there should be 3 replicates of a blank and a standard roughagein each run. The triplicates of the blank or the standard roughage should be dispersed among the syringes. Thus one of each is incubated as the first (No 1) and second (No 2) syringes, the second blank and standard will be in the middle, while the third replicate of the blank and standard roughage should be the last 2 syringes (Table 9.2). Samples are normally run in duplicate and a run is usually repeated 3-4 times.

Table 9.2. Layout of design for measuring gas production.

Syringe number	Sample	Fresh weight	Level of piston at zero time*	Gas production after 3h	6hr	96hr
1	Blank	Empty	about 30			
2	Standard	215±5 mg				
3	Sample A	"	"			
4	Sample B	"	"			
S	Sample C	"	"			
6	"	"	"			
	Sample n					
	Blank	Empty				
	Standard	215±5mg				
	Sample n					
"	"	"	"			
"	Sample C	"	"			
"	Sample B	"	"			
"	Sample A	"	"			
"	Blank	Empty	"			
n	Standard	215±5mg	"			

* After injecting rumen liquor:buffer mixture

Weigh about 215±5 mg of air-dry milled (1.0 mm) sample (gives approximately 200 mg DM if the DM content of the samples is about 90%) into a glass boat. Use an aluminium or metal rod to hold the glass boat containing the sample. Empty the content of the glass boat into the bottom of the glass syringe. Try not to get any particles or dust from the sample onto the high inner side of the syringe since this could affect the movement of the piston.

Lubricate the pistons with a small amount of Vaseline (pure petroleum jelly) to ease the sliding of pistons and prevent gas escape. Push the piston inside the glass syringe gently after opening the clip. Make sure the sample in the syringe is not blown up and that it does not get in touch with the piston.

At IFRU, weighing of samples into the syringes and lubrication with Vaseline are completed the night before the start of the incubation (run). The syringes are then prewarmed in an incubator 40°C overnight before the rumen liquor/buffer solution is injected into the syringe.

If a water bath is used, the heater should be turned on some time before the start of incubation (e.g., the night before).

Starting the incubation

It is important first to calculate roughly how much of the buffer mixture solution and rumen liquor is needed. This will depend on the number of syringes to be incubated. For instance if a total of 35 syringes are to be injected, then at least 30 x 35 = 1050 ml of rumen liquor:buffer solution will be needed. In order to be on the safe side it is better to prepare at least 1200 ml of the mixture and, for that, 800 ml buffer and 400 ml filtered rumen liquor is needed. The 800 ml of the buffer mixture solution is prepared first in a Wolf flask. The content of the flask is heated to 39 °C and then transferred to the small water bath. A submerged stream of CO_2 is pumped into the liquid until it becomes colour-less or very slightly pinkish. Then, lift the stream of CO_2 to a level above the surface of the liquid. It is important that the stream of CO_2 is lifted to prevent over-saturation of the buffer mixture with CO_2. If this is allowed to happen, more CO_2 gas will be released as a result of buffering the volatile fatty acids during fermentation and, as a result, the variability between runs will increase. The reduction solution is added minutes before the addition of the rumen liquor.

Rumen liquor

The donor animals could be cattle, sheep or goat but should be receiving a balanced roughage-based diet (at IFRU, 3 rumen cannulated sheep are used and they receive 1200 g of hay and dehydrated grass cubes (2:1) in two equal feeds per day). Before the morning feed, an equal amount of rumen liquor from each of the 3 sheep is pumped into plastic bottles and quickly stored in warmed Thermos flasks and taken to the laboratory. Then, the rumen liquor is stirred and filtered through 2 layers of muslin. The filtered rumen liquor is bubbled with a stream of CO_2 for 1-2 minutes. This is followed by adding the required amount of filtered rumen liquor (pH 6.3±0.15) while stirring the buffer solution in the flask. Remember that the proportion of rumen liquor to buffer is 1:2 (the pH of the rumen liquor/buffer mixture should be about 6.90±0.1).

Inoculation

Record the zero time (i.e., the time when injection of the rumen liquor:buffer mixture into the syringes is started) of the incubation.

Inject 30±1.0 ml of rumen liquor/buffer mixture into each syringe, followed by drawing most of the air from the syringe. Shake the syringe gently to make sure that all the substrate is mixed with liquid and then take out all remaining air or air bubbles from the syringe. Record the level of the piston (which should be around 30.0 ml) and incubate the syringe in a water bath (39±0.1°C).

Record the time when you finish filling the syringes with the rumen liquor-buffer mixture. The period of time needed to complete the filling of all syringes with the rumen liquor/buffer mixture should be as short as possible. At IFRU, it takes about 15-20 minutes to complete 54 syringes.

Shake the syringes gently 30 minutes after the start of incubation and then every hour during the first 8-10 hours of incubation. This is important for roughage with low rates of degradability, which tend to float. When gas production is recorded, shake the syringes after taking the reading.

