Standardization of Analytical Methodology for Feeds

Proceedings of a workshop held in Ottawa, Canada, 12–14 March 1979

Editors: W.J. Pigden, C.C. Balch, and Michael Graham

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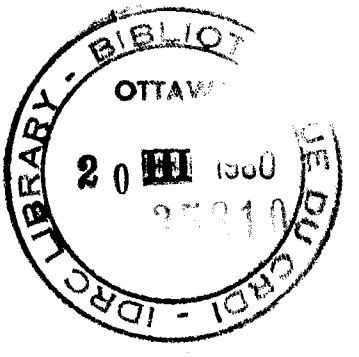

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Prediction of Energy Digestibility of Forages with In Vitro Rumen Fermentation and Fungal Enzyme Systems

Gordon C. Marten[1] and Robert F. Barnes[2]

Two-stage in vitro rumen fermentation (acid-pepsin or neutral-detergent second stage) has become the method of choice for estimating relative energy digestibility of all types of forages. This is true even though numerous sources of variation must be controlled by the individual laboratory to ensure accurate and repeatable in vitro values.

We present two specific recommended in vitro rumen fermentation procedures that were developed collaboratively by members of a North Central Regional Research Committee (NC-64). One of these procedures (A) is a modification of the Tilley and Terry (1963) procedure, principally in that it employs a smaller sample (250 mg rather than 500 mg) and has the option of addition of urea to the bicarbonate buffer to aid digestion of samples having large amounts of soluble carbohydrates. The second procedure (B) is strikingly modified in that it employs direct acidification, without centrifugation, at the end of stage one; this is made possible by substitution of a phosphate buffer (with or without urea) for the original bicarbonate buffer. Both procedures include the optional use of 24-h instead of the usual 48-h second stages (acid-pepsin); this option facilitates routine analysis of large numbers of samples by allowing completion of one or two in vitro runs within a normal work week.

The difficulty of standardizing in vitro rumen fermentation techniques among laboratories, and the expense associated with routine analyses, have resulted in limited application of these techniques for testing of farmers' samples.

Nylon bag in vivo rumen fermentation methods are useful in research programs that require an assessment of the influence of rumen conditions on digestion of limited numbers of samples. Although they have been successfully used for mass screening of forage samples, they have been largely supplanted by the easier-to-standardize in vitro methods.

Recent evidence suggests that fungal cellulase digestibility methods may be able to satisfactorily predict the in vivo digestibility of most forages (after pretreatment with either acid-pepsin or neutral detergent). Cellulase techniques are more convenient than in vitro rumen techniques in that they do not require a source of rumen fluid, and they may be more precise. However, the cellulase techniques appear to be more sensitive to forage species variation, and commercially available cellulases vary considerably in their digestive capacity. More studies are needed to confirm the merit of the cellulase procedures successfully used by several laboratories.

Establishment of In Vitro Rumen Fermentation as an Elite Technique for Forage Quality Evaluation

Pioneering research describing the use of rumen fluid in an "artificial rumen" technique (later called in vitro rumen fermentation) was conducted by Clark and Mott at Purdue University, and reported by Clark (1958). Pigden (Pigden and Bell 1955) was a Canadian pioneer of in vitro rumen fermentation research.

Most in vitro rumen fermentation methods used for forage evaluation are not designed to completely duplicate all rumen conditions, but rather to provide a final result that predicts in vivo parameters. Pigden (1969) outlined two variations of early in vitro rumen fermentation systems: (1) A one-stage digestion in rumen fluid followed by a cellulose determination of the residue to provide digestible cellulose values correlated

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with in vivo available energy; and (2) A short one-stage digestion followed by a cellulose determination on the residue to provide digestible cellulose values correlated with nutritive value index (an estimate of total digestible energy intake per unit of metabolic size of an animal; Donefer et al. 1960).

Although digestible cellulose was often estimated in initial in vitro rumen fermentation research, the almost universal acceptance of the Tilley and Terry (1963) two-stage procedure (or a vast array of modifications thereof) obviated use of cellulose measurements. This technique attempts to measure not only the digestible fibrous fraction of herbage, but the digestible soluble fraction as well. The second stage involves the solubilization of the residue from the first stage by acid-pepsin. The acid-pepsin acts to simulate the in vivo breakdown of feed and microbial protein by the digestive enzymes of the ruminant abomasum.

Also, addition of the second stage (pepsin digestion) to in vitro systems sometimes eliminates the need for development of regressions to predict dry matter loss in vivo from dry matter loss in vitro, in that the two stage in vitro values are often close to in vivo values. Pigden (1969) called the basis for this the "digestion ceiling" concept, because digestion of a forage normally proceeds in vivo until the available cellulose and hemicelluloses of the cell wall constituents are essentially exhausted (their availability being largely determined by the type and extent of lignification).

Van Soest et al. (1966) proposed the use of neutral-detergent solution (neutral-detergent fibre assay) as a substitute for acid-pepsin in the second stage of the Tilley and Terry (1963) procedure. The neutral-detergent solution solubilizes more total dry matter than does acid-pepsin because neutral-detergent solubilizes bacterial cell walls and other endogenous products in addition to protein. Therefore, the Van Soest modification predicts true digestibility rather than apparent digestibility.

