In Vitro Digestion—Sources of Within- and Between-Trial Variability

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Abstract

Procedures for, and conditions during, inoculum collection and preparation, digestion, and residue recovery stages of the in vitro digestion technique were investigated to determine sources of within- and between-trial variability in digestion coefficients for forages of different chemical composition. Digestion coefficients differed significantly among inoculum preparation times of 1, 2, and 4 hours and for a decline in rumen fluid temperature to 29° in transport. These differences were not uniform across forage species and did not correlate with forage digestibility. Digestion coefficients differed significantly among inoculums prepared from fibrous deer rumen fluids that were strained only, strained and layered, and blended in a Waring blender and filtered through glass wool but did not differ between strained-layered and blended-filtered inoculums of non-fibrous rumen fluid from a fistulated cow. Forage in vitro digestion in the absence of microbial activity (by solubility alone) indicated that forages having more soluble components were least affected by inoculums of different microbial activities, suggesting that between-trial differences be adjusted by a solubility, rather than a digestibility, factor. Inoculum nitrogen concentration did not correspond to between-trial differences in forage digestibility. Size of test tube, but not centrifugation versus filtration method of residue preparation, significantly affected digestion coefficients. However, because the standard large tube size cannot be centrifuged, the two methods of residue recovery would not be comparable unless the products of digestion were transferred from large tubes to centrifuge tubes. The end products of digestion must be stored under refrigeration if filtering proceeds for extended periods of time.

Developing sound management policy for land use requires methods for quantitatively evaluating the capacities of habitats to support domestic ruminants and wildlife populations. Habitat evaluation, which includes animal forage preferences and nutritional values of those forages, is a first step in development of carrying capacity estimates to facilitate management decisions for species that are forage limited. The in vitro digestible dry matter (IVDDM) technique has been extensively used as an inexpensive and rapid method for large-scale evaluation of range forage quality (Pearson 1970, Johnson 1966). We have encountered difficulties applying this technique to large scale habitat evaluations for wild ruminants. These difficulties arise from problems associated with using wild ruminants as inoculum donors, both when comparing digestion coefficients from one in vitro trial with those from another trial and when preparing and handling the inoculum and the digested residues.

Rumen microorganisms require a strictly anaerobic environment, controlled temperature, and energy and nutrients for their survival.

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Depressed IVDDM values occur if certain time and temperature criteria are not met in the inoculum preparation stage. Furthermore, nitrogen concentration (Pichard 1977) and microorganism content (P.J. Van Soest¹, pers. comm.) of rumen inoculum from the same animal on the same diet can vary considerably, thereby altering IVDDM coefficients. Electron microscopy reveals that the degradation of bundle sheath and epidermal cell walls by rumen microorganisms is preceded by their attachment to the plant material (Akin et al. 1974, Akin and Amos 1975, Patterson et al. 1975). Therefore, established methods of preparing rumen fluid for in vitro inoculum may not result in a representative sample of rumen microbial composition, which may vary with the amount of fiber present in both the rumen and the inoculum subsample. In vitro digestion residues of high viscosity do not consistently filter properly. The delays incurred, and the alternate means of recovering the digested dry matter through centrifugation, may also affect IVDDM coefficients. This study investigates aspects of the IVDDM technique that may affect within- and between-trial digestion coefficients and factors involved in across-trial comparisons.

Methods and Procedures

Rumen fluids were obtained by sacrificing a deer or vacuum pumping a fistulated cow that had been maintained for 2 weeks on a native meadow hay diet. To reduce variability of rumen fluid caused by recent drinking or eating and to allow particles in the rumen to break down so that large particles would not clog vacuum pump lines, feed and water were removed approximately 12 hours before rumen fluid was collected. Rumen fluid was flushed with CO₂ from a portable tank, tied off in a large plastic bag, and placed in a preheated thermal chest for transportation. Five control IVDDM trials followed the procedure of Tilly and Terry (1963), except that we "layer" the rumen fluid to prevent contamination of the test samples with rumen particulate matter and to prevent clogging of the autopipette one-way valves. Control rumen fluids were strained through 8 layers of cheese cloth, flushed with CO₂, and allowed to stand undisturbed for 15 minutes. The clear middle layer of the raw fluid was then siphoned into the pH- and temperature-adjusted buffer-nutrient solution to be used as inoculum. Trials were terminated by the addition of 12 ml sodium carbonate (53 g/liter H₂O). Digestion residues were vacuum filtered through Watman GFA paper.

