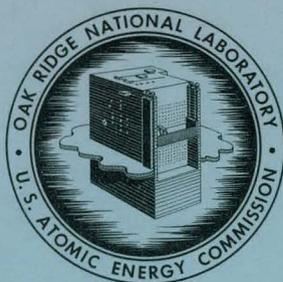


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VARIABLES AFFECTING IN VITRO RUMEN FERMENTATION STUDIES IN FORAGE  
EVALUATION: AN ANNOTED BIBLIOGRAPHY

G. M. Van Dyne

P. T. Haug

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G. M. Van Dyne and P. T. Haug

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VARIABLES AFFECTING IN VITRO RUMEN FERMENTATION STUDIES IN FORAGE  
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INTRODUCTION

Expanding human populations and the commensurate demand on croplands have increased the need for producing forage, the principal source of nutrients for ruminants, on land not suitable for cultivated crops. The increased importance of forage crops to livestock production has made it essential to understand the decomposition and utilization of forages by ruminants. In many other areas from the textile industry (Siu, 1951) to the continuous carbon-mineral cycle throughout the world ecosystem, decomposition of natural organic compounds is of primary importance. Approximately 40% of plant material is cellulose, the world's most plentiful naturally occurring organic compound (Halliwell, 1959). Cellulose is also relatively resistant to natural, non-enzymatic decomposition. Mammals do not produce cellulases or polyglucosidases and rely on symbiotic relationships with anaerobic bacteria and protozoa to release useable plant nutrients by degrading the enveloping cellulose. These bacteria and protozoa, as well as certain fungi, molds, actinomycetes, insect larvae, worms, wood-boring insects, snails, and limpets produce the necessary cellulases and polyglucosidases (Hungate, 1949 and 1966).

Because of this symbiosis, forages conventionally have been evaluated by in vivo digestion trials. Inherent disadvantages of this system are that trials require relatively large amounts of forage, they are expensive, and they do not yield particularly precise results because of large animal-to-animal and trial-to-trial variation. Other approaches have been by chemical analyses of proximate components and other nutrients and, more recently, by chemical solubility methods (Johnson et al., 1964). However, chemical analyses, although being improved (Van Soest, 1966), have yet to replace digestion trials as a means of evaluating forage. Early in vitro systems in the 1800s were used to study forage digestion but not specifically for forage evaluation. In vitro techniques designed for the quantitative nutritive evaluation of forages have been used since 1955 (Barnes, 1965).

In vitro variations are numerous. Sometimes these "artificial rumen" systems attempt to simulate conditions in the rumen, but more often rumen microorganisms are used simply as an "analytical reagent" without any attempt to simulate rumen conditions. Temperature, anaerobic conditions, and acidity are variously controlled, and rumen bacteria, nutrients or cofactors, and forage are added to the system. Digestive activity is evaluated mostly by measuring the disappearance of a given constituent (usually cellulose or dry matter) over time, but some manometric methods have been used.

Because there is no standard method for evaluating forage in vitro (Barnes, 1966), results vary widely. Several books and review papers have investigated this problem (Annison and Lewis, 1959; Bentley, 1960; Barnett and Reid, 1961; Lewis, 1961; Johnson, 1963; Barnes, 1965; Dougherty et al., 1965; and Hungate, 1966). R. F. Barnes of Purdue University has compiled a listing of references on in vitro studies in general. His listings show 383 references through 1964. The current bibliography was prepared in conjunction with a review of literature through 1966 that examined methods and interpretations of in vitro evaluations as well as sources of variation in these evaluations. In the current bibliography the emphasis primarily is on in vitro studies concerned with forage evaluation. Some additional general references or those concerned with variables affecting conduct or interpretation of in vitro studies are included. These sources of variation and suggestions for minimizing them are outlined below.

The rapid evolution of in vitro forage evaluation techniques has produced confusing and contrasting results in the literature. Analysis of the many variables producing these results is necessary to establish a common ground from which investigators can proceed. These errors or variables appear to be of four major types: (1) variations in microbial populations due to the diet of the host animal, animal-to-animal differences, and inoculum processing differences; (2) variations due to different storage, grinding, and processing techniques in sample preparation; (3) differences attributable to inoculum (sample ratio, buffer, and nutrient medium); and (4) procedural variations such as length of fermentation, criteria of digestibility, and laboratory errors.

From the literature reviewed it appears that variation and error could be decreased if the following procedures were standardized: (1) Use several fistulated animals as sources of inoculum. These animals should be selected for uniformity and "normality" in order to avoid extreme individuals. The animals could be chosen through comparative in vivo digestibility trials with appropriate statistical analyses before in vitro trials were begun. (2) Feed the source animals according to standard regimes similar to the forages being evaluated in vitro. The regime should be maintained for several days before in vitro trials are begun in order to allow rumen microbes to adjust to the diet. (3) Use a standard sampling regime at or near the maximum concentration of rumen microbes. (4) Use whole rumen fluid as inoculum. (5) In each trial, include standard forages for which in vivo digestibility data are available. These should be of both high and low quality and should approximate the same type of plants being evaluated in vitro. (6) Buffer the system near pH 6.9. (7) Omit complex nutrient media. These appear unnecessary and may actually introduce variability. (8) Calculate digestibility relative to the standards, and report the results only to the nearest whole percent. (9) Report measures of variation and include, where appropriate, variance components and standard errors of estimates.