While early reports (Simpkins and Baumgardt 1962; Pigden 1969) indicated that two-stage in vitro techniques were unsuitable for estimating in vivo digestibility of silages, Schmid et al. (1975) found that a modification of the Tilley and Terry method (which incorporates urea in the buffer as a nitrogen source) was the best of numerous biological and chemical methods for estimating in vivo digestibility of 51 corn and sorghum silages. Correlations between in vitro and in vivo digestible dry matter of $r = 0.83$ for corn silages and $r = 0.91$ for sorghum silages were obtained. Dowman and Collins (1977) also reported that the Tilley and Terry (1963) procedure accurately predicted the in vivo digestibility of perennial grass silages.

Barnes (1973) presented 16 sets of correlation coefficients and standard errors of estimate between in vitro rumen fermentation and in vivo measurements of digestibility or nutritive value index. In 12 of these cases, correlations (r) between in vitro and in vivo measurements were 0.87 or higher, and they were never lower than 0.71. Standard errors of estimate ($s_{y \cdot x}$) were commonly less than 3.0.

Two-stage in vitro rumen fermentations have become universally recognized as methods of choice to predict digestibility of all types of forages. They are often the standards of excellence against which other procedures are compared for estimating forage digestibility (Terry et al. 1978; McLeod and Minson 1979).

Sources of Variation in Digestibility Estimates via In Vitro Rumen Fermentation Methods

Barnes (1973) reviewed the voluminous literature concerning development, modification, and application of in vitro rumen fermentation methods for estimating forage quality. A brief summary of his systematic review and of more recent reports of the many sources of variation associated with in vitro fermentation systems follows.

Fermentation Vessel

Fermentation vessels that have been primarily used include various vented glass and plastic containers. However, sealed culture vessels have been successfully used because the end products of fermentation apparently do not deter microbial action. Sayre and Van Soest (1972) found that 122 × 28 mm glass centrifuge tubes provided lower in vitro dry matter digestibility (IVDMD) values than did 125 ml Erlenmeyer flasks or 200 × 25 mm glass screwcapped tubes, most likely because they had difficulty providing sufficient agitation of the centrifuge tubes during fermentation (some particles adhered to the rubber stopper during shaking). They also reported fermentation vessel × sample size interactions, and fermentation vessel × forage species interactions. Moore and Mott (1976) reported that polyethylene centrifuge tubes gave higher IVDMD values than did polycarbonate tubes; forage particles formed dense mats that were raised above the level of the media by entrapped gas only in the polycarbonate tubes. They also found that whereas vacuum infiltration of water into samples before inoculation (used by Minson and McLeod

(1972) to reduce floating in polycarbonate tubes) increased IVDMD with both types of tubes, omission of vacuum infiltration gave satisfactory and repeatable results in polyethylene tubes.

Buffer-Nutrient Solution

The buffer-nutrient solution controls pH and supplies nutrients for the rumen microorganisms during fermentation. Buffer-nutrients may include carbohydrates, N, and minerals, but the majority of in vitro procedures rely on "artificial sheep saliva" (McDougall 1948) as the primary buffer. Supplementation of the buffer with N (urea or ammonium sulfate) is recommended for feedstuffs having large quantities of available carbohydrate (Schmid et al. 1969). Nelson et al. (1972) found that rumen fluid efficiency was affected by the diet of the donor animal (legume hay, grass hay, or maize silage) unless both urea and glucose were added to the inocula. These additives had less effect on IVDMD values and on standard deviations when the diet of the donor, the substrate, or both contained a relatively high percentage of crude protein. We have found no need to supplement the buffer-nutrient solution with available energy for assay of any forage (our donor animals receive high-quality alfalfa hay as their primary ration).

Inoculum Source, Processing, and Amount Used

The inoculum represents the greatest source of uncontrolled variation in in vitro rumen fermentation systems. Standard herbage samples must be included in each in vitro run to measure variability among runs and to determine when an entire run should be discarded. Digestive capacity of rumen inoculum may be influenced by animal species, breeds within species, individuals, and within animal variation from time to time. Form or type of donor diet has frequently been found to influence inoculum efficiency. While some investigators have contended that variability in inoculum digestive capacity may be controlled by feeding the donor animal a ration similar to the substrate being tested, Nelson et al. (1972) reported that their study (including donor diets of legume or grass hays or maize silage) did not support this thesis. Also, Grant et al. (1974) found no differences among rumen fluid sources (Philippine water buffalo, Holstein-Red Sindhi cow in the Philippines, or Holstein cow in New York) in capacity to digest a great diversity of forages. On the other hand, the Philippine ruminants provided a more effective rumen fluid for digesting tropical grasses, rice straw, and pineapple pulp to which they were adapted and to which the New York cow was not adapted.

Slyter and Weaver (1972) reported that cellulolytic bacteria possessed less cellulase activity, but no reduction in numbers, when grain was added to the forage diet of the donor animal. Researchers generally agree that donor animals should not receive grain in their diets to achieve best in vitro digestion with minimum variability.

Rumen (cow) microbes were more efficient in digesting grass hays and wheat straw than were cecum (pony) microbes in both in vitro and nylon bag procedures (Koller et al. 1978); however, high-quality alfalfa hay was equally well digested by both inoculum sources.

Attempts have been made to improve the uniformity of rumen inoculum by various processing methods. However, simple straining of collected rumen fluid through cheesecloth is a satisfactory approach, and more complex procedures have not proved greatly advantageous.