We investigated the effect on digestibilities of delayed preparation or a temperature decline of rumen fluid used as inoculum. Before being transported, a subsample of thoroughly mixed rumen fluid was taken, flushed with CO₂, and capped but was not placed in a thermos. One-half hour later, in the lab, the control fluid was again mixed and flushed with CO₂, and two additional subsamples were taken. These raw fluid samples were then maintained at 39° C with a continuous supply of CO₂ for an additional 1 and 3 hours before being prepared as inoculum. The control fluid and the fluid not placed in a thermos reached temperature lows of 34 and 29° C,

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respectively. These fluids were pipetted into test tubes 1 hour after the rumen pump was started.

Two separate in vitro trials were performed to evaluate methods of inoculum preparation. The first run used deer inoculum. Entire rumen contents were collected, mixed, and divided into three samples. The treatments were (1) strained through eight layers of cheese cloth only, (2) strained through eight layers of cheese cloth and allowed to layer for 15 minutes before the clear middle layer was used as inoculum, and (3) raw fluid blended for 3 minutes in a CO₂-supplied Waring Blender, then filtered through glass wool by CO₂ pressure. Use of three pipetting teams allowed handling all the inoculum simultaneously.

Using inoculum from the entire deer rumen yielded a large quantity of fiber in the fluid, introducing problems similar to those we encountered with rumen fluid from free-ranging elk. Depressed digestion coefficients may result under these conditions (Hobbs et al. 1980). The deer used in this trial was a captive animal; therefore, time of rumen fluid transport and previous diet were identical to those of the second trial.

The second in vitro trial used inoculum from a fistulated cow. This fluid contained very little fiber. Techniques for preparing layered and blended rumen fluid, as described for deer fluid treatments 2 and 3, were evaluated.

We have often encountered problems in filtering in vitro residues and have had to transfer residues to smaller centrifuge tubes to spin down dry matter and suction off supernatant. The purpose of this experiment was to examine the effect both of using the centrifuge technique and of performing digestion in small centrifuge tubes. Tube sizes used were 100 ml × 30 mm × 200 mm versus 50 ml × 25 mm × 130 mm. Sample size was 0.5000 g forage in 50 ml inoculum (40 ml buffer + 10 ml layered cow rumen fluid) and 0.3000 g forage in 30 ml inoculum (24 ml buffer + 6 ml layered cow rumen fluid). Centrifuging was performed three times for 10 minutes at 1900 rpm. The last 2 centrifugations involved resuspending the solid material in water—i.e., three spin-downs, two washings.

Seasonal habitat evaluation on a large scale requires several in vitro trials and that comparisons be made between trials. Rumen inoculum, even from the same animal on the same feed, may vary considerably from one sample to the next. Using a single standard forage to adjust the results of one trial to another, as is often cited in the literature, is open to considerable criticism. In this investigation we evaluated digestibility in the absence of microbial fermentation to determine what proportion of the digestibility of various forages can be attributed to solubility. We also compared the effect of rumen fluid nitrogen level on digestibilities of various forage standards to evaluate the effects of nitrogen level on between-trial variability.

A subsample from thoroughly mixed raw rumen fluid was continuously and vigorously flushed with pure oxygen for 5 minutes to kill the strictly anaerobic microorganisms. This fluid was then processed in the same manner as the control fluid. Forage cell wall, acid detergent fiber, and sulfuric acid lignin components (Goering and Van Soest 1970) were compared with the solubility and fermentation digestion values to determine how forages of differing chemical compositions were affected by inoculums of differing microbial activities.

The nitrogen level of rumen inoculum varies from sample to sample, even when taken from the same animal on the same feeding regime (Pichard 1977). This can alter the digestibility of a given forage and prohibit between-trial comparisons (Pichard 1977). Nitrogen levels of the rumen fluids in the buffer-nutrient solution were analyzed for correlation to between-trial deviation from the mean of standards common to all trials. Standard Kjeldahl nitrogen analysis (A.O.A.C. 1965) was performed in duplicate on 10 ml of inoculum from each in vitro digestion trial.

When residue filtering is slow because of sample viscosity, up to 1 week may be required to filter 450 samples. Our experience has been that high-viscosity of in vitro residues occurs when whole-rumen contents from free-ranging animals are collected for inocu-

lum. We examined the effect of end product storage on IVDD coefficients by storing tubes at room temperature for 0, 4, and days. Another group was refrigerated for 8 days before being filtered.

Test forages were chosen to represent a broad range of chemical compositions and digestibilities. The base set included alfalfa hay, meadow hay, a native grass combination, and oak stems. Additional forage comparisons were desirable in some of the experiments. The other forages included alfalfa leaves and *Potentilla fruticosa*. Only meadow hay and alfalfa hay were used in the residue storage experiment. Five replications of each test forage were included in each experiment. However, unequal subclass frequencies resulted because of methane valve malfunctioning, filter paper breaking under suction, etc.