If the above procedures were applied, variation could probably be decreased by 50%, and the results would be 100% more useful.

The abstracts are listed alphabetically by author and are numbered serially. The numbers are referred to in the permuted index of the titles.

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## ABSTRACTS

1. **Adler, J. H., J. A. Dye, D. E. Boggs, and H. H. Williams.** 1958. Growth of rumen microorganisms in an *in vitro* continuous-flow system on a protein-free diet. *Cornell Vet.* 48: 53–66. — Best results in an *in vitro* system are obtained when rumen microorganisms are presented with a diet similar to that to which they were exposed in sheep.
2. **Alexander, R. H., and M. McGowan.** 1966. The routine determination of *in vitro* digestibility of organic matter in forages—an investigation of the problems associated with continuous large-scale operation. *J. Brit. Grassl. Soc.* 21:140–147. — Detailed descriptions and illustrations are given for equipment and methods utilized for handling 250–300 *in vitro* determinations of organic matter digestibility per week by 3 people. Inoculum is obtained from sheep maintained under a standard regime, being fed a uniform diet of medium quality, coarsely-chopped hay in three feedings of 1.25 lb/day. Mixing rumen liquor from various sheep had no detrimental effect, and the standard procedure is to use one liter from each sheep. Three sheep were utilized in the trial, but it is not stated whether or not this is the desired number. The influence of added nitrogen in the *in vitro* digestion depended upon the nitrogen content of the herbage being evaluated. There was an inverse, highly significant relation ( $P < .001$ ) between organic matter digestibility and crude protein content of the sample. Decreasing amounts of rumen liquor from 20 to 2 ml/0.5-g herbage sample had little effect on digestion of samples with about 18% crude protein but greatly affected samples with only 5% crude protein. Expressing results on an organic matter basis was considered preferable to a dry matter basis. There was little effect on *in vitro* digestibility of organic matter if samples were inoculated within 1 hr after collection of the rumen liquor. Five standard samples were included in duplicate at the beginning and end of each batch. The standard deviation of a single estimate of a standard was  $\pm 0.61$  units. There were no significant differences in the *in vitro-in vivo* relationships for grasses and legumes and that for hays. The standard error of estimate of their regression was  $\pm 2.33$  with a multiple correlation of 0.96 ( $P < .001$ ).
3. **Allison, M. J., and M. P. Bryant.** 1958. Volatile fatty acid growth factor for cellulolytic cocci of bovine rumen. *Science* 128: 474–475.
4. **Anderson, R., E. Cheng, and W. Burroughs.** 1956. A laboratory technique for measuring phosphorus availability of feed supplements fed to ruminants. *J. Anim. Sci.* 15: 489–495. — Cellulose digestion after a 24-hour fermentation period was measured in a series of small fermentation tubes to which graded amounts of phosphorus had been added. Bacteria used herein were washed suspensions of rumen microorganisms. A phosphorus-deficient medium was used to assay added phosphorus to the system. Three fistulated steers were used in obtaining samples. The rations fed differed somewhat from time to time. Each was fed twice daily, at 0900 and 1700. Rumen samples were collected prior to the morning feed.
5. **Arias, C., W. Burroughs, P. Gerlaugh, and R. M. Bethke.** 1951. The influence of different amounts and sources of energy upon *in vitro* urea utilization by rumen microorganisms. *J. Anim. Sci.* 10: 683–692. — Carbon dioxide was bubbled continuously through the fermentation contents of 1000-ml Erlenmeyer flasks connected by tubing. The purpose of the  $\text{CO}_2$  stream was to maintain

anaerobiosis and to stir the fermentation mass. Rumen contents were obtained from a rumen-fistulated steer that had been withheld from a high quality alfalfa hay ration 12–16 hr prior to sampling. A variety of cellulose sources including ground corncobs, Solkafloc, and ground filter paper was used.

