OPTIMUM COMBINATION OF TIME AND RUMEN FLUID IN THREE IN VITRO TECHNIQUES USED TO PREDICT FORAGE DIGESTIBILITY

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OPTIMUM COMBINATION OF TIME AND RUMEN FLUID IN THREE *IN VITRO* TECHNIQUES USED TO PREDICT FORAGE DIGESTIBILITY

INTRODUCTION

The problem of assessing to forages a nutritive value in relation to its usefullness or digestibility when fed to ruminant animals has intrigued animal scientists for several generations. Forages have been shown to vary in nutritive value not only between species but also at various stages of maturity, as well as in the different forms in which they are processed and fed such as pasture, hay or silage.

The problem of concern in this study is one of improving the estimation of forage digestibility with specific application to utilization by ruminant animals.

Underlying this problem is the variability in forage composition. Inherent factors influencing the composition include: environmental conditions such as soil type and climatic conditions; soil fertility; genotype; and stage of maturity. In addition, these factors are in a constant state of change which adds to the complexity of the problem. In view of the numerous species and strains of forages utilized by ruminant animals, the problem of differentiating forages as to digestibility becomes one of attempting

to separate the forages according to the intricacies of the chemical composition.

In vivo digestibility trials comprise the only reliable index at the present time for classifying forages as to nutrient availability in ruminant rations. Although this procedure is feasible with a small number of forages, its practical application is limited by the time and cost that would be involved in appraising the manifold forages utilized by ruminant animals.

Ration formulation requires a knowledge of the chemical composition of the feedstuff. An attempt has also been made to utilize this knowledge as in vitro method of predicting forage digestibility. The usual chemical analysis of a forage divides the nutrients into a few general classes which include: moisture content; ash; protein; fiber; fat; and nitrogen-free extract. Although in some individual studies, high correlations have been shown to exist between one or more of the in vitro groups and certain in vivo measurements, the use of chemical analysis has not been proven to provide a reliable estimate of forage digestibility.

Another approach to forage evaluation has involved incubation in the laboratory of rumen contents with various forage substrates.

The "artificial rumen" has provided a method of studying the digestibility of individual components of the forage as well as the dry matter digestibility. In vitro fermentations have also been utilized to study the various factors influencing digestion in the intact rumen. Because the artificial rumen does not exactly duplicate conditions existing in the normal rumen, certain limitations are placed on the relevance of the in vitro data to in vivo application. Precision, speed and cost have provided the impetus to the use of this method as a useable tool in forage evaluation.

An absolute method has not yet been devised that will duplicate those results occurring in vivo. Since the initiation of artificial rumen studies, many such systems, and subsequent variations, have been developed in an endeavor to improve the precision of predicting the digestibility of forages.

Since many variables are encountered in the diverse in vitro rumen fermentation procedures currently being used, this study was initiated to determine if there was a difference between three divergent in vitro systems and to resolve the optimum combination of rumen liquor and length of fermentation in order to predict the digestibility of the various roughages.

LITERATURE REVIEW

In Vitro Techniques

Although a diversity of in vitro systems has been employed to study fermentation in the laboratory, they all can be grouped into two main categories, which are: (1) the all-glass, impermeable system; and (2) the membranous, semipermeable system.

Pearson and Smith in 1943 (57, p. 142-148) incubated an undiluted sample of whole rumen contents in an impermeable system in one of the first reported in vitro procedures. Marston (81, p. 564-574), striving to more closely replicate conditions of the intact rumen, incubated rumen contents under anaerobic conditions, maintained a constant physiological pH and suspended the rumen liquor in a mineral solution.

Although at the time very little was known concerning the absorption of fermentation end-products, Louw et al. (80, p. 478-480) elaborated on this concept by utilizing a semipermeable membrane which allowed the end-products to diffuse out of the fermentation vessel. By approximating absorption from the rumen, it was assumed that any inhibitory end-products which would tend to reduce the in vitro activity of the microorganisms would be eliminated. This was one of the first semipermeable systems to be evolved; since then

this system has been used extensively to study rumen fermentation.

Numerous diversities and composites of these basic systems have been designed. A combination of the techniques described by Louw et al. (80, p. 478-480) and Marston (81, p. 566) were used by Wasserman et al. (103, p. 572) to study the effect of antibiotics on in vitro cellulose digestion. Huhtanen et al. (60, p. 328) proposed a simpler and less cumbersome apparatus for the study of fiber breakdown. Warner (102, p. 739-746) applied several criteria of normal rumen function to an in vitro system of the semipermeable type that met these criteria with reasonable success for periods of about 8 hours. Also with a semipermeable type of in vitro procedure, Huhtanen and Elliott (59, p. 1180) investigated several of the variables influencing in vitro cellulose digestion.

Burroughs et al. (27, p. 650-651; 28, p. 674; 29, p. 19-23; 30, p. 698; 31, p. 523) have worked extensively with the impermeable version of the artificial rumen. In this procedure the substrate was incubated with whole rumen liquor diluted with a mineral solution comparable to ruminant saliva. This system has been used to examine: factors influencing cellulose digestion (29, p. 9-24); urea utilization (27, p. 650-651); the effects of minerals (30, p. 693-705; and digestion of good and poor quality roughages (32, p. 513-522).

Bentley et al. (20, p. 585-590) fractionated rumen liquor in an effort to concentrate the cellulolytic-aiding factor(s) present in rumen

juice. This preliminary research led to a more complete evaluation of the cellulolytic-factor activity of short-chain fatty acids and their relationship to non-protein nitrogen utilization (19, p. 389-400). Similar procedures have been used by: Hershberger, Bentley and Moxon (18, p. 663) to study nitrogen availability; Baker et al. (6, p. 656-661) investigating the physical properties of cellulose; Hershberger et al. (57, p. 770-779) comparing forage digestibility in vitro and in vivo; and Dehority et al. (42, p. 1098-1109) studying the cellulolytic fraction of rumen bacteria.

In addition to the in vitro systems just discussed, manometric techniques have been conceived and utilized both with washed cell suspensions (44, p. 825-831; 94, p. 41-45) and whole rumen contents (83, p. 106-110). Basically the manometric technique is a divergence of the all-glass impermeable system, in which rumen contents are incubated under conditions similar to those of the rumen, but differs to the extent that gas production is used as a criteria of in vitro fermentation activity.

Olson (85, p. 349) found the gas phase from the rumen of a cow to be composed of 68.12% carbon dioxide, 17.20% methane, 12.73% nitrogen, 1.80% oxygen, 0.05% carbon monoxide and 0.10% miscellaneous. However, the composition of the gases has been found to be influenced by the kind and amount of ration consumed as well as varying progressively with time after feeding (65, p. 701-703).

Hungate et al. (67, p. 161-173) have estimated that a one-thousand pound bovine animal forms 1.2 to 2.0 liters of gas per minute during fermentation in the rumen and reticulum.

The various manometric techniques have utilized the total gas production (83, p. 106-110) and methane production (69, p. 196-201) as measurements of fermentation activity. Research involving manometric techniques has included: comparison of the fermentation rates in bloated and normal cattle (67, p. 161-173); comparison of the fermentation rates between species of cattle (69, p. 196-201; 91, p. 417-420); evaluation of the nutritional value of corn silage (26, p. 34-38); investigation of the fate of formate in rumen contents (34, p. 525-536); and a demonstration of the influence of low levels of antibiotics on the rumen microorganisms (68, p. 997-1002).

Without standardization of the innumerable in vitro systems, results produced from one laboratory may not always be readily reproduced in another laboratory using a comparable system. Consequently, research has been conducted in several laboratories contrasting the various in vitro procedures in an endeavor to evolve an artificial rumen that will produce analogous results in all laboratories.

Walker (101, p. 193-197) weighed the relative merits of several published methods, especially for simplicity, repeatability and accuracy to differentiate forage digestibilities. The method of

Huhtanen et al. (60, p. 328-335) was unsatisfactory due to: mixing difficulties; excessive gas formation in the membranous sac; variability; reported work could not be reproduced; unsuitable for dry matter digestion; and rupture of the dialysis sac with prolonged digestion over 48-72 hours. Warner's method (102, p. 733-748) varied considerably from one trial to another, possibly due to leaks in the apparatus and mixing difficulties. The procedure used by Lambert and Jacobson (75, p. 509-514) was rejected because of the difficulties encountered in obtaining repeatable results. The design finally resolved by Walker (101, p. 193-197) was a variation of the impermeable type. This procedure was selected as it enabled a more thorough mixing of the sample with the rumen fluid and salt solution, in addition to obtaining samples more representative of the whole mixture at the end of the digestion period. Even with the in vitro procedure finally adopted by Walker, oat straw produced extremely variable results; however, this variability was also reflected in the in vivo consumption of the straw.

Baumgardt, Cason and Taylor (15, p. 59-61) compared the relative accuracy of several in vitro methods. The method of Pigden and Bell (92, p. 1239) produced an estimate of the total digestible nutrients (TDN) considerably lower than the actual value, which made it necessary to develop new prediction equations for each forage tested. Moreover, the carbohydrate analysis was too tedious and the results

too variable for general acceptance of the method. Another method proposed by Thurman and Wehunt (100, p. 302) could not be correlated with any of the in vivo measures of animal performance. The mathematical equations of Schneider et al. (96, p. 77) for the estimation of TDN were not correlated with in vivo TDN; however, a significant correlation was obtained with each of the other in vivo values used in the comparison. From this preliminary study, Baumgardt, Taylor and Cason (16, p. 62-68) refined a procedure of the impermeable type for simplicity, standardization and repeatability. This is one of the three methods being studied in this report.