Data sets with unequal and disproportionate subclass frequencies were subjected to preliminary analyses of variance of pooled main effects to determine whether the interactions involved in the experiment were significant. Analyses of variance were then performed by the weighted squares of means method or the fitting constants method, based on the preliminary significant or non significant interaction, respectively (Bancroft 1968). Tukey's *q* values were used to compute least significant ranges (LSR) to identify significant differences between means (Sokal and Rohlf 1969).

Results

A significant inoculum preparation time delay ($P < 0.05$) and temperature decline ($P < 0.01$) by forage interaction occurred. A time delay of one hour significantly reduced digestion coefficient of all forages except alfalfa hay (Fig. 1). An additional 2-hour time delay further reduced the digestion coefficients of the grass combination and meadow hay forages. Declines in digestibility averaged 6.0 and 11.2 percentage units for 1-hour and 3-hour delays, respectively. The decline in the digestibility of individual forages ranged from 5.9 to 28.4% and did not correlate with their levels of digestion. Compared with the 34°C control inoculum, inoculum that had reached 29°C significantly lowered the digestibility of only one of the three forages tested.

The strain-layer, strain-only, and blend-filter through glass wool methods of preparing deer rumen fluid for in vitro inoculum produced significantly different ($P < 0.001$) digestion coefficients

Fig. 1. The effects of delays in inoculum preparation on the in vitro digestibility of different forages. Use LSR_{trt} to test for significant ($P = 0.05$) differences between treatment means within forage species and LSR_{sp} to compare species within treatments.

Fig. 2. In vitro digestibilities for three methods of preparing highly fibrous deer rumen fluid for inoculum.

and the response was not uniform across forages (Fig. 2). Blending and filtering deer rumen fluid significantly increased in vitro digestibilities of all five forages compared with either of the other two methods of inoculum preparation. Compared with the strain-layer method, the strain only method of preparing inoculum increased the digestibility of the grass combination, decreased the digestibility of the alfalfa hay, and did not significantly affect the digestibility of the other three forages. In this experiment we collected the entire deer rumen, which, as we found for rumens of free-ranging wild elk, contained a large amount of undigested fiber in the rumen fluid.

Strain-layer versus the blend-filter method of inoculum preparation was repeated with rumen fluid obtained from a fistulated cow, which contained very little fiber. Digestibilities for the five forages did not significantly differ between treatments.

A significant ($P < 0.01$) forage-by-treatment interaction was observed for the small tubes centrifuged, small tubes filtered, and large tubes filtered. LSR values indicate that the significance resulted primarily from the lower digestion coefficients obtained with the larger tube size rather than from differences associated with the centrifuge versus filtering method of dry matter recovery (Fig. 3). However, because the standard large tube size cannot be centrifuged, the two methods of residue recovery would not be comparable unless the products of digestion were transferred from large tubes to centrifuge tubes. Significant differences between

Fig. 3. The effect of tube size and the filter and centrifuge methods of dry matter recovery on in vitro digestion coefficients.

digestion coefficients obtained with small tubes centrifuged and large tubes filtered were indicated for all six forages tested.

The proportion of in vitro digestion attributable to solubility versus microbial activity was significantly ($P < 0.001$) affected by forage species (Table 1). Microbial digestion accounted for only 8.7% of the digestibility of alfalfa hay but for 52.3% of the digestibility of the grass combination. The decline in forage digestibility after the 3-hour inoculum preparation time delay (Fig. 1) followed a pattern similar to that of the proportion of forage digested by microbial activity—i.e., forages with high in vitro solubility were affected less by inoculum of different microbial activity. However, the wide range in in vitro bound solubles indicates poor correspondence between total cell solubles and in vitro solubility and appears to be related to forage lignin content (Table 1). In vitro bound solubles are considered the difference between total cell solubles, as determined by the Goering and Van Soest (1970) cell wall extraction procedure, and the soluble fraction, determined by in vitro digestion in the absence of microbial activity.

The within-trial deviation from a forages-across-trial mean digestibility was compared with the same calculations for inoculum protein level. No relationship was apparent between inoculum protein concentration and between-trial differences in digestion coefficients of the four cow-inoculum trials (Table 2). The layered

Table 1. Forage structural composition compared with various solubility parameters.