6. **Arroyo, A., J. P. Evans, and M. W. Taylor.** 1964. The artificial rumen technique of estimating the nutritive value of forages. *Puerto Rico J. Agr.* 47–48:169–179. — Nine forages of known nutritive value were digested *in vitro* for 12 and 24 hr. Correlations of relative intake and nutritive value index were made with 12-hr *in vitro* cellulose digestion values. The 24-hr *in vitro* cellulose digestion values were more closely related to measures of energy digestibility than were the 12-hr measures.
7. **Asplund, J. M., R. T. Berg, L. W. McElroy, and W. J. Pigden.** 1958. Dry matter loss and volatile fatty acid production in the artificial rumen as indices of forage quality. *J. Anim. Sci.* 38: 171–180. — Limited evidence suggested that the artificial rumen may be used to assess the relative quality or nutritive value of forages. These workers used 500 mg. of substrate ground through a 40-mesh sieve in a Wiley mill, 10 ml of inoculum, and a buffer solution with a slight amount of  $\text{CoCl}_2$ . Inocula were taken from ruminal-fistulated sheep 3–4 hr after they had been fed. A washed cell suspension was used. To study dry matter digestion, workers placed finely ground samples of forages in fermentation sacs, dried the forages and sacs for 21 hr, inoculated the sacs, and incubated them in the artificial rumen. After incubation the sacs were removed, rinsed with distilled water, and dialyzed in tap water at room temperature for 24 hr. Two filtrates were prepared by pooling rumen fluids from two sheep that had been fed mixed grass-alfalfa hay and from two sheep that had been fed straw. Crude protein and fiber in these samples, respectively, were 12.5 and 26.2% in the grass-alfalfa hay, 5.9 and 32.1% in oat straw, and 18.9 and 20.4 in an excellent alfalfa hay. Inocula taken from hay-fed sheep consistently digested the dry forage samples better than did the inocula from straw-fed animals. The digestibility differences for three types of dry matter were: alfalfa, 12%; mixed hay, 12%; and straw, 5%. Washed cell inocula, compared to filtered cell inocula, did not materially increase the degree of association between *in vitro* and *in vivo* data for dry matter digestibility. Also, there were interactions between filtered vs. washed cells and straw vs. hay feeding.
8. **Baker, F., and S. T. Harriss.** 1947. Microbial digestion in the rumen (and caecum), with special reference to the decomposition of structural cellulose. *Nutr. Abstr. Rev.* 17: 3–12. — Oligotrich ciliates have been found in all ruminants and in some nonruminants including the horse, elephant, rhinoceros, tapir, capybara, and wild guinea pig, as well as some anthropoids such as the chimpanzee and the gorilla. They have not been found in the domestic guinea pig and rabbit. Relatively few of the cellulosic particles in the rumen are small enough to be ingested by even the largest ciliates. Elimination of the protozoa in the rumen does not significantly decrease the amount of cellulose digested. This suggests that the protozoa are functionally insignificant in digesting cellulose in the normal animal. Also, it is difficult to culture protozoa independent of the ingested or symbiotic bacteria. Chewing by the ruminant detaches the cuticle, mechanically separates the epidermis from underlying sclerenchyma, bundles, and mesophyll, and exposes more surface area to attack. Detached cuticle and guard cells are resistant to decomposition by bacteria. Microscopic examination of filter paper and cotton wool taken from bags suspended in the rumen shows that attack commonly begins at the broken ends of the fibers. The amounts of cellulose digested depend upon the fineness of grinding. There is a reciprocal dependence of one microbial species upon the other.
9. **Baker, T. I., G. V. Quicke, O. G. Bentley, R. R. Johnson, and A. L. Moxon.** 1959. The influence of certain physical properties of purified celluloses and forage celluloses on their digestibility by rumen microorganisms *in vitro*. *J. Anim. Sci.* 18: 655–662. — Cellulose is composed of both

crystalline and amorphous structures. The degree of crystallinity of cellulose is inversely proportional to its rate of decomposition by cellulolytic organisms. Also, the average size of the glucose chains making up the cellulose polysaccharide influences the chemical behavior of the celluloses. Holocelluloses prepared from mature and immature forages showed no major difference in x-ray diffraction patterns. Comparison of the x-ray diffraction patterns of holocellulose and the untreated plant materials showed similarities. Apparently the molecular structure of cellulose in pasture and forage plants is not sufficiently organized to give strong peaks in x-ray patterns. The conclusion from this is that much of the cellulose is amorphous. Samples ground to pass a 40-mesh screen were sieved to get samples that would pass 40–60, 100–150, and 200-mesh sieves. No marked differences were observed in the digestibility of cellulose in the different fractions. However, if the material was ball-milled, digestibility increased slightly. These studies suggested that the activity of cellulases from rumen organisms are affected by cellulose structure in much the same way as the cellulases isolated from molds and soil organisms.