The amount of inoculum above a minimum does not affect in vitro values if its ratio with the substrate and buffer is held constant. The rate of fermentation may increase if inoculum amounts are increased without a commensurate increase in buffer-nutrient solution (McLeod and Minson 1969).

Anaerobiosis

Although the freshly collected inoculum can be aerated considerably without loss of activity, exposure to air and unnecessary delays in inoculation should be avoided. Most researchers recommend CO_2 gasing over the inoculated substrate before stoppering of the fermentation vessel with gas release valves or before sealing of screw cap culture tubes. The earlier-used continuous bubbling of CO_2 through the inoculated substrate during incubation is not needed.

pH

The volatile fatty acids produced during in vitro fermentation depress pH; however, pH will normally be maintained within recommended limits of 6.7–6.9 if the donor animal is fed hay. McLeod and Minson (1969) found the highest in vitro digestion at pH 6.7 and lowest at 6.1; a pH of 7.2 gave intermediate digestion. During the acid-pepsin second stage, a pH of 1.2 should be maintained for optimum results.

Temperature

Because the first stage of the in vitro rumen fermentation procedure is temperature-sensitive, incubation should be at 38.5 or 39 °C.

Sample Size

Numerous researchers have found that sample size can at times influence in vitro values. For example, Sayre and Van Soest (1972) found that increasing the substrate sample size from 250 to 500 mg did not change digestibility in centrifuge tubes, but digestibility increased with sample size in Erlenmeyer flasks while it decreased in screwcapped tubes. However, variation can be controlled if the concentrations of buffer-nutrient and rumen inoculum are maintained in constant ratio with the amount of substrate. Although the Tilley and Terry (1963) method calls for a 500-mg sample, many laboratories have successfully utilized 250-mg samples, and our recommended procedures include the smaller sample size.

Sample Preparation

Drying and grinding procedures influence in vitro rumen fermentation much as they do numerous other laboratory assays. Oven drying at excessively high temperatures produces indigestible artifacts. Usually, drying at 65 °C or less is recommended. Fine grinding (about 0.5 mm) is highly desirable, but 1-mm particle size is also satisfactory. McLeod and Minson (1969) found that digestibility of five grass species increased when particle size was reduced from 2 mm to 0.4 mm, except for *Setaria* spp. for which the largest particle size gave the highest digestibility.

Length of Incubation

Usually the curve for rate of digestion in stage one of in vitro rumen fermentation systems is sigmoid, with an initial lag phase of up to 12 h. The curve plateaus at 18–24 h and often becomes asymptotic at about 48 h. Therefore, a 48-h first stage is usually recommended. However, because of greater concentrations of soluble cell contents, the initial rates of digestion of legumes are faster than those of grasses, and the digestion ceiling is reached sooner for legumes. Also, Grant et al. (1974) reported that true DM digestion in vitro increased when incubation time was increased from 48 to 96 h when substrates were tropical grasses, rice straw, and pineapple pulp.

Reduction of incubation time during the second stage from 48 to 24 h has been proposed to facilitate scheduling for routine analyses. Slightly reduced IVDMD values are obtained from a 24-h second stage incubation; however, satisfactory in vitro – in vivo relationships have been reported (Barnes 1966; Larsen and Jones 1973).

[3]These procedures were developed jointly with other members of a subcommittee of NC-64; we have made further additions and modifications.

Recommended Procedures for Determination of In Vitro Dry Matter Digestibility[3]

One of the primary accomplishments of the NC-64 North Central Regional Research Committee in the United States was the documentation of an in vitro rumen fermentation system that could be recommended for estimating in vivo digestibility. The proposed methods outlined below are suggested as having potential for laboratories just initiating an in vitro system of forage evaluation and for use by on-going laboratories to compare with their current procedure. The methods are not claimed to be superior to others, because there is no perfect method adaptable to all circumstances. Indeed, we have already modified the original direct acidification method (method B) to best adapt it to the forage quality laboratory facility in the Agronomy and Plant Genetics Department at the University of Minnesota. However, the procedures described give reproducible in vitro dry matter and organic matter results, as verified by collaborative trials.

Any laboratory engaged in forage quality evaluation must establish its own "standard" procedure, which has been tested and proven reliable through the use of forage samples with known in vivo and in vitro results. Thus, methods other than those outlined below may be more appropriate for use in a specific laboratory.

Modification of the Two-Stage Tilley-Terry Method

Apparatus

(1) Polyethylene or glass 50-ml centrifuge tubes and appropriate racks to hold tubes upright.

(2) Rubber stoppers for item 1 fitted with a gas release valve (Tilley and Terry 1963; Harris 1970).

(3) Fritted glass filtering crucibles (coarse porosity, 40–60 microns and crucible holders for use during filtration.

(4) Permanent laboratory equipment, including pH meter preferably with combination electrodes (usable in centrifuge tubes), analytical balance, drying oven, muffle furnace, centrifuge, incubator, incubation bath, and oxygen-free CO_2 from a regulated source.

(5) Other expendable laboratory supplies such as beakers, Buchner funnel, cheesecloth, Erlenmeyer flasks, glass tubing, graduated cylinders, insulated flask, side arm suction flask, thermometer, and tongs.