Forage	Cell wall	Acid detergent fiber	Lignin	Solubles	IVDDM (%)	In vitro ¹ solubility	% of digestion ² by solubility	In vitro ³ Bound solubles
Grass Combination	70.9	38.4	5.2	29.1	50.0	20.7	47.7	8.4
Alfalfa Hay	43.8	30.9	6.5	56.2	63.1	49.8	91.3	6.4
Oak Stems	62.2	42.5	19.6	37.8	29.1	17.5	68.2	20.3
Meadow Hay	68.9	34.7	3.3	31.1	67.8	30.4	47.2	0.7
Alfalfa Leaves	31.0	20.6	8.1	69.0	—	53.1	—	15.9

¹In vitro digestion without microbial activity.

LSR = 3.5 for within species comparisons of this column to the IVDDM (%) column.

²100-value = % digested by microorganisms.

³[(100-CWC) - in vitro solubility].

Table 2. Inoculum crude protein level compared with between in vitro trial deviation in forage digestibility.

Trial	Deviation from ¹ IVDDM mean	Inoculum protein ² (%)	Deviation from protein mean
I (cow)	+1.8	0.029	-0.001
II (cow)	-1.0	0.047	+0.016
III (cow)	+1.5	0.021	-0.010
IV (cow)	-2.0	0.026	-0.005
V (Deer layered ³)	-7.1	0.030	-0.001
V (Deer blended ³)	12.3	0.055	+0.024

¹Average deviation of 5 forages.

²Inoculum protein determined on rumen fluid in the buffer nutrient solution.

³Digestion and inoculum protein means were calculated from the four cow inoculated trials only. Deviations from the means for the deer trials were calculated as deviations from the cow trial means.

deer inoculum had a protein concentration similar to the mean for the four cow inoculums but produced depressed digestion coefficients. The same deer inoculum prepared by blending had a high protein concentration and produced elevated digestion coefficients.

A filtering delay of 4 days for samples stored at room temperature did not significantly alter digestion coefficients (Fig. 4). However, the storage of samples at room temperature for 8 days significantly reduced digestion coefficients. The digestion coefficients of refrigerated samples were not affected by storage for 8 days.

Discussion

The inoculum preparation stage of in vitro digestion trials may influence digestion coefficients because of the difficulty both of meeting time and temperature criteria in rumen fluid transportation and of the technique of handling the rumen fluid before mixing it with the buffer solution. Schwartz and Nagy (1972) reported a significant decrease in digestibility with inoculum stored for 2 hours or more, whereas Prins and Geelen (1971) found storing rumen fluid up to 2 hours did not significantly change cellulolytic or amylolytic activity. Our data indicate that a temperature drop to 29° C or a time delay of 2-hours significantly reduces digestion coefficients. This decline is not uniform across forage species and does not correlate with forage digestibility. For example, the digestibilities of alfalfa hay and meadow hay were 63.1% and 67.8%, respectively; whereas the declines in digestion after a 4-hour inoculum-preparation delay were 5.85% and 28.9%, respectively. Forage solubility correlated better than total digestibility with the reductions in digestibility. The proportion of digestion that could be accounted for by solubility alone was 91.3, 68.2, 47.7, and 47.2 percent, compared with a decline in digestion of 5.9, 25.4, 28.5, and 28.9 percent after a 4-hour inoculum-preparation delay. Plants having the more soluble components are least affected by inoculums of different microbial activity, which may occur either through temperature decline or time delays in transporting inoculum, the method of preparing inoculum, or the microbial composition of rumen fluid at the time of sampling.

The results of one in vitro trial must often be compared with those of another. This is often accomplished by adjusting test forage values based on digestion coefficients of standards that have been included in both trials. Our results indicate that this procedure would bias relative digestibilities because two forages of equal digestibility can be affected differently by the fermentive capacities of inoculums because of different proportions of available digestible fiber. Solubility of forage appears to correlate better with differences in the fermentive capacities of inoculums because one parameter takes into account the complex interaction among cell solubles, digestible fiber, and lignin-bound fractions of a forage in the digestion process. Determining in vitro solubility requires additional effort, but fiber analysis of forages under evaluation is

Fig. 4. The effect of filtering delays and method of end product storage on in vitro digestion coefficients.

routinely performed. Cell solubles (the inverse of cell wall constituents) are the readily digestible portion of a forage, whereas lignin limits the extent of digestion. Therefore, it is plausible to assume that cell solubles and lignin may be two variables that could be used effectively to adjust for between-trial variation in digestibility.