El-Shazly, Dehority and Johnson (46, p. 1445-1451) contrasted the impermeable, semipermeable and the continuous flow procedures. They found no major differences between the various systems and concluded that the all-glass apparatus appeared to be advantageous particularly because of its simplicity.

The comprehensive studies of in vitro fermentation procedures, just cited, provide an indication as to the amount of research being executed to standardize a repeatable procedure that will allow a more meaningful interpretation of the results reported between laboratories.

Length of Fermentation

The fermentative nature of the rumen would suggest a considerable number of variables that may influence the digestive processes

at any given time. One such variable is the length of fermentation period required in in vitro fermentations.

Phillips et al. (91, p. 417-420) indicated that the amount of digestion in vivo is dependent upon the rate of digestion and the length of time the food remains in the rumen. By use of a manometric procedure described by Hungate et al. (69, p. 196-201) in conjunction with live animal digestibility trials, Phillips et al. (91, p. 417-420) estimated that 50 to 70% of the total dry matter disappearing from the ingested feed is accounted for by rumen fermentation. This estimate was similar to an earlier figure of 70% by Carroll and Hungate in 1954 (33, p. 205-214).

In most investigations, as might be expected, the total digestion of a particular substrate is directly related to length of fermentation.

Baumgardt et al. (16, p. 65-66) found that a longer fermentation period was necessary to obtain maximum digestion of grass hay than was required for alfalfa hay. This was in agreement with data presented by Quicke et al. (94, p. 282). Even though there was a slight increase in cellulose digestion by extending the fermentation period to 48 hours, Baumgardt et al. (16, p. 66) observed that the highest rate of digestion occurred between 12 and 24 hours. Furthermore, the 24-hour fermentation was significantly correlated to in vivo digestible energy. From these results, they concluded that no advantage would be gained by extending the fermentation period to 48 hours.

Donefer et al. (45, p. 547-548) determined the digestion of cellulose in fermentation intervals of 3, 6, 12, 24 and 48 hours. The data implied that the lag periods in the initial rate of digestion were directly associated with forage species, in as much as the relative rate of digestion after 12 hours did not differ significantly. Moreover, a high correlation existed between the 12-hour in vitro cellulose digestibility and relative intake in vivo. Crampton et al. (37, p. 538-544) discussed the relative intake as a basis from which the Nutritive Value Index of a forage was proposed. The significance of the 12-hour fermentation period to the rate of digestion and the correlation to in vivo data was further confirmed in studies by Johnson et al. (72, p. 250).

Lloyd et al. (79, p. 470) found that the lag period in fermentation rate also included an effect of maturity differences within a particular plant strain. Decrease of cellulose digestibility directly related to advancing maturity has also been found by Kamstra et al. (73, p. 203) and Quicke and Bentley (93, p. 368-369).

Recently, Dehority et al. (41, p. 510-511) observed that the most rapid rate of hemicellulose and pectin digestion occurred between 12 and 24 hours in the case of timothy and orchardgrass hay and up to 12 hours with the alfalfa hay. However, very little additional digestion was obtained after 24 hours with any of the three forages. The two constituents, hemicellulose and pectin, plus cellulose comprised

from 45 to 59% of the total composition of the alfalfa samples analyzed by Lagowski et al. (74, p. 310). Dehority et al. (41, p. 510) demonstrated that lignin may obstruct the digestion of these three constituents, in as much as both the rate and total digestion was increased by decreasing the particle size. Church and Petersen (36, p. 89) noted an opposite effect in a 48-hour fermentation period with alfalfa hay as a substrate, but no difference was observed with peavine hay as the substrate. These two studies may also serve to emphasize the disparities in digestibility among the various forages.

Concentration of Rumen Fluid

The concentration of rumen fluid used as inoculum in the fermentation flasks is a variable which has also received considerable attention. Huhtanen and Elliott (59, p. 1183) obtained no effect on the cellulose digestion of alfalfa meal by diluting the rumen fluid to one-fifth of the original volume; but below one-fifth, digestion was progressively decreased. Church and Petersen (36, p. 85) found that the percent dry matter and percent cellulose digestion both increased in a linear manner as rumen liquor levels were increased from 20 ml to 120 ml, using an alta fescue grass substrate. Walker (101, p. 195) could discern little difference in percent digestibility as a consequence of altering the concentration of rumen fluid.

The variables just discussed are but a few of those encountered in research involving the artificial rumen. Church and Petersen (36, p. 91) suggested standardization of not only concentration of rumen liquor, length of fermentation period, and the in vitro technique, but that also the source of rumen liquor, pH, quantity of substrate, mineral solution and particle size should be extensively studied with standardization of a repeatable procedure as the goal.

Criteria of In Vitro Function

Cellulose Digestion

Cellulose comprises a considerable portion of all forages; and accordingly, it has been studied quite extensively as a constituent of roughages, in addition to being utilized as a criterion of in vitro fermentation activity.

Lloyd et al. (79, p. 468-473) utilized the digestion of cellulose to discern the capabilities of the Nutritive Value Index to predict the digestibility of various maturity stages of timothy hay. Burroughs et al. (30, p. 703) reported the effect of minerals on cellulose digestion. Lambert and Jacobson (75, p. 509-514) investigated the influence of antibiotics on cellulose digestion.

Dry Matter Digestion

The in vitro digestion of dry matter has not been utilized to the same extent as the digestion of cellulose; nevertheless, dry matter digestion can make an important contribution to the overall evaluation of roughage digestibility.

Distinguishing among various criteria to measure in vitro activity, Walker (101, p. 194-195) concluded that the dry matter digestion yields more information than any other single estimate in comparing forage digestibilities.

A high correlation between in vitro dry matter and in vitro cellulose digestion was observed by Church and Petersen (36, p. 82). Bowden (23, p.69) confirmed and complemented the in vitro correlation of Church and Petersen with the report of a high correlation also between in vitro and in vivo dry matter digestion. Furthermore, in vitro dry matter digestion was just as accurate as in vitro cellulose digestion for appraising the in vivo digestibility of forages.

In the first of a sequential investigation, Baumgardt and Hill (17, p. 943) considered the factors influencing dry matter digestion. The ensuing comparison of various in vitro techniques by Baumgardt et al. (14, p. 1205) utilized as criteria the digestion of dry matter. Clark and Mott (35, p. 127) extended this work with forages of known nutritive value. A significant in vitro correlation was established

with fresh forage samples, but dried and stored samples of this same forage failed to yield a significant correlation six months later.

A high correlation between dry matter digestibility in vitro and in vivo was also corroborated by Asplund et al. (3, p. 177-179).

Gas Production

Reactions involving the mixed microorganism population as well as individual cultures from the rumen have been critically examined from the standpoint of the gas produced.

Hungate (65, p. 702) has suggested the production of methane as a useful measurement of fermentation activity. Moreover, he presents a method for extrapolating the quantity of methane produced to the amounts of acid and carbon dioxide produced. He demonstrated that this could be accomplished by calculating the methane produced from an earlier experiment in which the acids had been determined (33, p. 205-214) and compared this to his research in 1960 (91, p. 417-420). The actual methane produced was within about 1% of his calculations.

From a study in 1960, Hungate et al. (69, p. 196-201) proved that the production of methane could account for the diversity in the fermentation products produced by the least and by the most digestible materials, with the least digestible materials yielding a greater production of methane. The data was also suggestive of a direct

correlation between the percentage of acids produced and the rate of fermentation, but a negative correlation of carbon dioxide and methane with rate of fermentation.

Armstrong and Blaxter (2, p. 253) demonstrated that methane may very well indicate the extent of fermentative activity in the normal rumen. After 4 days of starvation, 2.03 liters of methane were produced from the rumen of starved sheep as compared to 26-28 liters produced by a fed animal. At the end of the 12th day of starvation, there were still 0.58 liters of methane production.

Bull (26, p. 35) utilized gas production as a guide to VFA production. He showed that the initial gas production reflected the quantity of available carbohydrates and that propionic acid production was directly related to gas production. The rate of gas production in this study reflected lag periods very similar to those reported by Lloyd (79, p. 468-473) which were directly related to stage of maturity. In both cases, the difference in the apparent digestibility of the carbohydrate fractions was offered as an explanation of the observed differences in the lag periods.

EXPERIMENTAL PROCEDURE

Forages

Three forages were used in this study: alfalfa and bromegrass supplied by Macdonald College (McGill University), Canada, and crested wheatgrass from the Squaw Butte Branch of the Oregon State Agricultural Experiment Station at Burns. These three forages were selected to give three distinctly different nutritive values.

Artificial Rumen Systems

Three similar in vitro systems were studied to determine if differences existed in their ability to digest the dry matter content of various forages.

A modification of the in vitro procedure reported by Barnett and Reid (11, p. 315-316) has been used in this laboratory by Church and Petersen (36, p. 81) and Bowden (23, p. 19-20). In this system, one gram of the substrate was incubated in 250 ml centrifuge bottles with 30 ml of an artificial saliva solution, the appropriate amount of rumen liquor and the total volume brought to 100 ml with distilled water. Before the rumen liquor was added, the fermentation flasks were allowed to pre-warm in a water bath at a temperature of 39° C, and were flushed with carbon dioxide for approximately an hour while the

rumen liquor was being prepared. After inoculation with the rumen fluid, the carbon dioxide was bubbled through the fermentation contents for the duration of the fermentation period. Hereafter this method will be referred to as the OSU method.

The second system was essentially the same as was reported by Baumgardt, Taylor and Cason (16, p. 63-64). This procedure differed from the first only in that there was no dilution with distilled water and the flasks were not bubbled with carbon dioxide during the fermentation period. However, they were initially flushed with carbon dioxide prior to inoculation. Hereafter this method will be referred to as the NJ Method.