Reagents

(1) Buffer-nutrient solution (McDougall 1948). The following quantities are used for 1 litre of

buffer: 9.8 g $NaHCO_3$; 7.0 g $Na_2HPO_4 \cdot 7H_20$ (3.71 g anhydrous); 0.6 g KCl; 0.5 g NaCl; 0.1 g $MgSO_4 \cdot 7H_20$; and 0.5 g urea (optional). Mix in ± 500 ml of distilled water in a 1 litre volumetric flask and stir until dissolved. Use distilled water to bring to volume, and then store. *Just prior* to use, add 0.04 g $CaCl_2$, keep at 39 °C and bubble CO_2 into the solution until pH is 6.8–7.0.

(2) 5% weight/volume mercuric chloride: add 5 g $HgCl_2$ to 100 ml volumetric flask and bring to volume with distilled water.

(3) 1 *N* Na_2CO_3: add 143 g $Na_2CO_3 \cdot 10\ H_2O$ to 1 litre volumetric flask and bring to volume with distilled water.

(4) 1 *N* HCl: add 86 ml concentrated HCl to 1 litre volumetric flask and bring to volume with distilled water.

(5) Acid-pepsin solution (must be freshly prepared for each run): add 2 g of 1:10 000 pepsin or equivalent and 100 ml of 1 *N* HCl to 1 litre flask and bring to volume with distilled water.

(6) Strained whole rumen fluid inoculum: a cow or steer fitted with a rumen fistula should be fed alfalfa hay, or a forage similar to the sample substrates, twice daily. Attention should be given to providing minerals and nitrogen if needed. Feed intake of the donor animal should be limited to approximately 1 kg hay/100 kg of liveweight per feeding. Time of daily feeding and the sampling of rumen contents should remain constant relative to time of feeding. Rumen contents should be obtained in a manner that is routine and standardized for each laboratory. The rumen contents should be processed by squeezing through four layers of cheesecloth and collecting the rumen fluid in a prewarmed insulated container. Rumen fluid should not drop below 39 °C and it should be exposed to an atmosphere of CO_2 whenever possible, preferably by bubbling the CO_2 throughout the fluid.

Inoculum blanks

The strained whole rumen fluid inoculum contains indigestible material that must be taken into account when calculating results. Therefore, inoculum blanks containing buffer-nutrient solution and rumen fluid inoculum are processed through both the fermentation and pepsin incubation stages. Six inoculum blanks interspersed throughout the forage samples are suggested for each in vitro run. The average dry matter residue of the inoculum blanks is used in calculating in vitro dry matter digestibility values.

Procedure

Weigh about 250 mg of sample on weighing paper or similar material and quantitatively transfer it to a 50-ml centrifuge tube. Weigh duplicate samples into dry tared containers for dry matter determination. The dry matter samples are dried for 24 h at 105 °C and hot weighed or weighed after cooling 30 min in a desiccator. Whenever possible, all samples for all runs should be weighed out within a short period of time. Add two 10-ml portions of buffer-nutrient solution to the centrifuge tube containing the 250 mg sample. Gently mix the contents between additions of buffer and wash down the sides of the tube with the second portion of buffer. Allow the tubes to stand at 39 °C for a short period to permit saturation of the substrate. Collect and prepare the rumen fluid inoculum during this time, which should not exceed 30 min. Care must be taken to maintain the pH of the buffer-nutrient solution between 6.8 and 7.0.

Add 5 ml rumen fluid inoculum per tube. Flush the surface of tube contents with CO_2 for approximately 10 sec before stoppering with the gas release valve. Incubate the tubes at 39 °C for 48 h.

Gently rotate the tubes at approximately 2, 4, 20, and 28 h after initiation of incubation to disperse the forage particles.

After 48-h incubation, add 1 ml $HgCl_2$ solution, 2 ml of Na_2CO_3 solution, and centrifuge for 15 min at 2000 × gravity to sediment the suspended dry matter. Decant supernatant carefully to avoid loss of dry matter.

Add 25 ml of acid-pepsin solution and mix gently. Incubate tubes without stoppers for 48 h at 39 °C. Gently rotate tubes to resuspend the residue at approximately 2, 4, 20 and 28 h after initiation of incubation.

After 48 h, filter the tube contents through a tared fritted glass crucible. Dry to constant weight at 105 °C. The residue retained on the filter is undigested dry matter. Crucibles should be weighed after cooling in a desiccator or weighed hot directly from the 105 °C oven on a single pan analytical balance.

Calculations

In vitro dry matter digestibility:

$$\text{IVDMD (\%)} = 100 \times \frac{\text{Samp. DM} - \left(\text{Resid. DM}_{\text{sample}} - \text{Mean resid. DM}_{\text{inoc. blank}}\right)}{\text{Sample DM}}$$

where DM = dry matter.

Verification

Nineteen laboratories participated in a study using this procedure to estimate in vivo digestibility of 12 hays of temperate species (*Medicago sativa*, *Phalaris arundinacea*, *Bromus inermis*, and *Festuca arundinacea*) having in vivo digesti-

bility ranging from 50 to 67% DMD. Average in vitro DMD ranged from 50 to 70%. The correlation coefficients between in vivo and in vitro DMD ranged from 0.79 to 0.97 for individual laboratories, with an average r value of 0.93 and a standard error of estimate of 2.5 (Barnes 1970).