10. **Baldwin, R. L., and D. L. Palmquist.** 1965. Effect of diet on the activity of several enzymes in extracts of rumen microorganisms. *Appl. Microbiol.* 13: 194–200. — One rumen-fistulated cow was fed barley straw, and the other was fed a concentrate mix of barley, milo, wheat, and beet pulp. The animals were restricted from water for a 2-hr period between 0900 and 1100 to reduce sampling errors. Rumen samples were extracted with a phosphate buffer, strained through cheesecloth, and centrifuged to get bacterial cells. These were washed in phosphate buffer before being disintegrated or disrupted with a sonic oscillator under a hydrogen atmosphere. The cell-free extract was used for the enzyme determinations. The rumen inoculum was sampled from the animals at 0700 (pre-feeding) and 1100 (4 hr post-feeding). Considerable amounts of additional work characterizing the enzymes of representative rumen microorganisms in pure cultures, resolving some of the biochemical problems related to anaerobic metabolism and anaerobic electron transport, and developing new enzyme assays must be carried out before enzymatic measurements can be employed effectively in defining the metabolic changes associated with changes in the rumen microbial population on various diets.
11. **Barnes, R. F.** 1962. Inter-laboratory *in vitro* study of standard forages. 4th *In Vitro* Evaluation Work Conference, Chicago, 14 p. — Alfalfa meal was purchased by Purdue University and distributed to 15 laboratories in the United States and Canada in 1960. In 1961 alfalfa and bromegrass hay were included from Macdonald College, Quebec, Canada. Results from various laboratories show variations in *in vitro* cellulose digestion of 40–51% at 24 hr and 51–65% for 48 hr. Further studies were made by nine laboratories; details were given on their individual techniques. In this study nine locations were studied (random), three runs within a location (random), three substrates (fixed), and two observations per cell (random). The mean cellulose digestion of three forage samples varied among stations from 41–50%.
12. **Barnes, R. F.** 1965. Use of *in vitro* rumen fermentation techniques for estimating forage digestibility and intake. *Agron. J.* 57: 213–216. — This is a general review of previous *in vitro* studies. Factors that can influence *in vitro* digestibility and hence its relationship to true digestibility include: substrate concentration, length of sample storage period, fineness of grind, composition and buffering capacity of the nutrient media, amount and preparation of the rumen fluid inoculum, and diet of the donor animal. A differential lag phase had been reported for various substrates. The initial digestion rates of alfalfa and other legumes have been greater, and the maximum level of digestion has been reached sooner than for grasses. The extent of digestion, however, may be greater for grasses than for legumes. This relationship was utilized by Donefer in developing the proposed nutritive value index (NVI). For utility in routine laboratory evaluation of forages, a method must (1) be relatively simple to permit rapid analysis of a large number of samples, (2) produce precise results, and (3) give an accurate estimate of forage quality. Errors associated with the *in vitro* procedure can be grouped into two groups: random errors and

biased errors. Random errors are associated with within- and between-trial variability. Greater variability exists for purified cellulose samples than for forage substrates. Greater variation between trials than within trials has generally been reported. Greater variation occurs during the initial stages of fermentation than in later stages. Two standard substrates included in a trial, one high quality and the other low, are useful in adjusting between-trial variations. Standard errors of the estimate of digestibility have ranged from 2.0 to 4.4 for the prediction of *in vivo* digestibility from *in vitro* results. To be of practical use, a regression equation should be capable of predicting apparent digestibility within a standard error of not greater than two digestibility units. Thus, the use of *in vitro* techniques for the prediction of forage are marked. Various constituents of the herbage may be preferentially utilized by *in vitro* microbial populations than *in vivo*. Thus, a forage with a high level of soluble carbohydrate or hemicellulose may be classified as being lower in nutritive value if cellulose digestion *in vitro* is used as the single criterion. This emphasizes the importance of chemical components of the plant and suggests that several measures may be necessary to develop an effective *in vitro* forage evaluation tool. Gas production generally is not a good measure, for it may evolve from a variety of substrates and its use for forages may be misleading. One of the greatest deterrents to accuracy of *in vitro* methods is the large inherent variability of *in vivo* measurements upon which the *in vitro* results must be based.

13. **Barnes, R. F.** 1966. Collaborative *in vitro* rumen fermentation studies on forage substrates. J. Animal Sci. (manuscript submitted). -- Research workers interested in the development of *in vitro* techniques for evaluating forages met initially on an informal basis in 1959. Since then various collaborative studies have been undertaken. In initial studies with dehydrated alfalfa meal, mean cellulose digestion after specified lengths of fermentation varied considerably among laboratories. The variance associated within and between runs also differed greatly among laboratories. These results led to a nested factorial experiment with different collaborators determining *in vitro* digestibility of three forage substrates. Each laboratory used its own current, routine *in vitro* technique. Variations existed in the respective *in vitro* systems, in the host animals used to obtain inoculas, in the diets of the host animals and in the buffer and nutrient solutions. Mean 2-hr cellulose digestion values for 14 laboratories was 47% (range 40 to 60). For most stations bromegrass had a lower initial rate of cellulose degradation but a higher rate of fermentation during the later periods. It eventually surpassed the alfalfa substrates. This fact illustrates the importance of length of fermentation in the evaluation of substrates. There was more variation among laboratories during early fermentation periods than at later stages. Of the 14 laboratories reporting 24-hr *in vitro* digestibility data, six had significant runs-by-substrate interactions. There was less variation among duplicates with increased fermentation times. There was also a trend for the variance associated with runs within laboratories to be less for 48-hr fermentation than for other time periods. The most commonly employed procedures involved a 90 ml centrifuge tube, 1 gram sample size, 30 ml McDougall's artificial saliva, 20 ml of strained whole rumen fluid obtained from fistulated cattle, and incubation with a gas release valve or continuous flushing with CO<sub>2</sub>. If a comparison between laboratories is desired, a standardized procedure could possibly be developed. The primary factors then remaining uncontrolled would be the source and activity of inoculum. Standard forages could be utilized to measure this variability and provide a reasonable degree of control over the digestive efficiency of various sources of the inoculum. The inherent variability of the *in vivo* measurement is as great as, or greater than, that of the *in vitro* measurement. The run-by-substrate interaction is an important factor because this error term generally is used to test substrates, and when it is large the chances of detecting differences between substrates are reduced. If such an interaction is detected, then there is a shift in relative fermentation curves of the various substrates between runs. This effect may be particularly marked when evaluating substrates of diverse types or of different species. For uniformity and ease of interpreting published *in vitro* results, variance components associated with runs, determinations within runs, and interactions of runs-by-substrates should be incorporated with the method outlined and reported in the paper.