The third system is a manometric technique similar to that reported by Bull (26, p. 23-24). The fermentation flasks were prepared as were those in the first procedure. After the flasks were inoculated with the rumen fluid, they were allowed to equilibrate for 15 minutes before being connected to the manometer tubes. The manometric tubes were prepared in this laboratory and were approximately 57.6 cm in length and one inch in diameter. They were calibrated in 10 ml divisions and filled with a solution of concentrated sulfuric acid diluted with distilled water to a pH of about 2.0, to which a dye was added for convenience of reading. Plastic intravenous tubing was used to connect the fermentation flasks to the manometer tubes. The gas produced pushed the solution down and out of the manometric tubes

through the same type of plastic tubing into 700 ml plasma bottles acting as collection receptacles. Just prior to reading, the plasma bottles were lowered to a height equal to that in the tubes so as to equalize the atmospheric and manometric pressure in all tubes. Hereafter this method will be referred to as the Gas method.

The apparatus used in this study can be observed in greater detail in the accompanying figures 1 and 2.

Rumen Fluid Inoculum

Rumen fluid was obtained from a steer fitted with a permanent rumen fistula. The steer was fed a maintenance ration of a medium quality hay composed of alfalfa and mixed grasses. Rumen contents were obtained and squeezed by hand into a previously-warmed quart thermos bottle. The collection was started at about 7:00 a.m. prior to the morning feed, and approximately 15 hours after the last feed. No attempt was made to control the water intake of the steer. A total of 3 liters of rumen fluid was collected to provide an adequate sampling of the rumen contents. The fluid was then taken back to the laboratory and strained through 4 layers of cheese cloth. The filtrate was allowed to stand for approximately 15 minutes during which time the large particles floated to the top of the sample. The bottom layer was removed by suction and was used immediately to inoculate the fermentation flasks.

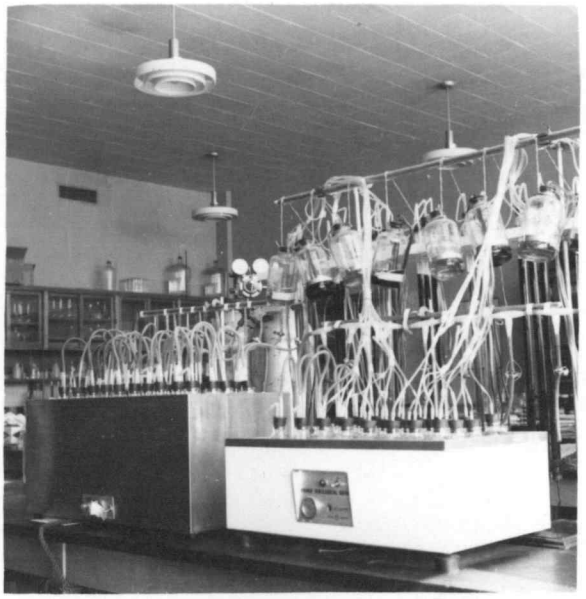

Figure 1. Apparatus for in vitro digestion. Note fermentation bottles, constant temperature water bath, connections to carbon dioxide tank and connections between fermentation bottles.

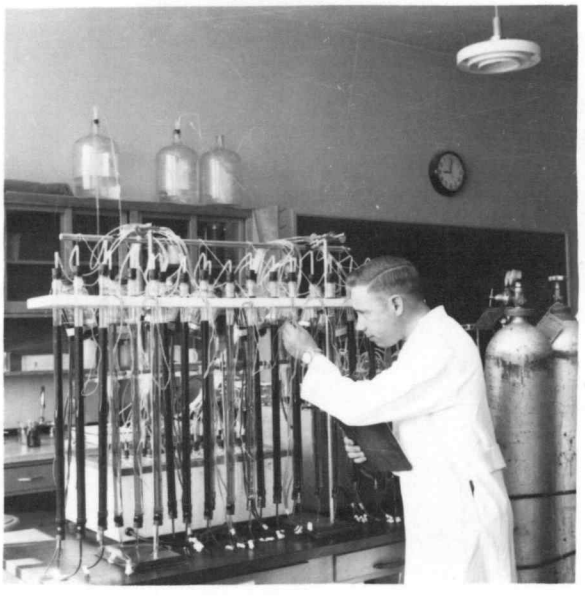

Figure 2. Apparatus for in vitro gas production. Notice especially the manometer tubes providing a measurement of the gas production from the fermentation flasks.

Artificial Saliva Solution

A mineral solution similar to that of McDougall's (84, p. 106) had been used by Baumgardt et al. (16, p. 63); therefore, it was decided to use a similar solution in this work. The mineral solution was composed of:

$NaHCO_3$	9.80 grams per liter of solution
$Na_2HPO_4 \cdot 12H_2O$	9.30 grams per liter of solution
NaCl	0.47 grams per liter of solution
KCl	0.57 grams per liter of solution
$CaSO_4 \cdot 2H_2O$	0.04 grams per liter of solution
$MgSO_4 \cdot 7H_2O$	0.06 grams per liter of solution
Urea	0.64 grams per liter of solution

A sufficient quantity of the mineral solution was prepared initially to furnish the required amount for the entire study. The pH of this solution was about 8.4, which differs very little from the pH of normal saliva reported by McDougall (84, p. 106).

RESULTS AND DISCUSSION

Variability of In Vitro Procedures

The rumen microorganisms serve as a basis for all artificial rumen procedures and, therefore, any inherent variability characteristic of the microbial population is reflected in the results of the in vitro procedures. However, it is not to be implied that variation of in vitro systems is only a result of the microbiota as a certain portion of this variability will be due to experimental error as well as to the inherent variability of the substrate. Any appraisal of artificial rumen procedures must be contingent upon the ability of the system to accurately express the magnitude of the growth and activity of the microbiota when subjected to various treatments.

Preliminary work (Table 1) serves to illustrate the variation that might reasonably be expected with in vitro fermentations. A total of 16 observations of a bromegrass substrate in 2 replications were used to estimate this variability. Within-trial standard deviations for the digestion of dry matter ranged from 2.50 to 3.93% and for the digestion of cellulose ranged from 1.63 to 2.87%. Coefficients of variation ranged from 5.00 to 7.83% for the dry matter digestion and from 2.78 to 4.95% for cellulose digestion. Pooling these data resulted in standard deviations of 3.18% and 5.68% and coefficients

Table 1: Comparison of the mean and variability of the dry matter and cellulose digestibility of Bromegrass.

Replication	Dry Matter Digestibility-%	Cellulose Digestibility-%
I	53.37	50.06
	51.70	47.47
	53.26	44.60
	48.40	44.18
	48.90	48.26
	50.83	46.61
	47.84	45.12
	46.83	43.40
Mean	50.14	46.21%
S. D.	2.50%	2.28%
C. V.	5.00%	4.95%
II	47.36	45.77
	42.77	44.77
	52.09	45.00
	49.15	43.83
	50.52	46.49
	55.35	47.45
	50.54	47.14
	53.86	46.75
Mean	50.20%	45.90%
S. D.	3.93%	1.62%
C. V.	7.83%	2.78%
OVERALL		
Mean	50.17%	46.05%
S. D.	3.18%	5.67%
C. V.	6.34%	12.33%

of variation of 6.34% and 12.33% for the dry matter and cellulose digestibilities, respectively.

A similar study previously conducted in this laboratory involved 52 observations in a comparison of cellulose and dry matter digestibility of an alfalfa substrate (23, p. 30). Analysis of pooled data from this study revealed standard deviations of 1.9 and 2.9% for the dry matter and cellulose digestibilities, respectively, and coefficients of variation of 3.3 and 5.3%, respectively. The pooled data of both studies would appear to indicate a marked tendency for cellulose digestion to be more variable between replications than is the dry matter digestion.

The latter work resulted in a lower overall variation for both the dry matter and cellulose digestibilities which may be a manifestation of forage species variability. The results of the present study may serve as a basis for this implication and will be discussed further as the results are presented.

Church and Petersen (36, p. 82) obtained coefficients of variation with an alta fescue substrate of 1.1 and 2.9% for the dry matter and cellulose digestibilities, respectively. Again, this study was conducted in this laboratory using similar apparatus, facilities and procedures to the two experiments previously cited.

In other laboratories, Baumgardt et al. (16, p. 65) adjusted the cellulose digestion of various forages between days to the digestion of

a standard forage and obtained a coefficient of variation of 1.59%. In an earlier study, Baumgardt and Hill (17, p. 943) reported within-trial coefficients of variation to be less than 2% for in vitro dry matter digestibilities. Pooled standard deviations of 3% and 2% for 12-hour and 24-hour cellulose digestibilities, respectively, were reported by Donefer (45, p. 551) using a variety of 10 substrates.

Rate of Digestion

The means, standard deviations and coefficients of variation of the rumen fluid levels, length of fermentation and substrate variables imposed on each of the in vitro systems in this study are shown in Tables 2, 3 and 4. The mean values in Tables 2 and 3 are the average of two observations each from different samplings of rumen contents. The mean values of Table 4 are an average of 4 observations with duplicates in each of the 2 replications. The values of Tables 2 and 3 are, therefore, a reflection of the day-to-day variation of the fermentative power of the rumen contents. The inclusion of at least duplicate observations within a trial is desirable, but was not possible in this study due to limitations of the available facilities.

Even though the digestibility of dry matter in Tables 2 and 3 reflects only between-trial variation, the variability encountered is, with few exceptions, about the magnitude that would be expected. As will be noted from the tables, the greater variability is apparent

Table 2: The effect of rumen liquor levels on the rate of percent dry matter digestion using the O.S.U. Method.