Direct Acidification Method

The direct acidification method is a modification of the method outlined above in that the centrifugation step following the initial 48-h fermentation is eliminated. The use of a phosphate buffer as outlined below greatly facilitates the procedure through avoidance of the excessive frothing that occurs with a bicarbonate buffer.

Reagents

(1) Buffer-nutrient solution (Kansas State buffer). Solutions A and B outlined below can be made in the volumes desired and stored separately for several weeks. *Just prior* to use, add 20 ml of solution B to each litre of solution A. The exact amount of solution B added to solution A should be adjusted so as to obtain a final pH as close to 6.8 as possible. No further adjustment of pH is necessary. Prewarming of solution A to 39 °C is recommended. Solution A (quantities in g/litre distilled water): KH_2PO_4 10.0; $Mg_2SO_4 \cdot 7H_2O$ 0.5; NaCl 0.5; $CaCl_2 \cdot 2H_2O$ 0.1; and Urea (reagent grade) 0.5 (optional). Solution B (quantities in g/100 ml distilled water): Na_2CO_3 15.0; and $Na_2S \cdot 9H_2O$ 1.0.

(2) 6 *N* HCl: add 516 ml concentrated HCl to 1 litre volumetric flask and bring to volume with distilled water.

(3) Pepsin powder: 1:10000 pepsin or equivalent.

Procedure

Using the phosphate buffer, follow the two-stage method outlined above until the end of the initial 48-h fermentation.

After 48-h fermentation, directly acidify by adding 1 ml 6 *N* HCl to each tube and mixing gently. Then add 0.1 g pepsin powder to each tube and mix. (A 1 ml HCl solution containing 0.1% pepsin may be used instead of adding acid and pepsin separately.) Incubate at 39 °C for 48 h without stoppers. Gently rotate tubes to resuspend the residue at about 2, 4, 20, and 28 h after starting incubation.

After 48-h incubation, filter the tube contents through tared fritted glass crucibles. The residue retained on the filter is undigested dry matter. Dry to a constant weight at 105 °C. Crucibles should be weighed after cooling in a desiccator under vacuum or weighed hot directly from the 105 °C oven on a single-pan analytical balance. Calculations remain the same as the two-stage method outlined above.

Verification

We compared methods A and B in Minnesota (using the urea reagent option in both cases) for predicting in vivo DMD of eight grass and alfalfa hays provided by the NC-64 committee and of 25 maize and 26 sorghum silages (Schmid et al. 1975). Mean DMD of the hays measured by in vivo, method A, and method B was 56, 54, and 58%, respectively. Correlations of $r = 0.85$ and 0.87 occurred between A or B and in vivo methods, respectively.

In the silage study, DMD means, standard deviations, and correlations (r) with in vivo DMD were as follows:

	Maize silages			Sorghum silages		
	Mean DMD (%)	S.D.	r	Mean DMD (%)	S.D.	r
In vivo	64	3	–	56	5	–
Method A	72	3	0.83	61	7	0.91
Method B	70	2	0.50	58	6	0.91

The correlations of A versus B values were $r = 0.67$ and 0.95 for maize and sorghum silages, respectively. While all correlations were statistically significant ($p \leq 0.05$), the relationship between method B and in vivo DMD for maize silages was too low for predictive purposes. Thus, we concluded that direct acidification in vitro rumen fermentation cannot be recommended for use with maize silages. We presented simple regression equations (Schmid et al. 1975) to predict in vivo DMD from in vitro DMD in the other three cases; standard errors of prediction ranged from 1.78 to 1.95.

Other Alternatives

Modification of the Filtering System

The use of filter paper and a filter funnel is suggested as an alternative to the use of fritted glass crucibles. Hardened filter paper such as Whatman No. 54[4] is recommended. A filter cone, such as the nickel filter cone (Sargent S-32615)[4] placed

[4]Mention of a trade name, proprietary product, or vendor does not constitute a guarantee or warranty, and does not imply their approval to the exclusion of other products or vendors that may also be suitable.

Table 1. Proposed schedule for routine two-stage in vitro rumen fermentation analyses (direct acidification and 24-h second-stage period).[a]

Day	Morning	Afternoon
Monday	Inoculation (1)	Weigh samples and place in oven (3)
	Weigh residue (0)	
	Calculate results (0)	
Tuesday	Inoculation (2)	Weigh samples and place in oven (3)
	Weigh sample (3)	
	Weigh oven-dry samples (3)	
Wednesday	Add HCl-pepsin (1)	Weigh samples and place in oven (3)
	Weigh oven-dry samples (3)	
Thursday	Add HCl-pepsin (2)	Weigh oven-dry samples (3)
	Filtration (1)	
Friday	Filtration (2)	Calculate results (1)
	Weigh residue (1)	

[a]Based on two runs per week of approximately 150 samples per run, where: 0 = previous week; 1 = run 1 of current week; 2 = run 2 of current week; and 3 = future week.

in a fluted filter funnel, may be used with 9-cm filter papers. Filter paper may be tared as follows: (1) break seal on box of 100 filter papers and allow them to equilibrate to lab conditions; (2) weigh (air dry) individual filter papers indicating weight on each paper; (3) select 10 filter papers at random, dry 24 h at 105 °C, and hot weigh to determine oven-dry weight; and (4) use the dry matter percentage of these 10 filter papers to calculate the oven-dry tare weight of the entire box of filter papers.