14. **Barnes, R. F.** 1966. The development and application of *in vitro* rumen fermentation techniques. Int. Grassl. Congr., Proc. 10: 434-438. -- Four methods were compared including: 50 ml buffer-nutrient solution; 50 ml buffer followed by a 24-hr incubation of the residue with 50 ml pepsin solution; 40 ml buffer solution plus 10 ml strained whole rumen fluid; and 40 ml buffer solution plus 10 ml strained whole rumen fluid, followed by a 24-hr incubation of the residue with 50 ml pepsin solution. Dry matter disappearance was determined at 0, 6, and 48 hr. Results were related to the *in vivo* digestible dry matter. Good predictions could be made of *in vivo* digestible dry matter, but attempts to relate *in vitro* or solubility results to voluntary intake were less successful. The most useful technique was an *in vitro* rumen fermentation technique utilizing a 48-hr incubation period plus an additional 24-hr incubation with pepsin. The technique was suitable for simultaneous evaluation of grasses and legumes.
15. **Barnes, R. F., G. O. Mott, L. V. Packett, and M. P. Plumlee.** 1964. Comparison of *in vitro* rumen fermentation methods. J. Anim. Sci. 23: 1061-1065. -- The inoculum was obtained from a fistulated cow receiving grain, hay, and corn silage. This inoculum was used in all the comparisons of techniques. Substrates were ground through a 40-mesh screen. Substrates included alfalfa and bromegrass hays and alfalfa meal. Fermentation time periods were 6, 12, 18, and 24 hr. Fermentation techniques were those outlined by Donefer: a complex nutrient buffer solution was used; a phosphate buffer extract of the rumen inoculum was added; and CO<sub>2</sub> was bubbled continuously through the fermentation, which was maintained at 29°C. A second method was that of Baumgardt: 20 ml of a CO<sub>2</sub>-saturated nutrient buffer solution were used; urea, glucose, and strained rumen fluid were added and the system was maintained at 39°C. The third and fourth methods were those of Tilley and Terry: gas release valves were used; the inoculum-nutrient-buffer solution and strained rumen fluid were flushed with CO<sub>2</sub> at the beginning and then fermented at 39°C. The latter method also involved a 48-hour pepsin digestion. In a second experiment 25 ml of a buffer-nutrient solution plus strained rumen fluid and small amounts of urea and glucose were added to 1-g samples in 75-ml centrifuge tubes fitted with gas release valves. Total liquid volume was reduced to prevent excessive frothing; CO<sub>2</sub> was flushed through the system; and it was fermented at 39°C. There were significant differences among methods, substrates, and fermentation times. There was also a method and time interaction. The various techniques, however, rank the substrates in the same manner. The coefficient of variation decreased with increasing times of fermentation. Both alfalfa substrates had higher digestibilities than did the bromegrass hay substrate. Variation within bromegrass was nearly twice that of alfalfa. Differences early in the fermentation may be due to non-cellulose constituents in the forages. The high initial activity of one method might be due to the constant bubbling of CO<sub>2</sub> through the flask and a more complex inoculum-buffer-nutrient solution than in the other systems. In summary, the primary factors that contributed to differences between the methods were size of substrate, nutrient medium, preparation of the inoculum, maintenance of anaerobiosis, and type of fermentation vessel.
16. **Barnett, A. J. G., and R. L. Reid.** 1957. Studies on the production of volatile fatty acids from grass by rumen liquor in an artificial rumen. J. Agr. Sci. 48: 315-321. -- It is impossible to use the same rumen liquor for all experiments in a series because rumen liquor loses potency within a week if stored under deep freeze conditions. Alternatives are to use the same fistulated animals kept under the same conditions or to incorporate with every experimental rumen a standard sample. All results then are compared back to the standard. These workers found variations in the volatile fatty acids produced from fresh and dried grasses. They suggest the variations are due to carbohydrate changes that occur during storage and drying of the grasses. Slaughtered sheep provide the rumen liquor. The diet of the sheep before slaughter was not reported.
17. **Barnett, A. J. G., and R. L. Reid.** 1961. Reactions in the rumen. Edward Arnold Ltd. 252 p. -- Page 39. An important function of the fistulated animal is, of course, as a source of rumen fluid or *in vitro* experimentation, but unless such material is pooled there is a possible danger of the

results obtained being "standard" only for one animal or for one animal on a particular diet.