Normally, the time required to inject the rumen liquor-buffer mixture into the syringes is longer than that required to read the volume of gas production during incubation. This difference, particularly if highly fermentable feeds are studied, can lead to an over-estimation of fermentation of this feeds that received the inoculum first compared with those which received the inoculum last. Therefore, when gas production is recorded at any incubation period, it is best that the time during which the readings are made is similar to that taken when the syringes were inoculated with rumen liquor-buffer. For example, if it took about 30 seconds to fill up each syringe with mixture, then you should allow about 30 seconds for reading the gas volume for each syringe.

Duration of incubation

The duration of incubation should be long enough to allow for the complete description of the curve of gas production (i.e., until the curve reaches a plateau, or until the difference in gas production between the last two incubation times is small). At IFRU, 96 hours of incubation is considered to be sufficient in most cases. The accumulating volume of gas is recorded after incubation periods of 3, 6, 12, 24, 48, 72 and 96 hours. If gas production exceeds 60 ml for a sample, take the syringe out of the water bath. Turn the syringe upwards, open the clip and push the

piston to release the gas. The piston could be pushed until it is close or back to the 35 ml position. Record the new level of piston and resume the incubation.

Calculation

Subtract the volume of gas produced from the blanks (average of 3 replicates) from the volume of gas produced from each sample. This will be the observed volume of gas per x amount of fresh sample. Then, knowing the DM content of each sample, the volume of gas per 200 mg DM can be calculated.

Data for gas production are then fitted to the exponential equation : $p = a + b\ (1\text{-}e^{ct})$ (Ørskov and McDonald, 1979); p represents gas production at time t, (a+b) the potential gas production, c the rate of gas production and a, b and c are constants in the exponential equation.

Note: It is acceptable to find that replicates of the blank or the standard roughage that were injected first with rumen liquor-buffer mixture produce slightly less gas (1-1.5 ml) compared with the ones that were injected last. It is not very clear why this happens. One possible explanation is that during injection of the rumen liquor-buffer mixture, more particles accumulate at the bottom of the flask as the content of the Wolf flask becomes smaller. Another more likely explanation is that the rumen liquor-buffer mixture becomes increasingly saturated with CO_2 towards the end of inoculating the syringes compared to the start. This is why it is important to place syringes of blank and standard at different positions in each run.

Limitations to the technique

- In order to use the technique reliably, it is essential to be sure of a constant supply of electricity in order to maintain a constant temperature during incubation.
- Like other *in vitro* techniques, it is a closed system in which end products accumulate and can inhibit fermentation or create an environment very dissimilar to the rumen.
- The technique will slightly under-estimate the nutritive value of feeds that are high in protein. This is due to the fact that protein fermentation contributes little to the total volume of gas production.

Theodorou's gas production technique

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Background

In principle, this technique is similar to the Menke *et al.* (1889) gas production procedure using ground particulate substrates, anaerobic media and rumen fluid inoculum. It differs, however, in that incubations are conducted in gas-tight culture bottles, thus enabling gases to accumulate in the head-space as the fermentation proceeds. A pressure transducer connected to a digital readout volumeter and a gas-tight syringe assembly is then used to measure and release the accumulated gas pressures from the incubated culture bottles. By repeating the "gas-measurements + gas- release" procedure at regular intervals, it is possible to construct gas accumulation profiles for feeds. The rate and extent of fermentation can also be calculated (Theodorou *et al.,* 1994).

The method was developed to get information on the fermentation kinetics of ruminant feeds based on long term end-point fermentations (166 hours). However, shorter fermentations (48 and 72 hours) have been also to evaluate tropical forages and rank them according to their fermentability. The method was developed with a nitrogen-rich (Theodorou) medium but the Menke medium can be used when a nitrogen free medium is needed. The protocol describes how to prepare both media. The technique has also been adapted to the biological evaluation of the effects of phenols on fermentation by adding binding agents such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and polyvinyl-polypyrrolidone (PVPP) (Rosales, M. and Wood, C., 1994, unpublished data).

Preparation of the sample (Thursday or earlier)

Grind substrate to pass through 1 mm dry sieve (if not already ground). Weigh out substrate. Generally use 1 g total substrate, weigh to tolerance of ±0.0020 g. Make up stock solutions for medium. Recipes for media are given below. Arrange serum bottles in order placing them on trays for easy handling.

Preparation of the medium (Friday)
Make up suitable amount of medium. Stir and gas with CO_2 for about 2 to 3 hours, then add a small volume of reducing agent (about 2 ml per litre buffer; 3 ml for Menke's medium). Continue gassing until the resazurin in the medium is pink. Dispense 90 ml medium into 125 ml serum bottles using pump and gassing with CO_2. Make 5 to 10 spare bottles for use in preparing the inoculum. Seal with butyl rubber stoppers, but do not crimp. Store at 4°C.