SUBSTRATE		6 hr	12 hr	18 hr	24 hr	30 hr	36 hr	42 hr	48 hr
Alfalfa									
5 ml	Mean-%	34.45	41.42	48.01	52.78	54.66	56.02	58.33	58.99
	S.C. -%[1]	0.81	0.42	0.16	1.00	3.08	1.20	1.63	2.45
	C.V.-%[2]	2.34	1.02	0.01	1.89	5.63	2.15	2.79	4.15
15 ml	Mean-%	37.61	47.36	52.33	54.14	56.15	56.77	59.05	59.72
	S.D. -%	0.38	0.90	1.19	1.98	1.17	0.28	1.82	2.09
	C.V.-%	1.01	1.90	2.27	3.66	2.08	0.49	3.08	3.50
25 ml	Mean-%	41.70	50.33	52.73	53.79	54.73	55.83	57.53	57.81
	S.C. -%	1.51	0.06	2.22	3.04	1.07	0.95	1.64	0.97
	C.V.-%	3.62	0.12	4.21	5.65	1.95	1.70	2.85	1.68
35 ml	Mean-%	42.23	50.33	51.75	53.35	54.48	55.39	56.93	57.27
	S.D. -%	1.27	0.99	4.02	2.70	2.16	1.20	1.91	1.30
	C.V.-%	3.01	1.97	7.77	5.06	3.96	2.17	3.35	2.27
Bromegrass									
5 ml	Mean-%	26.39	29.55	33.04	42.64	40.16	43.24	46.82	50.17
	S.D. -%	2.23	3.52	4.53	4.14	10.97	8.84	5.88	5.22
	C.V.-%	8.45	11.91	13.71	9.71	27.31	20.44	12.56	10.40
15 ml	Mean-%	27.24	33.09	42.54	48.71	51.36	54.07	55.52	57.73
	S.D. -%	2.57	6.70	5.34	0.54	4.13	0.47	2.96	2.04
	C.V.-%	9.43	20.25	12.55	1.11	8.02	0.87	5.33	3.53
25 ml	Mean-%	26.17	36.78	44.24	49.20	54.60	56.77	58.59	58.41
	S.D. -%	5.87	3.51	2.36	1.53	0.58	0.80	1.24	0.17
	C.V.-%	22.43	9.54	5.33	3.11	1.06	1.41	2.12	0.29
35 ml	Mean-%	26.94	39.87	45.85	50.03	55.54	58.00	60.27	60.25
	S.D. -%	5.53	4.21	2.63	2.79	1.23	0.47	0.37	2.09
	C.V.-%	20.53	10.56	5.74	5.58	2.21	0.81	0.61	3.47
Crested Wheatgrass									
5 ml	Mean-%	23.41	27.45	35.64	43.10	48.24	51.01	51.40	56.87
	S.D. -%	0.99	2.96	2.98	2.46	1.39	0.50	2.93	0.54
	C.V.-%	4.23	10.78	8.36	5.71	2.88	0.98	5.70	0.95
15 ml	Mean-%	24.93	32.73	43.74	48.77	51.67	54.23	57.83	58.06
	S.D. -%	3.65	4.91	3.79	1.39	1.92	1.43	0.76	0.76
	C.V.-%	14.64	15.00	8.66	2.85	3.72	2.64	1.31	1.31
25 ml	Mean-%	23.34	36.44	44.89	51.54	55.59	58.10	59.50	60.62
	S.D. -%	2.22	3.32	2.22	1.14	0.07	0.47	0.73	1.54
	C.V.-%	9.51	9.11	4.94	2.21	0.12	0.81	1.23	2.54
35 ml	Mean-%	25.81	39.14	48.01	52.55	57.38	58.76	59.45	61.11
	S.D. -%	3.08	5.44	5.46	4.60	3.03	0.86	2.39	1.13
	C.V.-%	11.93	13.90	11.37	8.75	5.28	1.46	4.02	1.84

[1] Standard deviation [2] Coefficient of variation

Table 3: The effect of rumen liquor levels on the rate of percent dry matter digestion using the N. J. Method.

SUBSTRATE		6 hr	12 hr	18 hr	24 hr	30 hr	36 hr	42 hr	48 hr
Alfalfa									
5 ml	Mean-%	34.66	39.92	46.08	49.24	48.16	50.03	49.88	49.76
	S.D. -%[1]	0.14	0.73	0.95	0.48	0.78	0.73	0.86	0.76
	C. V. -%[2]	0.40	1.83	2.06	0.97	1.62	1.46	1.72	1.53
15 ml	Mean-%	36.14	44.29	43.99	47.47	50.32	51.20	52.71	51.77
	S. D. -%	0.95	1.07	0.21	1.06	1.10	0.92	1.74	1.02
	C. V. -%	2.63	2.42	0.48	2.23	2.19	1.80	3.30	1.97
25 ml	Mean-%	40.71	43.19	42.54	43.26	45.85	48.06	48.56	49.84
	S.D. -%	1.48	1.12	1.71	0.14	1.03	1.29	1.47	2.11
	C. V. -%	3.63	2.59	4.02	0.32	2.25	2.68	3.03	4.23
35 ml	Mean-%	40.51	42.95	41.76	43.59	44.53	49.85	47.72	47.51
	S.D. -%	2.39	2.29	1.85	0.78	1.27	1.41	0.13	1.54
	C. V. -%	5.90	5.33	4.43	1.79	2.85	2.83	0.27	3.24
Bromegrass									
5 ml	Mean-%	25.62	30.25	37.03	45.77	51.60	53.82	56.13	54.81
	S. D. -%	1.57	2.04	2.49	1.27	2.04	3.28	2.28	2.96
	C. V. -%	6.13	6.74	6.72	2.77	3.95	6.09	4.06	5.40
15 ml	Mean-%	26.19	33.63	43.28	48.54	53.10	56.74	58.97	59.53
	S. D. -%	3.34	3.10	3.65	2.53	2.59	0.69	0.04	2.04
	C. V. -%	12.75	9.22	8.43	5.21	4.88	1.22	0.07	3.43
25 ml	Mean-%	28.09	35.52	43.03	48.98	53.43	56.99	58.77	59.17
	S.D. -%	0.19	5.43	3.12	0.23	2.79	0.93	2.60	2.04
	C. V. -%	0.68	15.29	7.25	0.47	5.22	1.63	4.42	3.45
35 ml	Mean-%	27.69	35.90	43.32	48.22	53.04	57.22	58.42	58.98
	S.D. -%	2.49	5.36	5.67	3.00	2.66	1.37	0.86	3.24
	C. V. -%	8.99	14.93	13.09	6.22	5.01	2.39	1.47	5.49
Crested Wheatgrass									
5 ml	Mean-%	22.13	25.80	29.76	35.07	43.94	46.53	50.24	51.04
	S.D. -%	1.10	1.57	4.85	1.16	2.62	3.48	2.99	3.21
	C. V. -%	4.97	6.08	16.30	3.31	5.96	7.48	5.95	6.29
15 ml	Mean-%	22.34	26.93	35.31	38.58	45.25	49.07	53.27	53.26
	S.D. -%	1.23	4.98	1.46	1.39	1.00	2.52	3.73	4.21
	C. V. -%	5.51	18.49	4.13	3.60	2.21	5.14	7.00	7.90
25 ml	Mean-%	21.73	29.15	34.99	38.65	45.84	50.84	51.23	54.89
	S. D. -%	0.47	1.61	0.68	1.41	1.57	1.61	1.03	4.06
	C. V. -%	2.16	5.52	1.94	3.65	3.42	3.17	2.01	7.40
35 ml	Mean-%	23.87	30.72	36.76	41.04	45.84	51.00	52.48	52.95
	S. D. -%	2.73	2.93	1.37	1.07	1.77	1.40	3.41	4.50
	C. V. -%	11.44	9.54	3.73	2.61	3.86	2.74	6.49	8.50

[1] Standard deviation [2] Coefficient of variation

Table 4: The effect of rumen liquor levels on the rate of milliliters of gas produced using the Gas Production Method.