Page 40. Simple incubation of rumen liquor is likely only to provide useful information if the reaction period involved is of short duration.

18. **Barth, J., and S. S. Hansard.** 1961. Comparative effects of calcium to phosphorus ratios and levels upon *in vitro* availability of phytin and inorganic phosphorus. *J. Anim. Sci.* 20(2): 396.
  
19. **Baumgardt, B. R., and H. K. Oh.** 1964. Evaluation of forages in the laboratory. IV. Within and among trial variability of the Wisconsin artificial rumen procedure. *J. Dairy Sci.* 47: 263-266. -- The optimum time for fermentation studies should be established within each laboratory since there may be variation in the source and activity of rumen fluid inoculum. This time should be chosen to maximize the correlation of *in vitro* and *in vivo* data and minimize within- and among-trial variation. These workers used a 100-ml tall-form beaker. If this beaker is used, no transfer is involved, since the entire procedure, even the final ashing (2.5 hr at 500°C), is accomplished in the beaker. Forages ground through a 60-mesh screen had a significantly higher apparent cellulose digestibility than when ground through a 40-mesh screen. Additionally, there was a forage x mesh interaction because the effect of grind fineness was much greater on grasses than on alfalfa. One method, however, does not appear better than the other when compared to the *in vivo* data. More finely ground forage tends to produce a repeatable sample more easily. Increasing the number of trials decreases the standard error more than increasing the number of replicates within a trial. In these studies, the workers include a control hay, but if the digestibility of the control hay deviates less than five digestibility units, no correction is made. If the deviation is more than five units, the entire trial is considered abnormal, and the data are discarded. This was done because variability did not decrease after the data were adjusted according to the digestibility of a control forage. Day-to-day variation was significantly less for the 40 hr fermentation than for any of the shorter times.
  
20. **Baumgardt, B. R., M. W. Taylor, and J. L. Cason.** 1962. Evaluation of forages in the laboratory II. Simplified artificial rumen procedure for obtaining repeatable estimates of forage nutritive value. *J. Dairy Sci.* 45: 62-68. -- Use of the artificial rumen specifically for the quantitative estimation of nutritive value was first recorded by Pigden and Bell in 1955 in an abstract in the *Journal of Animal Science*. In one series of trials they found digestibility *in vitro* to be significantly correlated with that *in vivo* whereas in another series of trials it was not. Variations in the diet of the fistulated, inoculum-donor cow could possibly account for these differences. These workers emphasize simplicity, standardization, and repeatability in developing their methods. Each of these factors is highly important if a routine method is to give consistently reliable estimates of nutritive value. An all-glass system capped with a Bunsen valve was used. This system gave results comparable to a semi-permeable membrane system for cellulose digestion and volatile fatty acid patterns. Rumen fluid from a single cow on an all-hay diet supplemented with trace-mineralized salt, bone meal, and water was used to digest several grass, alfalfa, and trefoil hay samples. The amount of cellulose in the rumen fluid inoculum was subtracted to correct results. A standard forage was included at each date to correct for inoculum variations. They examined forage substrate levels of 0.5, 1.9, and 1.5 grams in 30 ml of buffer mineral solution and 25 ml of rumen fluid. The percent cellulose digested was not affected. They used a 1-gm sample as a standard. Samples on four days during a two-month period from a cow on the same diet and the same cellulose source yielded a cellulose digestibility coefficient of variation of only 1.7%.
  
21. **Belasco, I. J.** 1954. Comparison of urea and protein meals as nitrogen sources for rumen microorganisms: urea utilization and cellulose digestion. *J. Anim. Sci.* 13: 739-747.