Place the samples into the bottles (Monday)
Make up a suitable amount of reducing agent in fume cupboard, keeping it stirred and under an atmosphere of nitrogen. Using a small wide bore funnel transfer the substrates into their bottles and add 4 ml reducing agent. Samples are normally run in triplicate. Remember to add reducing agent to the spare bottles. Keep gassing with CO_2. Reseal with butyl rubber stoppers and crimp with aluminium caps. Replace in incubator at 4°C and programme it to switch to 39°C at about 2 am.

Prepare inoculum (Tuesday)
A minimum of 2, and preferably 3, people are required to inoculate the bottles.

Collect rumen fluid starting at about 8.15 am and keep it warm in a Thermos flask. Filter fluid through 4 layers of course cotton muslin and collect in beaker (with volumes marked) under atmosphere of CO_2. Keep liquid stirred (not too vigorously). Note approximate volume of filtered liquid. Transfer solids to a blender and add a volume of medium (using the spare bottles prepared earlier) approximately equal to the volume of filtered liquid. Blend for about 30 seconds and filter through muslin into the beaker with filtered liquid to pool with original filtered rumen fluid. Keep stirred and under CO_2. The inoculum is now ready for use.

Inoculation of bottles (Tuesday)
While the inoculum is being prepared, the serum bottles must be adjusted to atmospheric pressure. This is done by using the "taking gas readings" procedure described below, but the gas volumes produced are not normally noted. Bottles are returned to the incubator at 39°C.

Using a 10 ml syringe and 21 gauge 1.5 in (0.8 x 40 mm) needles

(colour code green), 5 ml of inoculum is injected into each bottle. Shake bottles and return to incubator.

Starting at 10 am, the bottles are readjusted to atmospheric pressure, shaken and returned to the incubator. This is taken as the starting point (time = 0) of the experiment.

Taking gas readings

Readings are then normally taken at the following times:

Time	Hrs after start	Day
13.00	3	Tuesday, day 1
16.00	6	
19.00	9	
22.00	12	
02.00	16	Wednesday, day 2
06.00	20	
10.00	24	
14.00	28	
19.00	33	
01.00	39	Thursday, day 3
07.00	45	
14.00	52	
22.00	60	
08.00	70	Friday, day 4
20.00	82	
08.00	94	Saturday, day 5
20.00	106	
08.00	118	Sunday, day 6
08.00	142	Monday, day 7
08.00	166 (end)	Tuesday, day 8

A pressure transducer (Bailey and Mackey Ltd, Birmingham B42 1DE, UK) is used to measure headspace pressure in the bottles. The transducer should have a range of 0 - 25 psi, accuracy of 0.1±2%, readings calibrated to read in units of psi. It is connected to a disposable Luer lock 3-way tap allowing a needle (23 gauge 1 in, 0.6 x 25 mm; colour-coded blue) and syringe to be fitted to the other outlets.

Gas pressure is read by removing bottles tray by tray from the incubator, inserting the needle through the butyl rubber stopper into the

headspace above the culture medium. Note pressure. Adjust the pressure to atmospheric by removing gas into the syringe and note volume of gas removed (read from syringe). Take readings for all of the bottles in the tray, shake the bottles, and return them to the incubator.

Determination of dry matter disappearance (DMD)
At the end of the gas production run, vacuum filter through pre-weighed filter crucibles (Sintaglass, porosity 1 - regraded P160). Wash bottle with water to remove residues and wash residues on the filter. Oven dry overnight at 105°C. then allow to cool in desiccator and weigh. Express DMD as a proportion of the initial dry matter in the substrate.

Theodorou medium
(otherwise called Basal Medium D by Theodorou)

Component solutions
1. Micro-mineral solution (g per 100 ml)
This is made up in 100 ml lots and stored in the refrigerator as a stock solution.

$CaCl_2.2H_2O$	13.2
$MnCl_2.4H_2O$	10.0
$CoCl_2.6H_2O$	1.0
$FeCl_3.6H_2O$	8.0

2. Buffer solution (g per litre)
This is made up in variable quantities and can be stored in a fridge. Calculate how much is required for each run.

NH_4HCO_3	4
$NaHCO_3$	35

3. Macromineral solution (g per litre)
This is made up in variable quantities and can be stored in the refrigerator. Calculate how much is required for each run. The same volume of buffer and macromineral solution is required in the medium.

$Na_2HPO_4.12H_2O$	9.45
KH_2PO_4	6.20
$MgSO_4.7H_2O$	0.60

4. Resazurin solution

Resazurin 0.1g/100 ml water

To make medium: mix the component solutions in the following amounts to make about 900 ml medium. Use same proportions if more buffer is required (which will usually be the case).