SUBSTRATE		6 hr	12 hr	18 hr	24 hr	30 hr	36 hr	42 hr	48 hr
Alfalfa									
5 ml	Mean-%	20.37	33.25	44.87	56.25	62.75	68.37	71.50	74.37
	S.D. -%[1]	9.20	12.48	13.85	13.91	13.02	13.66	12.67	13.44
	C.V. -%[2]	45.16	37.53	30.87	24.73	20.75	19.98	17.72	18.07
15 ml	Mean-%	32.12	47.87	62.50	74.50	82.62	86.37	89.25	91.37
	S.D. -%	8.44	2.59	8.96	10.99	8.92	9.64	6.43	7.27
	C.V. -%	26.28	5.41	14.34	14.75	10.80	11.16	7.20	7.96
25 ml	Mean-%	42.62	66.62	82.12	91.87	96.87	100.00	100.75	101.87
	S.D. -%	6.93	8.26	11.52	13.14	10.88	10.04	9.00	10.27
	C.V. -%	16.26	12.40	14.03	14.30	11.23	10.04	8.93	10.08
35 ml	Mean-%	42.00	66.75	83.12	92.12	96.62	99.12	99.50	100.62
	S.D. -%	3.00	6.88	13.02	14.35	12.02	11.71	10.50	12.33
	C.V. -%	7.14	10.31	15.66	15.58	12.44	11.81	10.55	12.25
Bromegrass									
5 ml	Mean-%	18.50	19.37	24.50	32.12	38.62	42.25	44.87	47.00
	S.D. -%	4.24	6.15	2.86	2.53	3.90	5.31	6.30	6.52
	C.V. -%	22.92	31.75	11.67	7.88	10.10	12.57	14.04	13.87
15 ml	Mean-%	25.00	33.25	51.00	64.50	71.75	75.00	77.25	78.50
	S.D. -%	1.41	12.78	12.29	11.39	10.09	7.31	4.91	4.41
	C.V. -%	5.64	38.44	24.10	17.66	14.06	9.75	6.36	5.62
25 ml	Mean-%	34.50	42.75	61.75	75.62	83.12	86.25	88.00	89.00
	S.D. -%	5.66	21.22	21.46	21.30	20.45	18.40	16.01	15.52
	C.V. -%	16.41	49.64	34.75	28.17	24.60	21.33	18.19	17.44
35 ml	Mean-%	36.00	47.25	69.12	84.12	92.37	96.12	97.75	98.62
	S.D. -%	1.41	20.60	22.27	23.31	22.73	21.28	17.95	17.79
	C.V. -%	3.92	43.60	32.22	27.71	24.61	22.14	18.36	18.04
Crested Wheatgrass									
5 ml	Mean-%	10.25	19.00	26.62	33.00	40.87	46.75	50.87	52.00
	S.D. -%	2.10	2.61	2.81	1.78	1.70	1.26	0.63	1.08
	C.V. -%	20.49	13.74	10.56	5.39	4.16	2.69	1.24	2.08
15 ml	Mean-%	16.37	29.75	40.50	48.37	57.62	66.62	72.50	74.37
	S.D. -%	3.82	6.10	9.08	11.07	12.16	11.88	10.14	8.46
	C.V. -%	23.33	20.50	22.42	22.89	21.10	17.83	13.99	11.37
25 ml	Mean-%	18.50	34.75	46.50	55.12	64.87	75.25	82.00	83.75
	S.D. -%	1.91	5.30	8.43	10.32	12.04	11.06	8.44	6.56
	C.V. -%	10.32	15.25	18.13	18.72	18.56	14.70	10.29	7.83
35 ml	Mean-%	20.37	39.00	52.50	62.00	73.12	83.75	90.25	91.87
	S.D. -%	2.87	6.12	9.03	11.01	12.33	11.06	7.93	5.50
	C.V. -%	14.09	15.69	17.20	17.76	16.86	13.21	8.79	5.99

[1] Standard deviation

[2] Coefficient of variation

largely in the first 2 or 3 time periods. This may reflect to some extent inadequate mixing of the rumen liquor used for inoculum. If this were the case, varying periods required for adjustment by the microorganisms could reflect a greater variability until the adjustment had been made.

It will also be noted that the variability is greater with the bromegrass and crested wheatgrass substrates than with the alfalfa substrate. This fact adds support to the statement made earlier that the variability may also be an expression of the particular substrate. Although some difficulty was encountered in the filtering process with bromegrass, it was no less apparent with the alfalfa substrate and no difficulty whatsoever was encountered with the crested wheatgrass. Therefore, the explanation for this phenomenon must be from other sources.

An additional fact that is evident from these data is that the legume substrate had a definite tendency to be initially digested at a more rapid rate than the two grass substrates. After approximately 12 to 18 hours of digestion, little additional digestion was obtained with the alfalfa. However, this was not the case for the grass substrates in which a lower initial digestion was observed with a continual increase in dry matter digestion with each succeeding fermentation period. This effect will be easily noted in Figures 3 and 4 which illustrate graphically the data presented in Tables 2 and 3.

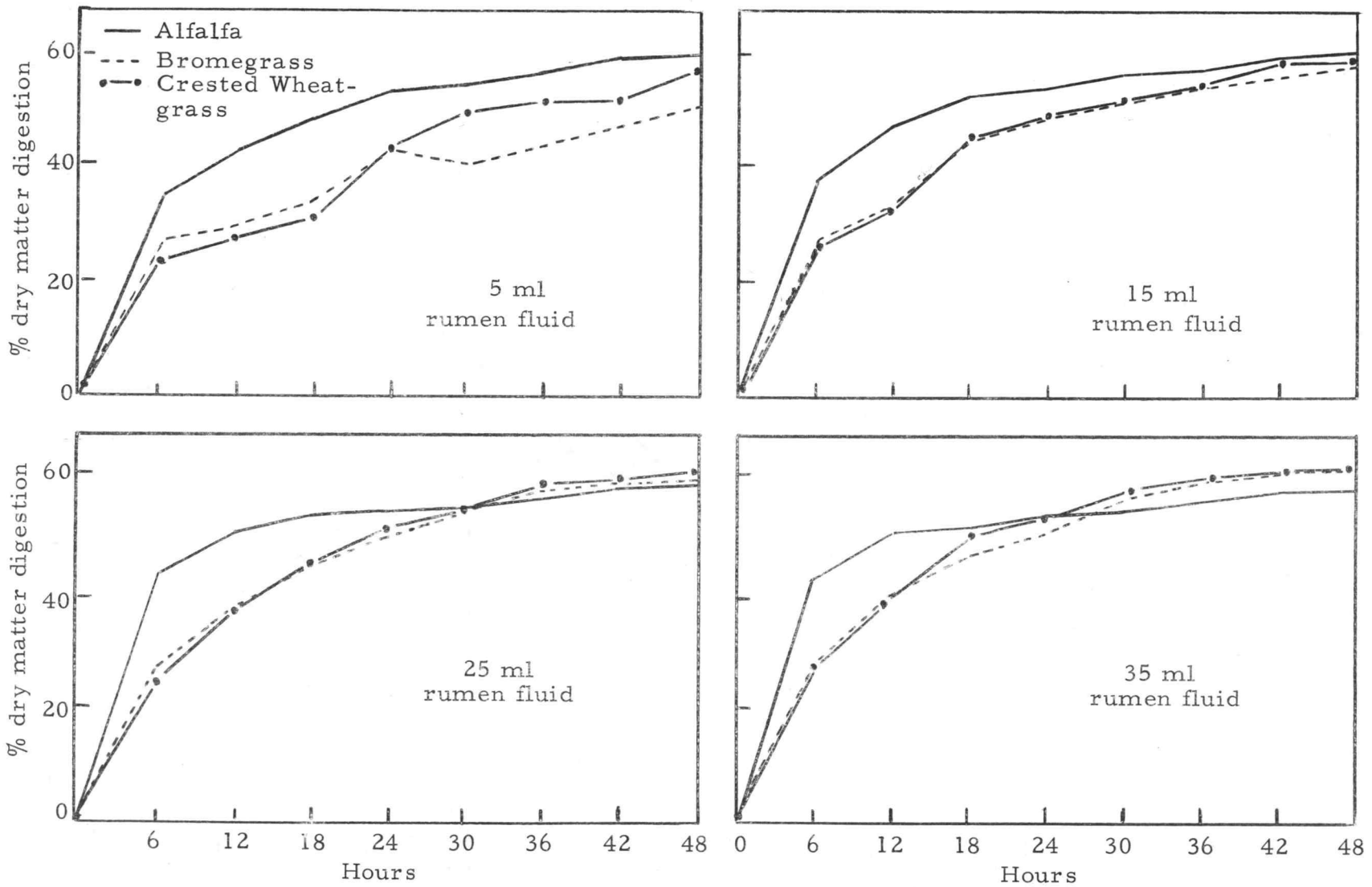

Figure 3. Rate of digestion of 4 levels of rumen fluid with "O.S.U." Method.