Hot weighing individual filter papers after drying reduces the variation in tare weights that may occur from the absorption of moisture by papers during the process of weighing. This is more of a problem when large numbers must be weighed under conditions of high humidity.

Modification of Length of Second-Stage Incubation

Reducing the length of incubation in the acid-pepsin stage from 48 to 24 h is suggested as an alternative for individual laboratories to consider. Such a modification greatly facilitates scheduling for routine analysis of large numbers of samples by allowing in vitro runs to be completed within one normal work week.

Laboratories attempting to adapt such a modification should verify the reliability of their in vitro results, particularly if interlaboratory comparisons are contemplated.

Proposed Schedule

The proposed schedule for routine two-stage in vitro rumen fermentation analyses (direct acidification and 24-h second-stage incubation period) is given in Table 1.

Use of Standard or Index Samples

Standard or index forage substrates in duplicate or triplicate are recommended for inclusion in each in vitro run. Standard forage samples of known high and low in vivo digestibility relative to the forages being tested should be used.

The digestive efficiency of the rumen fluid inoculum and pepsin solution used in a given in vitro run may be assessed with such standards. Procedures for adjustment of in vitro values to correct for run-to-run variation have been suggested

(Baumgardt et al. 1962; Minson and McLeod 1972; Tilley and Terry 1963).

Nitrogen Supplementation of In Vitro Medium

Nitrogen supplementation in the form of urea is included as an optional part of the buffer-nutrient solution in the above procedures. Nitrogen supplementation is particularly recommended when studying feedstuffs containing large quantities of readily available carbohydrates (Schmid et al. 1969). It may also reduce the analytical error associated with variations in rumen inoculum source and amount (Alexander and McGowan 1966). However, N supplementation should be used with caution when evaluating forages and feedstuffs that are not supplemented under practical feeding conditions.

Application of In Vitro Rumen Fermentation Methods

We agree with Pigden (1969) that estimation of absolute digestibility is not the primary application of in vitro rumen fermentation procedures. Rather, these procedures are unparalleled for predicting relative digestibility differences among a wide range of forage species and types. Plant breeders and forage production and management researchers find in vitro rumen fermentation to be a valuable technique, as do ruminant nutritionists who need to predict forage quality of vast numbers or greatly varying forage samples grown in limited quantity. The wide acceptance of in vitro rumen fermentation methods by researchers is evidenced by our recent title search of journal publications. Over 300 publications since 1970 include "in vitro digestibility" or "in vitro dry matter disappearance" in their titles.

Burton and Monson (1978) provided a recent example of success in using in vitro rumen fermentation in a forage breeding program (numerous other examples could be cited). They released 'Tifton 44' Bermuda grass partly on the basis of its 5–6% greater IVDMD than the cultivar 'Costal'. Tifton 44 gave 19% better average daily gain of steers than did Coastal when both were grazed or fed as pellets.

Richardson et al. (1976) reported that the in vitro effect of monensin was reproducible in vivo when they fed both concentrate and forage rations to cattle. When maize cobs and alfalfa were used as substrates in either case, propionic acid production increased while acetic and butyric acid production decreased.

In the Plant Science Research Unit (USDA-SEA-AR) at the University of Minnesota we have published more than 20 journal articles based on use of in vitro rumen fermentation to predict forage digestibility in grazing, plant breeding, and forage management trials.

We agree with McQueen (1978a) that in vitro rumen fermentation has only limited application to routine quality testing of producer samples, because most feed analysis laboratories consider the method to be too time-consuming and too difficult and expensive to use for farm feeds. The difficulty in getting commercial laboratory analysis via the in vitro technique has led the American Forage and Grassland Council's Hay Marketing Task Force to recommend acid-detergent fibre for routine prediction of forage digestibility of farmers' samples.

Status of Nylon Bag Methods for Forage Quality Evaluation

The "nylon bag" methods involve placement of forage substrate, in bags made of indigestible fabrics such as nylon, Dacron, or silk, directly in the rumen (in vivo). Both rate and extent of substrate digestion may be measured by loss of dry matter or specific nutrients after specific incubation periods. Barnes (1973) and Pigden (1969) reviewed the literature concerning development and application of nylon bag methods. Both pointed out that a major shortcoming of these methods is that they are difficult to standardize and that they are subject to considerable variability. Another disadvantage is that they require a large sample size (about 10 g dry matter).

However, several researchers reported high correlations between nylon bag in vivo digestibility and conventional in vivo digestibility and/or in vitro rumen fermentation values of a variety of forages (Barnes 1973; Monson et al. 1969). Also, Burton et al. (1967) released 'Coastcross-1' Bermuda grass largely because of its 12% greater digestibility than 'Coastal,' and the digestibility improvement was accomplished via a selection program using a nylon bag method.

Status of Fungal Enzyme Methods for Forage Digestibility Evaluation

Establishment of Cellulase and Related Techniques

Donefer et al. (1963) compared the efficacy of using the purified enzyme "Cellulase 36" (Rohm and Haas, Philadelphia), aqueous solutions, or both to replace the relatively inconvenient and difficult-to-standardize in vitro rumen fermentation systems for predicting energy digestibility of forages. They used either cellulase or cellulase + pepsin in KH phthalate solution (pH control) as

Table 2. Results obtained by Donefer et al. (1963) using eight temperate legumes and six grasses as substrates.