1. Microminerals	0.1 ml
2. Buffer	200 ml
3. Macrominerals	200 ml
4. Resazurin	1 ml
5. Distilled water	500 ml

The medium is kept mixed and CO_2 bubbled through it.

Theodorou reducing agent

Cysteine $HCl.1H_2O$	625 mg
Distilled water	95 ml
1M NaOH	4 ml
Sodium sulphide	625 mg

Add ingredients together in fume cupboard and stir under a stream of N_2.

Menke style N free media

Component solutions

1. Solution A (same as Theodorou Micromineral solution) (g per 100 ml)

This is made up in 100 ml lots and stored in fridge as a stock solution.

$CaCl_2.2H_2O$	13.2
$MnCl_2.4H_2O$	10.0
$CoCl_2.6H_2O$	1.0
$FeCl_3.6H_2O$	8.0

2. Solution B (g per l) Calculate how much is required for each run.

$NaHCO_3$	39

3. Solution C (g per l) Calculate how much is required for each run.

Na_2HPO_4 (anhydrous)	5.7
KH_2PO_4	6.2
$MgSO_4.7H_2O$	0.6

4. Resazurin solution (same as for Theodorou medium) (Resazurin 0.1g/100 ml water)

Medium prepared by mixing:

Solution A	0.1 ml
Solution B	200 ml
Solution C	200 ml
Resazurin	1 ml
Distilled water	400 ml*

* *Note:* Theodorou medium similar but less concentrated generally as more water is added. The Menke buffer has the composition described by Menke *et al.* (1988).

The medium is gassed with CO_2 and small volume of Menke reducing agent added to reduce it prior to bottling, similar to the treatment of the Theodorou medium.

Menke reducing agent

Distilled water	95 ml
1M NaOH	4 ml
Sodium sulphide	625 mg

(same as Theodorou reducing agent without cysteine).
Add ingredients together in fume cupboard and stir under a stream of N_2.

Curve fitting

A single pool model can be fitted:
$y = a + b(1 - e^{-ct})$
excluding data collected over the first 9 hours.
Using STATGRAPHICS, non-linear correlation:
PARM[1] + PARM[2]*(1-e$^{(-1 \text{ x time x PARM[3]})}$)
Parameter vectors: -10, 280, 0.035

Estimation of microbial protein supply to ruminants based on urinary excretion of purine derivatives

The details of this method were contributed by X.B. Chen, Rowett Research Institute, Scotland

Background

Microbial protein formed as a result of rumen fermentation of carbohydrate under anaerobic conditions is the major source of protein for ruminants. On many basal diets derived from tropical feed resources, that are low in nitrogen (e.g., crop residues, sugar cane, molasses and cassava roots), microbial protein is virtually the only source of protein. While this fact has been known for a long time it has been extremely difficult to determine the microbial protein contribution.

The method usually used are based on determinations of microbial markers, such as RNA, DAPA and 35S. These methods involve complicated procedures of measuring digesta flow and require usually the use of animals fitted with post-ruminal cannulae. It is therefore difficult in practice to conduct extensive *in vivo* studies on microbial protein synthesis. The method based on measurement of purine derivatives in urine overcomes the disadvantages of the above methods. It is simple (it requires only total collection of urine) and non-invasive (no surgical preparations are required). It is particularly appropriate as a simple tool to study factors affecting microbial production in the rumen. It has the potential to be further simplified for use under farm conditions.

The principle

Ruminant feeds usually contain small amounts of purines, most of which undergo extensive degradation in the rumen as a result of microbial fermentation. Absorbed nucleic acid purines are degraded and excreted in the urine as their derivatives: hypoxanthine, xanthine, uric acid and allantoin. The excretion of purine derivatives is directly related with the purine absorption. With knowledge of the purine:protein ratio in microbial biomass, microbial protein absorption can be calculated from the amount of purine absorbed which in turn is estimated from urinary excretion of purine derivatives.

Procedure
Some important experimental details are given as follows:

Urine collection
It is essential to ensure a complete collection of urine and separation of urine from faeces. To obtain a realistic estimation of daily excretion of purine derivatives, urine collection needs to be made for more than 5 days. This helps to reduce the error due to the 'end-of-collection' variation in urine output of the animal. Collection can also be made as a bulk for the whole period. However, where analytical facilities allow, it is better to make the urine collection daily, to obtain additional information on the variability of the daily measurements. In general, this day-to-day variation is about 10%.

Urine is collected into a container, with an appropriate amount of 10% H_2SO_4so that the final pH of the urine is below 3 to prevent bacterial destruction of the purine derivatives in the urine. Since the purine derivatives concentration in urine is very high and precipitation (particularly of uric acid) can occur during storage, dilution by about 5 times prior to storage will prevent the occurrence of precipitation.