22. **Belasco, I. J.** 1956. The role of carbohydrates in urea utilization, cellulose digestion, and fatty acid formation. *J. Anim. Sci.* 15: 496–508. — A culture was subdivided at 24-hr intervals for four successive periods. Urea utilization by rumen microorganisms *in vitro* was dependent on the amount and type of carbohydrate used as the energy source. Urea utilization was slightly greater with starch than with cellulose. The end-products of digestion suggest different metabolic pathways for the various carbohydrate substrates or a dynamic microbial population changing in number and type with changes in substrate.
23. **Bentley, O. G.** 1960. A comparison of artificial rumen techniques, p. 181–185. *In Oklahoma conference on radioisotopes in agriculture.* USAEC. TID 7578. — Principal substrates in artificial rumen studies are energy sources, nitrogen, minerals, buffers, and growth promotants. The pH is usually held at 6.8 to 6.9. Anaerobic conditions are maintained by bubbling CO<sub>2</sub> or CO<sub>2</sub> + N<sub>2</sub> thru the mixture. The optimum substrate level should be determined for each system, for the lack of substrate can lead to erroneous conclusions about the activity of a system. Washing of cells or even centrifuging to separate cells from the rumen juice seems unnecessary if one is studying cellulose digestion of a forage. It would seem unwise to use an inoculum from an animal fed a high-grain ration for studies on cellulose digestion in the artificial rumen or to use an inoculum prepared from animals on an unknown dietary regime.
24. **Bentley, O. G., A. Lehmkuhl, R. R. Johnson, T. V. Hershberger, and A. L. Moxon.** 1954. The “cellulolytic factor” activity of certain short chained fatty acids. *Amer. Chem. Soc. J.* 76: 5000–5001.
25. **Bezeau, L. M.** 1965. Effect of source of inoculum on digestibility of substrate in *in vitro* digestion trials. *J. Anim. Sci.* 24: 823–825. — Three alfalfa hays, a mixed grass hay, orchard grass hay, and a native prairie hay were fed to two fistulated donor cows in order of descending protein content of the hay. These cows provided the inoculum used for each substrate. The *in vitro* system included 90-ml tubes, each with 1 gm of substrate, 29 ml buffer solution, small amounts of glucose and urea, 25 ml of strained rumen fluid, and CO<sub>2</sub> bubbled continuously through the solution for the 24-hr fermentation. The period of digestion was determined by the time required to reach the recommended level of digestible cellulose of the three standard forage samples. (Supposedly these were three standard forages submitted by the forage evaluation group, who found 24 hr was near sufficient.) The inoculum of the native prairie hay was significantly more variable than were the other hays. Inoculum from alfalfa was the least variable. Digestibility of the substrate was not significantly different when the inoculum was from the same hay or when the inoculum and substrate were from different hays. The hays contained 7–18% crude protein and 26–37% cellulose. The digestibility of cellulose in the native and grass hays was higher than in the alfalfa hays. There was a highly significant difference in the activity of the inocula from the two donor animals. This may have been due to age, for one cow did not come to feed as quickly as the other when hay was changed, nor did the former cow have as large an appetite. When the same inoculum and substrate were used digestibility was about 51%; with different inoculum and substrate, digestibility was about 49%. Perhaps the lack of significant differences of an inoculum effect is due to the fact that there was relatively good buffer and nutrient solution. Another oddity in this trial is that the donor cows were removed from feed at 1200 on a given day, but inocula samples were not taken until 830 the following morning. Further there was only a ten-day preliminary period for each forage. Some studies have indicated longer than ten days are required for microbial populations to equilibrate to the new feeding conditions.
26. **Blake, J. T., R. S. Allen, and N. L. Jacobson.** 1957. The influence of various factors upon surface tension and pH of rumen fluid. *J. Anim. Sci.* 16: 190–200. — The pH of samples collected orally from steers differed from samples collected by ruminal fistulas from the semisolid stratum ventral and medial to the fistula. Surface tension values of the rumen fluid collected by these two routes were similar.

27. **Bonsembiante, M.** 1958. L'azione del saccarosio, in vitro e in vivo, sulla attività cclulosolitical dei batteri del rumine. *Rivista di Zootecnia*. Vol. 31: pp. 23-25.
28. **Bortree, A. L., K. M. Dunn, R. E. Ely, and C. F. Huffman.** 1946. A preliminary report on the study of factors influencing rumen microflora. *J. Dairy Sci.* 29: 542-543.
29. **Bowden, D. M., and D. C. Church.** 1962. Artificial rumen investigations. I. Variability of dry matter and cellulose digestibility and production of volatile fatty acids. *J. Dairy Sci.* 45: 972-979. — These workers bubbled CO<sub>2</sub> through 250-ml centrifuge bottles containing a 1-gm sample of forage, 70 ml of distilled water, 30 ml of artificial saliva, and 2.5 ml of sodium carbonate solution. Inoculum was obtained two to four hours after feeding from a single fistulated steer receiving a low-quality grass hay and mixed alfalfa-barley pellets. Rumen liquor was strained through cheesecloth and allowed to stand about 30 minutes. The bottom liquid was then siphoned off for inoculation. Letting the coarse material rise to the top of the container reduced within-treatment variability. Dry matter determinations were made by filtering the contents of each flask under suction through a fritted glass disc crucible with a 40- $\mu$  pore size. The amount of material added by the inoculum was subtracted. Crucibles were dried at 110°C for 24 hr, cooled, and weighed. The residue was then removed and weighed into a 50-ml centrifuge tube for cellulose determination. Crucibles were easily cleaned with a sulphuric acid-dichromate cleaning solution. The variable intake of water by the fistulated steer may have affected between-trial variability. No effort was made to control the water intake of the steer prior to collection of rumen liquor. There was a greater between-trial variability in cellulose digestion than in dry digestion. Their results suggest that some interaction between the substrate and inoculum results in a greater or lesser digestibility of some fraction of the dry matter at the expense of cellulose. *In vitro* digestibility of frozen forages and dried forages differed little. The frozen forages had higher between-trial variability, probably due to the difficulty of obtaining representative small samples from large samples of frozen green material. They note that samples dried at high temperature (by other workers) had lower dry matter digestibilities than when freeze-dried. Digestibility variance between trials in which a standard alfalfa was used indicated differences in the digesting capacity of rumen liquor collected from the same steer on different days.
30. **Bowden, D. M., and D. C. Church.** 1962. Artificial rumen investigations. II. Correlations between in vitro and in vivo measures of digestibility and chemical components of forages. *J. Dairy Sci.* 45: 980-985. — They ran correlations between *in vivo* digestibility determined with sheep to *in vitro* digestibility determined with cattle. They briefly reviewed the literature concerning this point giving reference to several other articles and concluded that "it seems advisable for most accurate estimates to use inocula from the animal which is on a diet of nutritional ingredients as close as possible to that being digested." There is an indication that differences between grasses and legumes may be particularly marked in this respect. Their correlations between *in vitro* digestibilities and crude protein were sufficiently low that they would require marked differences between forages to be detected by such techniques.
31. **Bowie, W. C.** 1962. *In vitro* studies of rumen microorganisms, using a continuous flow system. *Am. J. Vet. Res.* 23(95): 858-868.
32. **Brown, W. H.** 1959. Studies of the *in vitro* techniques and the effects of diet upon the production and recycling of rumen volatile fatty acids. Ph.D. Thesis. Univ. Maryland. 128 p. — In studies with washed cell suspensions of bacteria from rumen fluid, a fermentation period of less than 1 hr gave products from radioactive glucose in which the relative composition of volatile fatty acids was similar to that found in whole rumen fluid.
33. **Bryant, M. P.** 1960. Some aspects of ruminal metabolism revealed by pure culture studies of the indigenous flora. *Int. Grassl. Congr., Proc.* 8: 518-522.