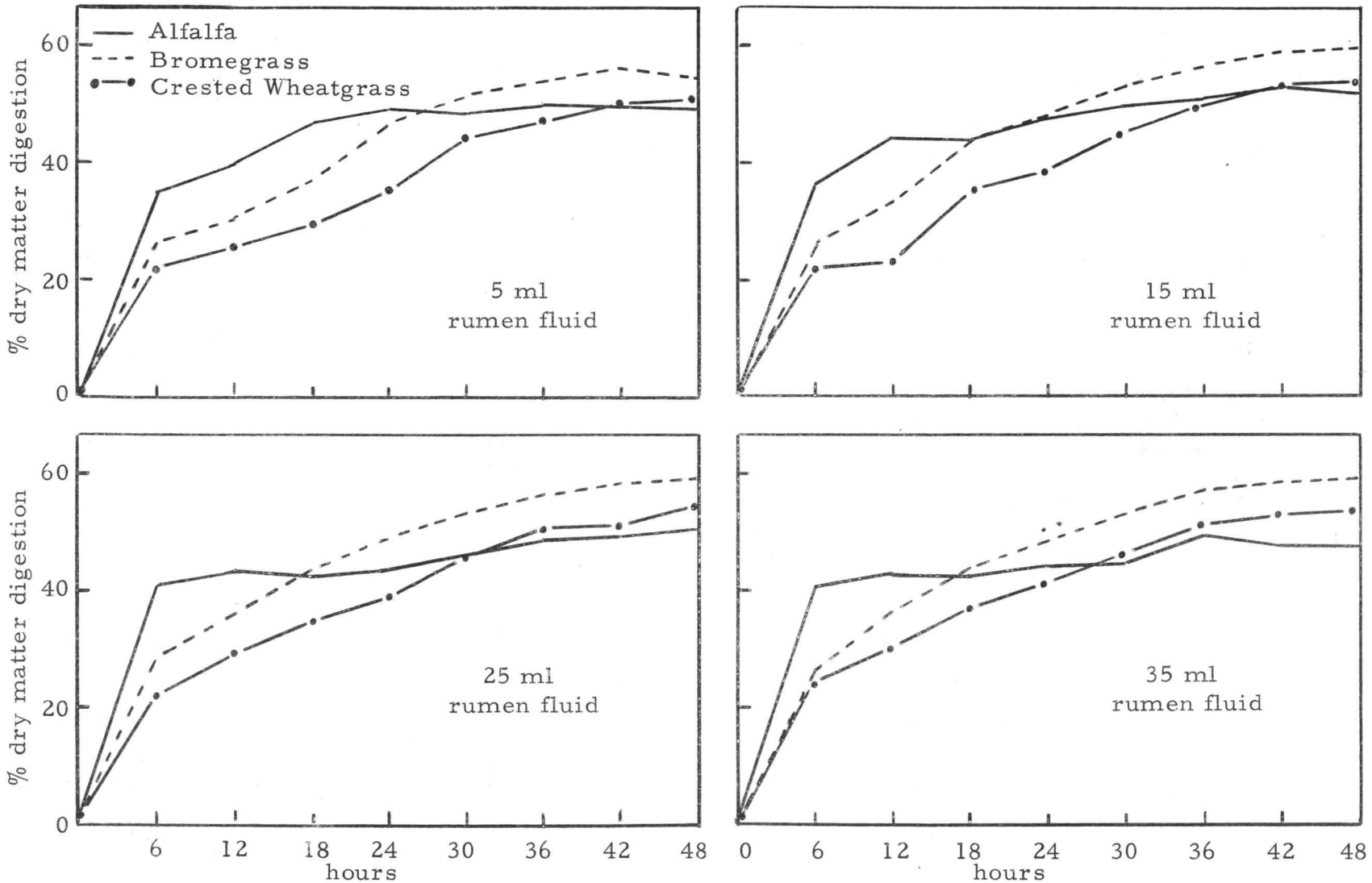

Figure 4. Rate of digestion of 4 levels of rumen fluid with "N. J." Method

This effect was also reflected to a lesser extent with the gas production (Figure 5). The rate of gas production began to decrease much more quickly in the case of the alfalfa substrate than with the grass substrate.

Although the general trend of the rate of gas production was in accord with the rate of dry matter digestion, interpretations that can be drawn from the data are more limited due to the greater within-treatment variation (Table 4). It may be that gas production is a more variable phenomenon than dry matter or cellulose digestion, but it is the author's opinion that too many experimental errors may have influenced these results. The apparatus, as utilized in this experimentation, was extremely sensitive to temperature changes. The equilibration time will have to be studied more extensively as a matter of 5 minutes will result in a tremendous difference in the overall gas production and, of course, in the initial rate of gas production.

The rate of dry matter digestion was about what would be expected. The rates of dry matter digestion are illustrated in Figures 3 and 4 and the rate of gas production in Figure 5 with respect to the 4 levels of rumen fluid used in this study. Figure 6 illustrates the overall rate of dry matter digestion and gas production with regard only to the in vitro method.

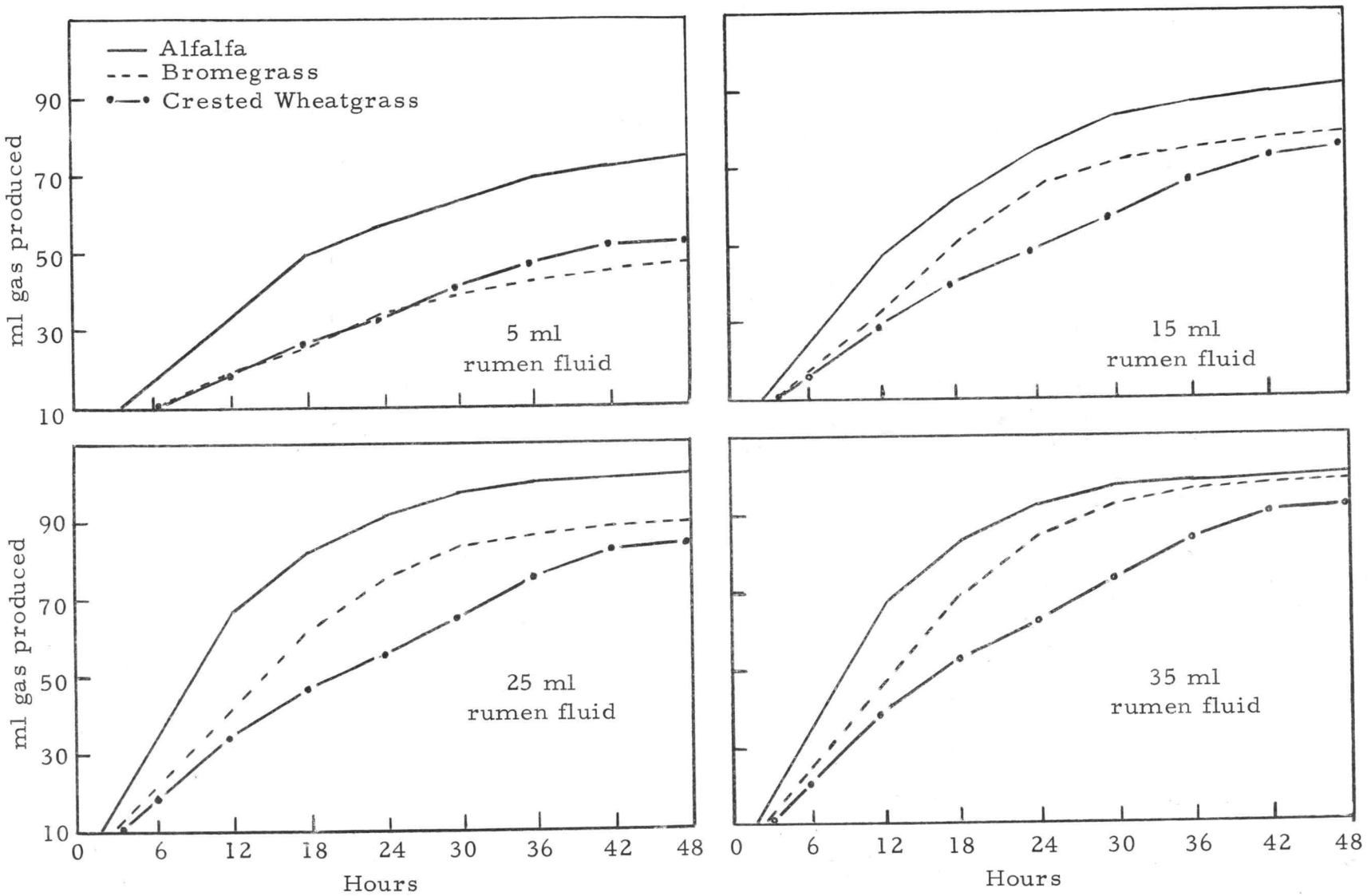

Figure 5. Rate of digestion of 4 levels of rumen fluid with "Gas" Method.

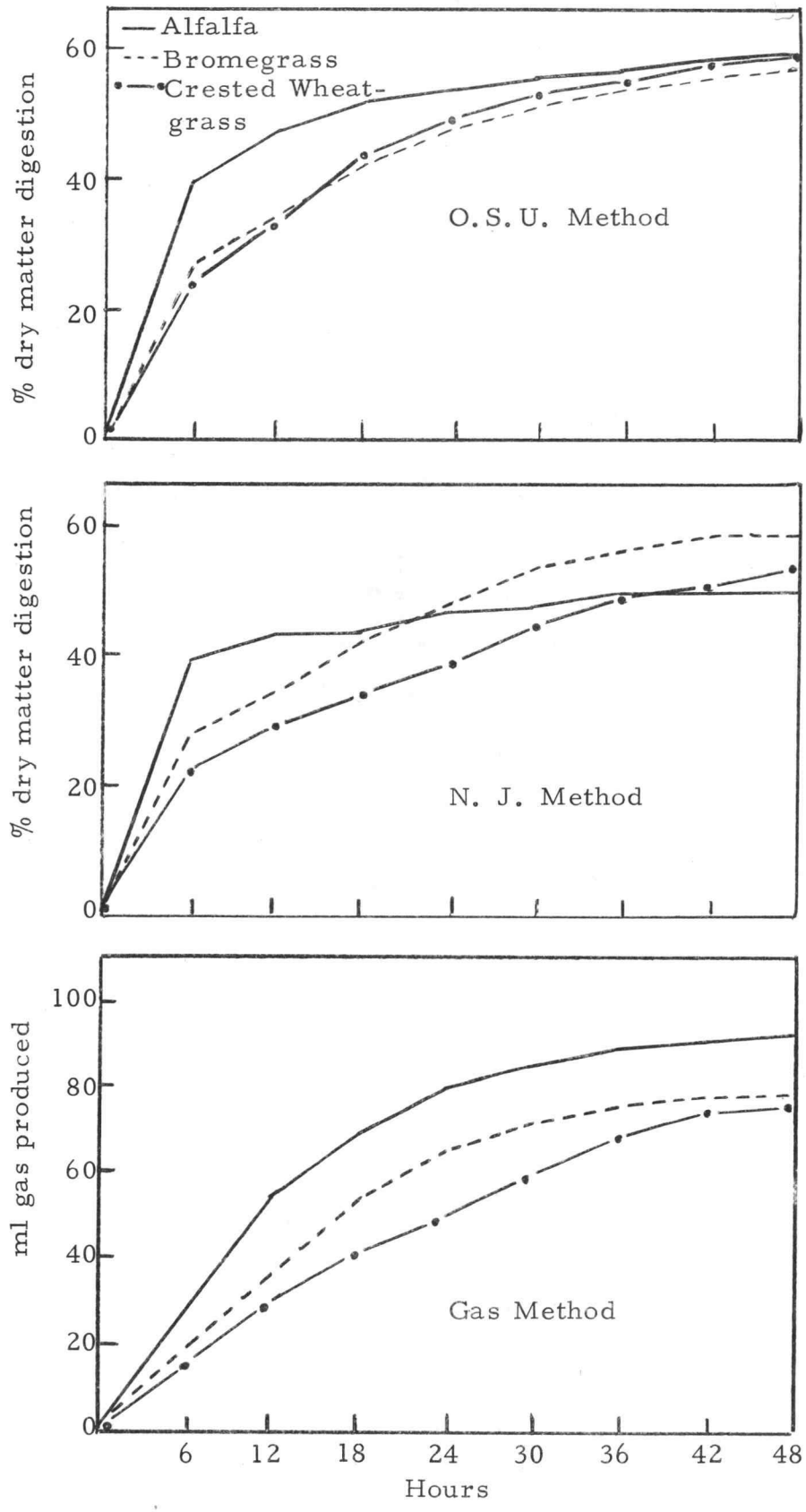

Figure 6. Comparison of 3 in vitro methods.