Determination of purine derivatives
In sheep, all four compounds are present in the urine. The proportions are approximately: allantoin 60-80%, uric acid 30-10% and xanthine plus hypoxanthine 10-5%. the proportion of allantoin increases with the daily excretion of purine derivatives. For this reason, the sum of four compounds, instead of allantoin alone, is used as the parameter to measure microbial protein supply.

Cattle urine contains allantoin and uric acid (allantoin 80-85%; and uric acid 20-15%). Within the same animal, the proportions of allantoin and uric acid are very constant, but there seems to be variation between animals. In dairy cows, allantoin and uric acid are also secreted in milk. The daily amount secreted in milk is equivalent to less than 5% of that excreted in the urine, and purine derivatives concentrations in milk appears to be constant. Correction for the output of purine derivatives *via* milk based on analyses done on several milk samples will be useful.

Methods for the chemical analysis of purine derivatives using various instruments, spectrophotometer, autoanalyser and high performance liquid chromatography (HPLC) are given in Table 9.3.

Table 9.3. Methods for determination of urine derivatives.

Spectrophotometer	Autoanalyser	HPLC	Authors
A			Young & Conway, 1942
AU			Fujihara *et al.,* 1987
U X	A		Pentz, 1969
X	A U X		Chen *et al.*, 1990
	A		Chen *et al.,* 1993
	A U X		Balcells *et al.*, 1992
	A		Diez *et al.,* 1992
A			Borchers, 1977
U			Fossati *et al.*, 1980
	A		Lindberg & Jansson, 1989
		A U	Lux *et al.*, 1992

A Allantoin; U Uric acid; X Xanthine

Calculation of intestinal flow of microbial N from excretion of purine derivatives

The daily excretion of total purine derivatives (allantoin, uric acid and xanthine plus hypoxanthine for sheep; and allantoin and uric acid for cattle) is calculated and expressed in mmol/day.

Based on experimental results published in the literature on the quantitative relationship between excretion of purine derivatives and purine absorption, the amount of microbial purines absorbed by the animal is estimated. Here it should be noted that different models are used for sheep and cattle, as discussed by Chen *et al.* (1991). Published work on models relating excretion of purine derivatives to purine uptake in sheep: Chen *et al.* (1990a,b), Balcells *et al.* (1991); and in cattle: Verbic *et al.* (1990).

The following factors can be used for the calculation of intestinal flow of microbial N from the microbial purines absorbed.

- Digestibility of microbial purines is assumed to be 0.83. This is taken as the mean digestibility value for microbial nucleic acids based on observations reported in the literature.
- The N content of purines is 70 mgN/mmol

- The ratio of purine-N:total-N in mixed rumen microbes is taken as 11.6:100.

The parameters given above are based on information available so far and need to further defined in the future. For this reason, the results should not be taken as absolute values.

Expression of results

The calculated microbial protein supply is expressed as g microbial N per day and/or as g microbial N per kg digestible organic matter(DOMI). The latter is effectively an expression of the efficiency of microbial protein supply. The efficiency can also be expressed as g microbial N per kg digestible organic matter apparently fermented in the rumen (DOMR) to facilitate comparisons with the reports in the literature. DOMR may be assumed to be 0.65 of the DOMI, based on ARC (1984).

Limitation

- The results are not absolute as stated previously although they show good agreement with those obtained by other procedures. Nevertheless, the method is best used to compare differences in intestinal microbial N flows between treatments in the same experiment.
- In the calculation it is assumed that there is little dietary nucleic acid reaching the small intestine. This could be true with most diets but may not be so when animals are fed with rations containing large quantities of fish meal.
- The calculation of microbial N from purine content assumes that the ratio of purine-N:total-N in mixed microbial populations is constant.
- It has been shown that sheep, cattle and possibly other species differ in their purine metabolism. The implication is that different models should be used for these species to relate excretion of purine derivatives with intestinal flow of microbial protein. So far, most information is available for cattle and sheep and very little for other species.

Secondary compounds in tropical trees

This section was contributed by Mauricio Rosales, Centro de Investigación en Sistemas Sostenibles de Producción Agropecuaria (CIPAV), Apartado 2019, Cali, Colombia

The term "secondary compounds" is used to describe a group of chemical constituents in plants thought not to be involved in the biochemical processes of plant growth and reproduction (Palmer *et al.*, 1990). These secondary metabolites are thought to have a defensive role that ensures survival of the plant (Coley *et al.,* 1985), by protecting against insect predation or by restricting grazing by hervivores (Swain, 1979). These secondary compounds have been implicated in limiting the utilization of many tropical feed resources, particularly trees and shrubs. They can inhibit digestion, have toxic effects, inhibit some enzymes and/or metabolic processes, or act as precursors of anti-nutritional compounds (Palo, 1987).