Pichard (1977) found surprisingly high variability in various nitrogen fractions of inoculum samples taken under exactly the same conditions. The nitrogen level of inoculums in this study ranged from only 0.021% to 0.047% and did not correspond with between-trial variability in digestion coefficients. Protein level of blended deer inoculum was only 0.024 percentage units above the mean protein level for cow inoculums but produced much higher digestion coefficients because of the high microbial activity of blended fibrous rumen fluid. Nitrogen in rumen fluid may be in many forms and either of plant or microbial origin. The microbial activity of inoculum is thus more important than total nitrogen as a factor in in vitro between-trial variability.

Van Soest² (pers. comm.) observed that blending rumen fluid in a Waring blender and straining it through glass wool reduces the variability among in vitro trials. The purpose of this procedure is to dislodge microorganisms that attach to the forage fiber and would otherwise be discarded when the fluid is strained. Electron microscopy indicates that the degradation of bundle sheath and epidermal cell walls is preceded by attachment of bacteria to the plant cell (Akin et al. 1974, Akin and Amos 1975, Patterson et al. 1975). The much higher digestion coefficients obtained with the highly fibrous deer fluid that was blended, compared with the same fluid prepared by the strain-only or layered technique, suggest that significant numbers of microorganisms were discarded in the filtrate of the last two techniques. Blending rumen fluid to dislodge microorganisms from the fiber can be used to obtain a more active and representative sample of rumen condition at the time of sampling.

Because we have obtained depressed digestion coefficients with inoculum from wild elk rumens containing large amount of fiber, the use of the blending technique at first appeared especially applicable to wildlife species. Discrepancies occur, however, when comparisons of the layer and the blend methods of inoculum preparation are made between in vitro trials inoculated with deer rumen fluid and cow rumen fluid. Digestibilities with blended deer fluid were considerably higher than those from either of the cow-

²P.J. Van Soest. Animal Science Department, Cornell University, Ithaca, New York.

inoculated trials. Digestibilities with layered deer fluid were both higher and lower than the cow-inoculated trials. The grass combination, oak stems, and meadow hay digestibilities were consistently lower, but alfalfa hay and alfalfa leaf digestibilities were equal or higher. The change in digestibilities corresponded with the solubility of these forages. Forages having more available digestible fiber and less soluble components gave depressed values when inoculated with fibrous fluid that had not been blended. However, blending fibrous rumen fluid produced inflated digestion coefficients. Although blended rumen fluid is more representative of rumen condition *at the time of sampling*, it does not represent the mean *in vivo* digestibility of a forage over time. *In vivo* digestion trials proceed for 7 to 10 days.

Repeatability and correlation to *in vivo* digestion are the important factors in *in vitro* digestions. Consider the following: (1) differences in the digestive capacity of inoculums are the result of sampling time after feeding and diet composition (Troelson and Hanel 1966, Bryant and Robinson 1968, Pichard 1977). We have observed large differences in digestion coefficients for wild elk inoculated in *in vitro* trials where the fiber content of the rums varied; (2) it does not matter what animal species is used as a source of *in vitro* inoculum as long as it correlates well with the *in vivo* digestion of the particular ruminant species being studied. Therefore, if the *in vitro* to *in vivo* regression is developed using cow-inoculated *in vitro* trials and elk *in vivo* digestion trials, cow inoculum can be used in later *in vitro* trials for correlation to the regression that has been established. The advantage is that one can control more closely feeding regime, time of inoculum collection after feeding and drinking, and time of transportation of inoculum to the lab. Although blended rumen fluid is a better representation of the microbial population in the rumen at a particular time, these values are inflated compared with *in vivo* digestion coefficients that represent a mean digestion over a 7- to 10-day trial. Repeatability and correlation with *in vivo* digestion improves with the use of cow rumen fluid obtained through a fistula. We allow raw rumen fluid to layer to reduce the variability among replications and to eliminate contamination by particulate matter. This provides a more homogenous, clean inoculum and prevents clogging and/or improper closing of autopipette one-way valves.

The differences in digestion coefficients for different tube size, and therefore the filter versus centrifuge technique of residue prep-

arations, indicates the need for strict adherence to one procedure. *In vitro* digestion comparisons between laboratories cannot be made until standard procedures are adopted for all stages of the *in vitro* digestion technique.

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GRADUATE RESEARCH ASSISTANT

GRADUATE RESEARCH ASSISTANT: (M.S. In Entomology) Field and laboratory studies of the effects of prescribed burning on insects associated with range plants. Involves intensive population sampling of insects in the field and simulation of fire stress on host plants in the lab. Salary \$6,000/year, out-of-state tuition waived. Available May 1982. Interested persons with Bachelor's degree in Biology, Entomology, Range Science or related areas encouraged to apply. Write or call Dr. James K. Wangberg, Department of Entomology, Texas Tech University, Lubbock, Texas 79409, (806) 742-2828.