Laboratory treatment	Correlation (r) with in vivo energy digestibility (%)	Range of absolute digestibility (%)
Cellulase	0.68	22–45
Cellulase + pepsin	0.70	23–49
Acid-pepsin	0.73	24–46
Distilled water	0.61	20–33
12-h in vitro cellulose digestion (rumen fermentation)	0.73	27–55

cellulolytic media, and obtained the results given in Table 2 with eight temperate legumes and six grasses as substrates.

The in vivo energy digestibility of these forages ranged from 53 to 67%. Obviously, the cellulase treatments were not accomplishing anything beyond that of acid-pepsin alone.

In contrast, Jarrige et al. (1970) in France and Guggolz et al. (1971) in Nebraska reported correlations between solubility in cellulase and in vivo DOM or DDM of up to 0.92 and 0.90, respectively, for mixed temperate forages. Jarrige et al. (1970) used a one-stage (24-h) procedure and cellulase (*Basidiomycete* source) supplied by a French company; they found a better prediction of in vivo digestibility via their cellulase procedure than via the Tilley and Terry (1963) in vitro procedure. Guggolz et al. (1971) used a two-stage procedure that employed Onozuka SS cellulase in stage one (72-h) and "Pronase" (protease) in stage two (overnight); they found a poorer prediction of in vivo digestibility by this method than by a modified Tilley and Terry in vitro method. In Minnesota, Schmid et al. (1975) obtained variable correlations (r= –0.42 and +0.72) between solubility of maize silages and sorghum silages, respectively, in cellulase (48-h stage one) and acid-pepsin (24-h stage two) compared to in vivo digestibility. We used Onozuka SS cellulase; our modified Tilley and Terry in vitro method predicted in vivo digestibility of both types of silages far better than did cellulase-acid pepsin. However, Autrey et al. (1975) found that cellulose content of maize silage that had been ensiled with *Trichoderma viride* cellulase was lower (31%) than that of untreated silage (34%).

Other recent reports have substantiated the merits of cellulase techniques for estimating digestibility of many forages. Jones and Hayward (1973) described a one-stage procedure based on a *T. viride* preparation (BDH Ltd., Poole, Dorset, England) that had cellulase, hemicellulase, and proteolytic activity. They reported very satisfactory prediction of in vivo DMD with this assay for five temperate grass species (Table 3), although absolute digestion values were more than 20 percentage units lower than those for in vitro or in vivo DMD. Pulli (1976) confirmed the merits of this approach for grass and a grass-clover mixture in Finland (Table 3). Dowman and Collins (1977) modified the one-stage procedure of Jones and Hayward (1973) by using a finer sample grind (0.75 mm), by increasing the concentration of cellulase, and by reducing the digestion time from 48 to 24 h. This modified method was as good a predictor of DOMD of grass silages as was Tilley and Terry (1963) in vitro, and it was highly correlated with in vivo DOMD (Table 3).

McQueen and Van Soest (1975) found a significant correlation between enzyme digestion and in vivo digestion of 18 grass and legume hays (Table 3), but their endorsement of enzymatic procedures was restricted by the need for separate estimates for individual species or groups of species. They also reported that enzyme sources varied in digestive capacity.

Jones and Hayward (1975) modified their 1973 method to include pretreatment of herbage with acid-pepsin before digestion in cellulase; this method allowed similar prediction equations for both grasses and legumes (Table 3). They tested four fungi sources, and concluded that *T. viride* (BDH Ltd.) was the most active on both herbage and cellulose paper. Adegbola and Paladines (1977) confirmed the observation of Jones and Hayward (1975) that predigestion with acid pepsin improves the solubility of herbage DM in cellulase solutions (Table 3); they used a *T. viride* cellulase from a New Jersey source to digest 11 tropical grasses and legumes. Goto and Minson (1977), using Onozuka SS cellulase and 48-h pepsin pretreatment (rather than the 24 h used by Jones and Hayward 1975), also concluded that in vivo DMD of both tropical and temperate grasses could be accurately predicted by the pepsin-cellulase assay (Table 3).

Terry et al. (1978) further tested the pepsin-cellulase (BDH Ltd.) procedure of Jones and Hayward (1975). They confirmed the reliability, accuracy, and precision of the pepsin-cellulase method for predicting in vivo DMD of grasses (Table 3). However, Terry et al. (1978) agreed

Table 3. Procedures and digestibility relationships for selected fungal enzyme assays of forages reported between 1973 and 1979.