As summarized by Barry and Blaney (1987), secondary compounds can be toxic to animals or cause reduction in their productivity by reducing feed intake. These plant constituents do not affect all herbivores equally; there are examples of plants being toxic for monogastric species but not for ruminants, because the toxin is rendered harmless by the rumen bacteria (Dobson, 1959).

There are more than 1200 classes of secondary compounds. These include among others, polyphenols, cyanogenetic glycosides, alkaloids, saponins, steroids, toxic proteins and amino acids, non-protein amino acids, phytohemagglutinins, triterpenes and oxalic acid (Kumar, 1992; Liener, 1980).

Tannins

Discussion here will be restricted to tannins which are the most common secondary compounds in tropical fodder trees. Vegetable tannins are water soluble polyphenolic compounds having relative molecular mass between 500 and 3000 (Haslam, 1981). Besides giving the natural, usual phenol reactions, they have some special properties such as the capacity to bind strongly with proteins, polysaccharides, nucleic acids, steroids, alkaloids and saponins (Mueller-Harvey and McAllan, 1992; Haslam,

1981). The mechanism of vegetable tannage is generally accepted to be the formation of a hydrogen-bonded network between hydroxyl groups of vegetable tannins and relevant groups in collagen, and hydrophobic interactions between vegetable tannins and certain regions or groups of the collagen polymer (Spencer *et al.,* 1988).

Traditionally, tannins have been divided into two groups: the condensed and hydrolyzable tannins. However, a new group, the complex tannins has been proposed (Tang *et al.,* 1992). It is generally thought that condensed tannins are less harmful than hydrolyzable tannins, although both have the capacity to bind protein.

Nutritional effects of tannins

Studies of the effects of tannins in animal nutrition have involved a wide range of plants and have covered a wide variety of animal species. In the vast majority of cases, there has been little or no characterization of tannins present in the feedstuffs used (Mueller-Harvey *et al.,* 1987). In general, tannins cause growth depression and an adverse effect on protein and dry matter digestibility (Liener, 1980). They can also produce liver necrosis, act as a pectinase inhibitor and as carcinogenic agents (NAS, 1973).

Tannins are known to impart an astringent or bitter taste and, at a certain level in the diet, may therefore reduce the palatability. However the effects of tannin may also be quite negligible or indeed they may even enhance intake (Mueller-Harvey and McAllan, 1992).

Levels between 0.2 and 2% (DM basis) of tannins have been shown to depress dry matter, protein and amino acid digestion, reduce energy utilization and growth and lead to poorer feed efficiency ratios in poultry. Leg abnormalities have been found in chicks fed sorghum grain of high tannin content. Histopathological effects in chicks include decreases in blood haemoglobin, red and white cell counts and necrosis of the kidney and liver. Decreases in egg production and yolk discolouration have also been reported (Mueller-Harvey and McAllan, 1992).

In pigs, a reduction in dry matter digestibility has been reported. Physiological abnormalities resulting from continuous ingestion of free gossypol by young pigs include anorexia, dyspnoea, hydrothorax and

oedema of lungs, hepatic degeneration and dilation of the heart. In general, feeding with high tannin diets results in poor performance, particularly on feed conversion efficiency (Liener, 1980).

The effect of tannins in ruminant feeding is not consistent and there are reports of possible harmful and beneficial effects (Zelter *et al.,* 1990; Barry and Duncan, 1984). The two types of tannins differ in their nutritional and toxic effects. The condensed tannnins have a more profound digestibility-reducing effect than hydrolyzable tannins, whereas the latter may cause varied toxic manifestations due to hydrolysis in the rumen.

Rumen microbes have been shown to degrade flavonoids. Strains of *Butyrovibrio* and *Peptostreptococcus* are prominent in the cleavage of heterocyclic rings. However, there are little or no data available on the degradation of tannins by the rumen microflora (Deschamps *et al.,* 1983; Field and Lettinga, 1992). Since rumen micro-organisms may modify or metabolize ingested tannins, the extensive adaptation of rumen microflora to different plant constituents could be of particular importance in reducing the potential toxicity of ingested tannins. These ingested tannins may act in the rumen in a number of ways such as:

- Affect the species and composition of the microflora.
- Complex with and inhibit extracellular enzymes produced by the microflora.
- Complex and render unavailable dietary nutrients.
- They (or metabolic products) may be absorbed from the rumen and prove toxic at the tissue level (Mueller-Harvey *et al.*, 1992).

Evidence is increasing that tannins can have some benefits. Tannins have been found to have bloat-safeguarding properties. It has been suggested that tannins inhibit the production of stable foam in the rumen helping to control bloat (Lees, 1992). Low concentrations (2% in dry matter) of tannins from *Lotus* have been shown to reduce the carcass fatness in growing lambs, whereas with high tannin diets increased levels of growth hormone were found in sheep blood (Barry and Manley, 1986). Dietary condensed tannins from *Lotus pendiculatus* (2-3% in DM) have been shown to impart beneficial effects because they reduce the protein degradation in the rumen by the formation of a protein-tannin complex (Barry and Blaney, 1987).