The lag phase in the initial periods of digestion appears to support the suggestions mentioned in the literature review that it is related to forage species. These curves are very similar to those shown by Baumgardt et al. (16, p. 66), Lloyd et al. (79, p. 471) and Donefer et al. (45, p. 548). In all three reports, it would appear that the grass species had not reached the maximum digestion by 48 hours. However, it was also noted by Baumgardt and Donefer that the legume species reached the point of maximum digestion at a much earlier time. These three reports were concerned with cellulose digestion, but with the high correlations of in vitro dry matter and cellulose digestion reported by Bowden (23, p.70), the overall rate of digestion does not appear to differ markedly.

Length of Fermentation

Four of the time periods were analyzed according to the method presented by Li (78, p. 316-318) for factorial experiments. The individual degrees of freedom were calculated on the 4 levels of rumen fluid and the three substrates, as described by Li (78, p. 226-233). The mean squares of the variables with the degrees of freedom for the 12, 24, 36 and 48-hour fermentation periods are presented in the following tables.

The analysis for the 12-hour period is presented in Tables 5 and 6. Table 5 is the analysis of data obtained by the method

Table 5: Analysis of variance of the % dry matter digestion obtained at the 12-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Method	1	178.1396**
Substrate	2	848.2416**
Alfalfa vs. Grasses	1	1,610.4816**
Bromegrass vs. Crested Wheatgrass	1	86.0016**
Rumen Fluid	3	127.2647**
5 ml vs. 15, 25 and 35 ml	1	307.1548**
15 ml vs. 25 and 35 ml	1	65.2272**
25 ml vs. 35 ml	1	9.4125
Method X Substrate	2	25.4980*
Method X Rumen Fluid	3	19.7625
Substrate X Rumen Fluid	6	3.6163
Method X Substrate X Rumen Fluid	6	1.1928
Replication	1	130.3173**
Error	23	6.5475
Total	47	

Table 6: Analysis of variance of the milliliters of gas produced at the 12-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Day-to-Day Variation	1	574.0830**
Substrate	2	2,337.0205**
Alfalfa vs. Grasses	1	4,469.0104**
Bromegrass vs. Crested Wheatgrass	1	205.0312*
Rumen Fluid	3	1,823.4097**
5 ml vs. 15, 25 and 35 ml	1	4,149.5069**
15 ml vs. 25 and 35 ml	1	1,266.7222**
25 ml vs. 35 ml	1	54.0000
Day X Substrate	2	1,175.2710**
Day X Rumen Fluid	3	95.7223*
Substrate X Rumen Fluid	6	68.9932
Day X Substrate X Rumen Fluid	6	63.6597
Replication	1	105.0200
Error	23	29.3252
Total	47	

* $P<0.05$

** $P<0.01$

presently in use at Oregon State University (OSU) and that proposed by Baumgardt et al. (16, p. 62-68) (NJ). The gas production method had to be analyzed separately as the units of measurement were expressed as milliliters of gas, whereas, the dry matter digestion was expressed as a percent of the total substrate.

No significant difference was observed between the 25 and 35 ml levels of rumen fluid. However, statistically significant differences ($P<0.01$) were found between the 5 and 15 ml levels and the 15 and 25 ml levels. The magnitude of the digestion directly followed the concentration of rumen fluid. Differences between the three substrates were also significant ($P<0.01$). Similar results were obtained from the analysis of the 12-hour gas production data.

The analysis of the gas production data for the 12-hour period is shown in Table 6. Because duplicate samples were used with this method, the day-to-day variation was separated from the replication effect. As can be noted in the analysis, removal of the day-to-day variation resulted in no significant differences between replicates.

Sufficient forages with in vivo data were not available for in vitro and in vivo correlations. However, the in vivo digestible energy values for the 3 forages used were available. The in vivo digestible energy was 60.0% for the alfalfa; 54.4% for the bromegrass; and 49.8% for the crested wheatgrass. The ratios of these three values were calculated and used as a rough guide in selecting

the optimum combination of time, rumen fluid and method.

Certain inferences can be made from the 12-hour data with the in vivo digestible energy figures given above.

The OSU method distinguished between the bromegrass and the crested wheatgrass only with the 5 and 15 milliliter levels of rumen fluid. However, all 4 levels of rumen fluid with the NJ method would appear to give the same approximate relationship between the 3 forages as the in vivo values. Probably any one of the 4 levels of rumen fluid could be used successfully, although the 5 ml level shows a tendency toward less variation.

The gas production method with the 15 ml level of rumen fluid also appeared to distinguish between the forages in accordance with the in vivo relationship, although a much greater variability existed with this method. As was previously mentioned, a refinement of this technique may provide less variable estimates and, thereby, increase the value of this method.

The observations from these data tend to suggest that a rumen fluid concentration of 35 ml with the NJ method would provide a high correlation with in vivo digestibility, on the basis of the in vitro and in vivo ratios of the three forages. Of course, this is a speculative supposition and would have to be verified with a study involving more forages of known in vivo digestibility. However, it does tend to lend support to the high in vitro and in vivo correlations reported by

Donefer (45, p. 551) between the 12-hour in vitro cellulose digestibility and the Nutritive Value Index. The Nutritive Value Index is an in vivo relationship of relative intake with digestible energy. Even though the criterion used was that of percent cellulose digestion the data in this study tend to indicate that percent dry matter digestion would be just as useful in predicting the Nutritive Value Index.

The analysis of the 24-hour fermentation period is shown in Tables 7 and 8. The data show some marked changes from the 12-hour digestion. Only one of the 4 levels of rumen fluid using the NJ method indicated a tendency to distinguish between all 3 forages, which was the 5 milliliter level. The OSU method failed to estimate a difference between the brome and the crested wheatgrass and the NJ method overestimated the bromegrass to the extent that it was valued higher than the alfalfa.

The OSU method produced a greater digestion with the alfalfa and crested wheatgrass than the NJ method which would account for the method-substrate interaction.

No significant differences could be shown between the 15, 25 and 35 ml levels of rumen fluid; however, the 5 ml level produced results that were significantly ($P < 0.01$) different from the results of the other levels of rumen fluid. Digestion decreased progressively with alfalfa from the lowest level to the highest level of rumen fluid; whereas the opposite was true with the other two substrates.

Table 7: Analysis of variance of the % dry matter digestion obtained at the 24-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Method	1	434.8848**
Substrate	2	152.3601**
Alfalfa vs. Grasses	1	170.2403**
Bromegrass vs. Crested Wheatgrass	1	134.4800**
Rumen Fluid	3	28.3397**
5 ml vs. 15, 25 and 35 ml	1	83.4482**
15 ml vs. 25 and 35 ml	1	0.1861
25 ml vs. 35 ml	1	2.0416
Method X Substrate	2	126.3865**
Method X Rumen Fluid	3	16.6262*
Substrate X Rumen Fluid	6	22.9528**
Method X Substrate X Rumen Fluid	6	1.0558
Replication	1	14.6081
Error	23	4.1196
Total	47	

Table 8: Analysis of variance of the milliliters of gas produced at the 24-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Day-to-Day Variation	1	1,059.380**
Substrate	2	3,378.536**
Alfalfa vs. Grasses	1	5,082.3151**
Bromegrass vs. Crested Wheatgrass	1	1,674.7578**
Rumen Fluid	3	3,593.588**
5 ml vs. 15, 25 and 35 ml	1	8,906.6406**
15 ml vs. 25 and 35 ml	1	1,725.7813**
25 ml vs. 35 ml	1	162.7604*
Day X Substrate	2	1,728.938**
Day X Rumen Fluid	3	103.172*
Substrate X Rumen Fluid	6	131.995**
Day X Substrate X Rumen Fluid	6	163.194**
Replication	1	49.005
Error	23	29.7443
Total	47	

* $P<0.05$

** $P<0.01$

The analysis of the 24-hour gas production data is shown in Table 8. In this method, a significant difference ($P< 0.01$, except 25 ml and 35 ml $P< 0.05$) existed between all levels of rumen fluid. In addition, the substrate-rumen fluid interaction is also present in this method, but it is primarily due to the bromegrass rather than to the alfalfa. Referring to Figure 4, it can be easily seen that each succeeding concentration of rumen fluid increases the distinction between the bromegrass and crested wheatgrass, and the opposite is true with the bromegrass and alfalfa.

The data from the 24-hour fermentation would suggest that only the NJ method with the 5 milliliter level of rumen fluid of the two methods utilizing dry matter digestion as criteria would appear to approximate the in vivo relationship of these forages. The gas production method with 25 ml of rumen fluid, however, would appear to merit further study.