Reference	Forage type	Primary type of digestion	Correlation with in vivo or in vitro digestibility (r)	Prediction equation (y=% in vivo DMD; x=% enzyme DMD)	Error expression
Jones & Hayward (1973)	Temperate grasses	Cellulase	0.92 (vivo)	$y = 0.72x + 33.0$	RSD 2.5
Pulli (1976)	Temperate grass	Cellulase (Jones & Hayward 1973)	0.99 (vitro)	—	RSD 1.3
	Grass & red clover		0.99 (vitro)	—	RSD 0.9
Dowman & Collins (1977)	Grass silage	Cellulase (modified Jones & Hayward 1973)	0.89 (vivo)	$y = 0.58x + 31.6$ (organic matter)	RSD 2.3
McQueen & Van Soest (1975)	Temperate grasses & legumes	Cellulase + hemicellulase	0.80 (vivo)	—	S.E. 6.0
Jones & Hayward (1975)	Temperate grasses	Pepsin + cellulase	0.96 (vitro)	$y = 0.61x + 30.4$	RSD 2.4
	Temperate legumes		0.94 (vitro)	$y = 0.60x + 31.6$	RSD 2.7
Adegbola & Paladines (1977)	Tropical grasses & legumes	Pepsin + cellulase (Jones & Hayward 1975)	0.98 (vivo)	—	$s_{y \cdot x}$ 2.3
Goto & Minson (1977)	Tropical & temperate grasses	Pepsin + cellulase (modified Jones & Hayward 1975)	0.94 (vivo)	$y = 0.69x + 20.3$	RSD 2.7
Terry et al. (1978)	Temperate grasses	Pepsin + cellulase (Jones & Hayward 1975)	0.92 (vivo)	$y = 0.56x + 34.7$	RSD 1.8
McLeod & Minson (1979)	Tropical & temperate grasses	Pepsin + cellulase (Goto & Minson 1977)	0.94 (vivo)	$y = 0.70x + 18.2$	RSD 2.6
	Tropical & temperate legumes		0.91 (vivo)	$y = 0.60x + 22.2$	RSD 3.1
Roughan & Holland (1977)	Temperate grasses & legumes	Neutral-detergent fibre + cellulase	0.98 (vivo)	$y = 0.98x - 10.12$	RSD 2.8

with McQueen and Van Soest (1975) when they reported that the pepsin-cellulase method was decidedly less accurate than the Tilley and Terry (1963) in vitro method for predicting digestibility of temperate legumes or grass-legume mixtures (separate regression equations were needed for each legume species). On the other hand, McLeod and Minson (1979) found that their modified Jones and Hayward (1975) pepsin-cellulase method could be used to estimate the in vivo DMD of legumes with an error only slightly higher than that for grasses, and that the regressions for legumes and grasses were similar (Table 3). They concluded that their results may have differed from those of Terry et al. (1978) in that they used much higher concentrations of cellulase and a superior "broad spectrum Onozuka cellulase." McLeod and Minson (1979) also concluded that in vivo DMD of legumes and grasses can be predicted by the pepsin-cellulase method while using the same equation; however, to eliminate bias they suggested that samples of known digestibility similar to those being tested should be included as standards in each run (some species may require completely different regressions).

Further tests by McLeod and Minson (1979) indicated that fineness of sample grind (1 mm or 0.4 mm), incubation temperature (39 or 50 °C), cellulase concentration (2.5% or 0.625% w/v cellulase solution), and incubation time (24 h or 48 h for each stage) had very little effect on pepsin-cellulase prediction of digestibility. Use of a 0.5 g, rather than 0.2 g, sample size provided lower standard deviations.

Roughan and Holland (1977) in New Zealand claimed that none of the cellulase methods proposed in the literature (including that of Jones and Hayward 1975) solubilized nearly as much organic matter as is digested in vivo, so they decided to try a different approach; this approach was to take the cellulase digestion to completion by using a highly active enzyme preparation. They selected a "potent cellulase solution" prepared from culture filtrates of an artificially-produced mutant of *Trichoderma* identified as the new species, *T. reesei* Simmons (obtained from NLABS Culture Collection of Fungi, Department of Botany, University of Massachusetts). While cell walls of untreated whole, dried forage were either not attacked by this cellulase or only very slowly, cell walls isolated by neutral detergent extraction were readily hydrolyzed. Thus, they substituted neutral detergent for the acid-pepsin of earlier methods (much as Van Soest et al. 1966, substituted neutral detergent solution for acid-pepsin in the Tilley and Terry in vitro rumen fermentation procedure).

This two-stage neutral detergent extraction followed by "exhaustive hydrolysis" with standardized cellulase (Roughan and Holland 1977) was highly correlated with in vivo DMD of grasses and legumes (Table 3). This procedure gave absolute values higher than in vivo DMD! Because they decided that the best way to ensure a continuing supply of active enzyme was to produce it from fungal cultures grown in their own laboratory, they described a detailed procedure for producing the cellulase.

Conclusions Regarding Fungal Enzyme Techniques

We have made the following conclusions regarding the use of fungal enzymes for predicting energy digestibility of forages:

1. Recent evidence indicates that fungal cellulases are often able to predict the digestibility of forages (after pretreatment with either acid-pepsin or neutral detergent) nearly as well as in vitro rumen fermentation.

2. The application of the pepsin-cellulase method described by Jones and Hayward (1975) and the modifications thereof by Goto and Minson (1977) and McLeod and Minson (1979) to legumes as well as grasses needs confirmation in other laboratories. Fungal cellulases appear to be more sensitive to forage species variations than are rumen inocula.

3. The added benefits of use of neutral detergent pretreatment of substrates followed by a standardized "potent cellulase solution" such as described by Roughan and Holland (1977), in order to take the digestion by cellulase to completion, need confirmation.

4. Because some forage species respond differently than others to cellulase enzymes, standard samples of each species under test with known digestibility should be included in each cellulase assay.

5. The activity of the selected cellulase should be measured before routine use via incubation of standard forage samples and/or cellulose paper at several enzyme concentrations.

6. Because cellulases may vary greatly in their capacity to digest forage fibre, further research is needed to standardize the activity of marketed cellulase preparations. Production of standardized cellulases within each analytical laboratory may also resolve this problem.