Tannins and By-pass Protein

Protein that is slowly degradable in the rumen may provide amino acids and peptides for microbial growth in addition to providing by-pass protein. Tannins are known to protect dietary protein against microbial attack in the rumen. Thus if a freshly harvested tropical legume given as a supplement is to provide by-pass protein then it should be selected for a relative high content of tannins, even though this may depress fibre digestibility. The benefits of including by-pass protein in the diet have been widely documented (Preston and Leng, 1987).

The tannin-protein complexes have maximum stability in the pH range 4-7. Above and below this pH range the complex is readily dissociated. In this way the tannin-protein complex, after escaping from the rumen fermentation (about pH 5-7), would be digested readily by the enzymes in the gastric (about pH 2.5) and pancreatic (about pH 8-9) secretions (Palo, 1987).

Chemical determination of secondary compounds

The traditional chemical analyses of feeds do not take account of the secondary compounds or their anti-nutritive effects. But the key to assessing the nutritive value of tree leaves lies in the ability to estimate the presence and effects of these compounds.

There is a wide range of analytical techniques that have been developed to analyze particular plant secondary compounds. High Performance Liquid Chromatography (HPLC) is perhaps the most sophisticated tool for the rapid screening of plant materials. It is however comparatively expensive and not readily accessible to research scientists in developing countries. HPLC can characterize the secondary compounds profile of the plant, but gives no estimate of action on the animal (Gill *et al.,* 1992).

The main difficulty is that there is a wide variety of such compounds and each requires a different analytical method. It is not practical to consider analyzing each species for each compound. The diversity in chemical structure is extremely problematic when choosing an appropriate method of analysis. However, the presence of antinutritional factors can be determined qualitatively using the preliminary phytochemical tests described below.

Phytochemical preliminary tests

The presence of anti-nutritional factors can be determined qualitatively according to the methods described by Larrahondo (1985) and Rosales *et al.* (1989). An extract is prepared and colour changes observed following addition of various reagents, giving an indication of the presence of phenols, steroids, alkaloids and saponins.

Preparation and extraction of the leaf material

Weigh 10 g of fresh leaves, place them in a mortar and grind them after the addition of 30 ml of ether and 30 ml of a 9:1 methanol-water solution. Filter the resulting solution, place it in a separating funnel and leave it to stand until two separate layers can be identified. The lower layer is the methanol-water polar fraction, the top non- polar fraction being formed with ether. Both phases are used in subsequent analysis.

Saponins

9 ml of water are added to 1 ml of the methanol fraction and then filtered. 1 ml of this solution is shaken in a small test tube for 30 seconds. After 15 minutes, the height of foam in the tube is measured, giving an indication of the levels of saponins in the forage:

Height of foam:

5 mm or less = Negative.
5 - 9 mm = Low content of saponins.
10 - 14mm = Medium content of saponins.
15 mm or more = Forage high in saponins.

Phenols

Three drops of the methanolic fraction are placed in a five division ceramic test plate. Drops of distilled water are added to each division to give a yellow colour. One drop of $FeCl_3$ is added to the solution in the first division, two drops in the second, and so on. The last division is left as a control. Changes in colour indicate the presence of phenolic compounds as follows:

No change = No phenols or tannins
Dark Blue = Water soluble tannins or phenols.
Dark Green = Flavonoids or condensed tannins.

Steroids (Lieberman-Buchard)
1 ml of the non polar-fraction is evaporated in a basin and 4 drops of chloroform added. One drop of the resulting solution is placed in each division of the test plate plus 2 drops of acetic anhydride and one of concentrated sulphuric acid. Changes in colour indicate the presence of steroids as follows:
Blue or Green = Steroids.
Red, Pink or purple = Triterpenoids
Light Yellow = Saturated Steroids or triterpenoids.

Alkaloids (Dragendorff)
4 drops of ammonia (NH_4OH) are added to 3 ml of the methanolic fraction. The sample is reduced by evaporation in order to concentrate the compound of interest. Then 3 drops of acetic acid and one of distilled water are added. The solution is evaporated again and drops of the final residue are placed on a filter paper. These drops are covered with drops of the Dragendorff reagent, a colour change to red or pink indicates the presence of alkaloids.

An example of the results of a preliminary test is shown in Table 9.4

Table 9.4. Results of qualitative contents of antinutritional factors in the some forage trees Source: Rosales *et al.,* 1989).