The analysis of the 36-hour fermentation data is shown in Tables 9 and 10 and the analysis of the 48-hour digestion data is shown in Tables 11 and 12. These two fermentation periods showed very little overall difference in the ability of the methods with any level of rumen fluid to differentiate between the 3 forages. A significant difference ($P<0.01$) existed between the 5 ml level of rumen fluid and the other levels of rumen fluid in both the OSU and the NJ methods and in both the 36 and 48-hour time periods, but no difference was found between

Table 9: Analysis of variance of the % dry matter digestion obtained at the 36-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Method	1	113.037**
Substrate	2	20.8525**
Alfalfa vs. Grasses	1	4.2294
Bromegrass vs. Crested Wheatgrass	1	37.4761**
Rumen Fluid	3	58.5470**
5 ml vs. 15, 25 and 35 ml	1	164.7372**
15 ml vs. 25 and 35 ml	1	8.9394
25 ml vs. 35 ml	1	2.7803
Method X Substrate	2	116.8775**
Method X Rumen Fluid	3	15.9320*
Substrate X Rumen Fluid	6	22.8178**
Method X Substrate X Rumen Fluid	6	7.1170
Replication	1	21.8160*
Error	23	4.5991
Total	47	

Table 10: Analysis of variance of the milliliters of gas produced at the 36-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Day-to-Day Variation	1	543.3802**
Substrate	2	1,721.3125**
Alfalfa vs. Grasses	1	3,071.3438**
Bromegrass vs. Crested Wheatgrass	1	371.2813**
Rumen Fluid	3	3,850.2274**
5 ml vs. 15, 25 and 35 ml	1	9,759.7934**
15 ml vs. 25 and 35 ml	1	1,586.7222**
25 ml vs. 35 ml	1	204.1666
Day X Substrate	2	1,395.6455**
Day X Rumen Fluid	3	80.8522
Substrate X Rumen Fluid	6	110.9305**
Day X Substrate X Rumen Fluid	6	188.1808**
Replication	1	11.5052
Error	23	26.8422
Total	47	

* $P<0.05$

** $P<0.01$

Table 11: Analysis of variance of the % dry matter digestion obtained at the 48-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Method	1	238.4760**
Substrate	2	44.1965**
Alfalfa vs. Grasses	1	75.2604**
Bromegrass vs. Crested Wheatgrass	1	13.1328
Rumen Fluid	3	27.3620**
5 ml vs. 15, 25 and 35 ml	1	80.8051**
15 ml vs. 25 and 35 ml	1	0.0975
25 ml vs. 35 ml	1	1.1837
Method vs. Substrate	2	112.6085**
Method vs. Rumen Fluid	3	5.4213
Substrate vs. Rumen Fluid	6	17.3953*
Method vs. Substrate vs. Rumen Fluid	6	1.7558
Replication	1	32.5540*
Error	23	5.3976
Total	47	

Table 12: Analysis of variance of the milliliters of gas produced at the 48-hour fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Day-to-Day Variation	1	619.9218**
Substrate	2	1,258.5989**
Alfalfa vs. Grasses	1	2,455.3151**
Bromegrass vs. Crested Wheatgrass	1	61.8828
Rumen Fluid	3	3,614.6719**
5 ml vs. 15, 25 and 35 ml	1	9,661.2517**
15 ml vs. 25 and 35 ml	1	1,005.0138**
25 ml vs. 35 ml	1	6.7810*
Day X Substrate	2	670.9219**
Day X Rumen Fluid	3	125.3525*
Substrate X Rumen Fluid	6	112.5573**
Day X Substrate X Rumen Fluid	6	135.9358**
Replication	1	0.0052
Error	23	26.7661
Total	47	

* $P<0.05$

** $P<0.01$

the 15, 25 and 35 ml levels. The NJ method in both time periods overestimated the value of bromegrass as was also noted in the 24-hour digestion period.

The inability of either the OSU or the NJ method at any level of rumen fluid to differentiate all 3 forages according to the in vivo values would tend to reject the usefulness of these 2 periods at least in predicting the digestible energy value of forages.

The gas production method significantly separated all three forages in the 36-hour fermentation period, but failed to distinguish between the bromegrass and crested wheatgrass in the 48-hour fermentation period. The data suggest that either the 25 or 35 ml level of rumen fluid in the 36-hour fermentative period may produce the appropriate relationship of the 3 forages.

At the end of the 48-hour fermentation period, the contents of the gas production flasks were filtered and the dry matter digestion was calculated to provide a means of comparison with the other two methods. The analysis (Table 13) did not reveal replication differences when the day-to-day variation was removed from the replicate effects. This was also shown with the analysis of the gas production data, but the dry matter analysis of the 48-hour gas production data would apply directly to the dry matter digestion of the other two methods. The analysis also revealed a tendency to overestimate the bromegrass as was the case with the two dry matter methods.

Table 13: Analysis of variance of the % dry matter digestion of the 48-hour gas production fermentation period.

Source of Variation	Degrees of Freedom	Mean Square
Day-to-Day Variation	1	4.8769*
Substrate	2	30.7599**
Alfalfa vs. Grasses	1	7.1068*
Bromegrass vs. Crested Wheatgrass	1	55.8096**
Rumen Fluid	3	51.3526**
5 ml vs. 15, 25 and 35 ml	1	152.6048**
15 ml vs. 25 and 35 ml	1	0.3133
25 ml vs. 35 ml	1	1.1397
Day X Substrate	2	15.1060**
Day X Rumen Fluid	3	0.3243
Substrate X Rumen Fluid	6	21.7793**
Day X Substrate X Rumen Fluid	6	4.1749**
Replication	1	0.8164
Error	23	1.0227
Total	47	

* $P<0.05$
** $P<0.01$

Comparison of the mean dry matter digestion by the 3 methods suggested a tendency for the 15 ml level of rumen fluid to produce the greatest digestion in the case of alfalfa, but this characteristic was not applicable to the other 2 substrates. The only other similarity was with the 5 ml level which produced a digestion comparable to that of the 15 ml concentration with the alfalfa in all 3 methods, but the lowest digestion with the other 2 substrates in all 3 methods. However, none of the 3 methods at any concentration of rumen fluid appeared to approximate the in vivo relationship of the digestible energy values.

In general, the data appear to suggest that the 12-hour fermentation period best approximates the in vivo digestible energy relationship. At least in respect to the in vivo digestible energy relationship, the fermentation periods over 24 hours overestimate the bromegrass and in some cases the crested wheatgrass. Further, the NJ method appears to differentiate between the bromegrass and the crested wheatgrass more in accordance with the in vivo relationship. It is difficult to draw any definite conclusions from the gas production method in view of the variability encountered with this method. It would appear to be useful in evaluating digestible energy through the 24-hour fermentation period. Its usefulness, however, is dependent upon further refinement of the technique as utilized in this study. Any one of the four levels of rumen fluid seems to work

equally as well in the NJ method and in the gas production method with the exception of the 5 ml concentration, which was not able to distinguish between the bromegrass and the crested wheatgrass in the gas production method.

SUMMARY

The objective of this study was to compare three in vitro fermentation procedures and to determine the optimum concentration of rumen fluid and length of fermentation in order to predict the in vivo digestibility of forages used in ruminant rations. Basically, the procedure in all three fermentation methods involved incubating forage samples with varying levels and dilutions of rumen fluid and 30 milliliters of a mineral solution in glass centrifuge bottles which were immersed in a water bath held to a constant temperature of 40° C.

The percent dry matter digestion was utilized in two of the in vitro methods and the milliliters of gas produced was utilized in the third method as criteria of in vitro function. Dry matter digestion and gas production were determined after time intervals of 6, 12, 18, 24, 30, 36, 42 and 48 hours of fermentation in order to determine the rate of digestion of the three forages of known in vivo digestibility used in this study.

The mean, standard deviation and coefficient of variation were calculated for each of the 288 treatment combinations of time, rumen fluid, forage and method. The results of these calculations showed the gas production method to be the most variable method. In addition the data suggested a tendency for the bromegrass substrate to be more variable than either the alfalfa or the crested wheatgrass. The

six and twelve-hour fermentation periods, in general, were the most variable throughout the eight time periods.

The most rapid rate of digestion occurred through the 12-hour fermentation period for the alfalfa samples and through 18 to 24 hours for the bromegrass and crested wheatgrass samples. Although a sufficient number of forages of known in vivo value were not available for in vitro and in vivo correlations, the data would suggest that of the eight time periods the 12-hour fermentation period more closely approximated the in vivo relationship of the three forages.

Results of the analysis of the data obtained from the four levels of rumen fluid suggested a marked tendency for the 5 ml level to produce a lower digestion than the other four levels of rumen fluid. The 15 milliliter level was usually intermediate between the 5 milliliter and the 25 milliliter levels; but, with few exceptions, no differences existed between the 25 and 35 milliliter levels of rumen fluid.

Each of the three in vitro procedures compared in this research exhibited some specific characteristics. The OSU procedure could not differentiate between at least two of the three forage samples in any of the eight time periods with the exception of the 12-hour period utilizing the 5 or 15 milliliter levels of rumen fluid. The NJ procedure over-valued the bromegrass to the extent of valuing it higher than the alfalfa after 24 hours of digestion. The gas production data were extremely more variable than those of the other two procedures.

Ratio comparisons of the in vitro data with the in vivo values suggested that the NJ procedure utilizing the 12-hour fermentation period with any of the four levels of rumen fluid would produce forage digestibilities more in accordance with known in vivo digestibilities.

The ratio of the mean values of the three forages also suggest the OSU method utilizing the 12-hour fermentation period with either 5 or 15 milliliters of rumen fluid may provide a means of assessing the digestibility of forages.

The mean values of the gas production data suggested that a high correlation may exist with the in vivo digestibility values. In the author's opinion, much of the variability encountered with this method as utilized in this study may be overcome by further refinement of the apparatus and procedure in order to reduce the experimental errors. A less variable method of this type offers distinct advantages over the other methods that measure gross digestion, in that it is much simpler to obtain the results from the fermentations and less time is required for the overall procedure. This method certainly merits further study as an in vitro procedure in evaluating the in vivo digestibilities of forages.

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