Tree species	Phenols	Steroids	Alkaloids	Saponins
Trichanthera gigantea	+	++	-	+
Gliricidia sepium	+	+	-	++
Inga spectabis	+++	++	-	++

Chemical determination of tannins
The chemical diversity of tannins makes their analysis difficult. Several methods have been developed, especially to measure chemical groups or structures. Of these the most widely used are the Folin or Prussian blue method for the determination of total phenols (see Price and Butler,

1977) and the acid-butanol method for the determination of condensed tannins (see Porter *et al.,* 1986). Hagerman and Butler (1989) have made a critical review of analytical techniques.

An alternative approach is to assess the biological effect of the tannins, for example in relation to their capacity to precipitate proteins. Several methods for assays of this type have been developed, the radial diffusion assay of Hagerman (1987) being the most recent and probably the easiest to perform. A modification of this method is described below. This method measures condensed and hydrolyzable tannins, but is unable to distinguish between them.

Radial Diffusion Method for Phenolics

Adapted by C Wood (Natural Resources Institute, Chatham, ME4 4TB, Kent, UK) from the method of Hagerman (1987)

Equipment

1. 8.5cm petri dishes
2. Water bath at 45-50°C

Reagents

1. Buffer A
   Dissolve 10.6mg of ascorbic acid in a small amount of distilled water. Add 2.9ml of glacial acetic acid and make up to just under 1 litre with distilled water. Adjust to pH 5 using NaOH pellets (about 14 are required). Make up to 1 litre with distilled water.
2. Agarose (Type 1, Sigma)
3. Bovine haemoglobin (Sigma M2500)
4. 70% aqueous acetone
   350ml acetone made up to 500ml with distilled water

Procedure

The assay is normally conducted in duplicate on different plates. Samples are usually extracted in duplicate. Therefore each sample requires 2 wells on each of 2 plates.

Preparation of agarose plates

1. Prepare a 1% (w/v) solution of agarose by suspending the agarose in a small volume of cold buffer A and add to boiling buffer whilst stirring.
2. When all the agarose is dissolved transfer to a waterbath at 45-50°C and allow to cool.
3. Add 0.1% haemoglobin whilst stirring with a glass rod until fully dissolved.
4. Rapidly pipette 9.5ml of the warm agarose/haemoglobin solution into a petri dish using an open-ended pipette.
5. Allow to stand on a flat surface to cool, and refrigerate.
6. Do not store for more than a week before using.
7. Mark the underside of the dish with a line to mark the origin (so that the numbering sequence of wells can be determined) and dots to mark where the well are to be located. Wells must be at least 15mm apart and a similar distance from the sides. Generally 8 wells are cut using a 4mm cork borer and the gel core removed.

Preparation of the leaf extracts

1. Accurately weigh approximately 500mg (±10mg) of dried sample (ground to pass a 1mm screen) into a 10ml glass beaker.
2. Add 5ml of 70% aqueous acetone and homogenize for 1 min using an ultraturrex at medium power.
3. Transfer to a 15ml coned centrifuge tube and centrifuge at 2000rpm for 10 mins.

Plating out of extract

1. Add 15 l of the extract to a well.
2. Apply the extracts in a clockwise sequence around the plate. Duplicate extracts are plated out in duplicate onto two different plates, thus giving four plates per sample.
3. When fully loaded, the plates are left until the extract penetrates the gel.
4. Seal the petri dish with parafilm and incubate at 30°C for 4-5 days.
5. Measure the diameter of the rings of precipitated protein with a vernier scale to the nearest 0.05 mm and then measure again at a 90° angle to the first measurement.

Calculation

Duplicate a

Average diameter (da) = $\dfrac{(daA1 + daA2 + daB1 + daB2)}{4}$

Average ring area (a) = $da^2 - 16$

Radial diffusion RD/g = $\dfrac{\text{area (a)} \times 3.33}{\text{sample wt. a (g)}}$

Duplicate b

Average diameter (db) = $\dfrac{(dbA1 + dbA2 + dbB1 + dbB2)}{4}$

Average ring area (b) = $db^2 - 16$

Radial diffusion RD/g = $\dfrac{\text{area (b)} \times 3.33}{\text{sample wt. b (g)}}$

RD/g (DM basis) = $\dfrac{100}{\%DM} \cdot \dfrac{[RD/g\ (a) + RD/g\ (b)]}{2}$

However, to suggest that protein precipitation assays are the sole recommended technique is perhaps an over-simplification. Low molecular weight phenolic compounds are potentially very toxic to animals and it is not certain whether they will precipitate proteins. What is also uncertain is whether low molecular weight phenolics are extensively degraded in the rumen or are the symptoms of toxicity caused by phenolics that themselves are the product of hydrolysis or oxidation of other larger phenolics or tannins.

The gas production methods can also be used to assess the effects of the secondary compounds on the fermentation.

Given the lack of knowledge on the effects of specific tannins, the best approach, at least in the short-term, is to subject samples of fodder trees to a number of methods for analyzing tannins and to combine these data with records of the animal production responses to these species (Gill *et al.* 1992).