34. **Bryant, M. P.** 1963. Symposium on microbial digestion in ruminants: identification of groups of anaerobic bacteria active in the rumen. *J. Anim. Sci.* 22: 801-813. -- This is a comprehensive review with major sections on methodology and on some features and differentiation of species. Earlier review articles are cited, especially the one of Hungate (1960) in which the ecological analyses of rumen populations are discussed. Other articles referred to discuss various rumen microbial species and their culture and enumeration, metabolism, and nutrition. Other cited articles concern utility of pure-culture studies and the philosophy of bacterial classification. The author concludes that a complete ecological analysis of the rumen microbial population is necessary for a more complete understanding of ruminant metabolism. The identification of individual species is an important part of this analysis, but this identification is difficult because the species are difficult to grow and because there are many unknown variations in morphological and physiological characters. Furthermore, many workers have used poor methods and inadequate controls in establishing known features. Some of the more useful identifying features include morphology, fermentation products, energy sources, and certain nutritional, cultural, serological, and other physiological characteristics. Consultation with experienced workers will greatly expedite bacterial isolation and identification. No major group is now working on long-term classification and identification of rumen non-sporeforming anaerobic bacteria. This situation needs correction.
35. **Bryant, M. P., and L. A. Burkey.** 1953. Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. *J. Dairy Sci.* 36: 218-224. -- Fistulated animals were fed diets of alfalfa hay concentrate, wheat straw, alfalfa hay, and concentrate. Protein varied from 3.8% for wheat straw to 15.4% for concentrate. Bacteria were examined from colonies grown in culture tubes. The level of feeding of hay-concentrate ration had little effect on the numbers of bacteria grown. The number of starch-hydrolyzing bacteria were found lowest on straw ration, intermediate on alfalfa and alfalfa-concentrate, and highest on concentrate. The numbers of cellulose-digesting bacteria isolated varies with the amount of crude fiber in the ration. The numbers of bacteria which would produce acid from xylose were highest in samples taken from animals on the straw ration. There were differences between animals not accounted for by ration effects. The cellulolytic bacteria constituted 5% of all bacteria on concentrate rations and 28% of bacteria on wheat straw rations. Although a higher proportion of cellulolytic bacteria were found on the poor quality, low protein wheat straw than on the alfalfa hay of alfalfa hay concentrate rations, all contained about the same amount of bacteria.
36. **Bryant, M. P., and R. N. Doetsch.** 1955. Factors necessary for the growth of *Bacteroides succinogenes* in the volatile acid fraction of rumen fluid. *J. Dairy Sci.* 38: 340-350.
37. **Bryant, M. P., and I. M. Robinson.** 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. *J. Dairy Sci.* 44: 1446-1456. -- Significant differences appeared in nonselective viable counts of ruminal bacteria obtained from samples of rumen contents collected at different times during the day from a cow fed alfalfa or grain mixture once daily. Differences also were due to the two different rations. On a hay ration, numbers of bacteria (expressed as colony counts  $\times 10^9/g$ ) varied from about 1 to 3.2, with daily extremes at 0900 and 1900. On a grain ration, extremes varied from about 2.2 at 0800 to about 11 at 1100. The count dropped between 0500 and 0700. This corresponded to the first water intake each day. The bacteria decreased further during the first one to two hours after feeding, possibly due to a dilution of the contents with feed and saliva. The numbers then rapidly increased for two to four hours, after which they declined on the grain ration or plateaued on the hay ration to near the early morning levels before water and feed had been consumed.
38. **Burroughs, W., N. A. Frank, P. Gerlaugh, and R. M. Bethke.** 1950. Preliminary observations upon factors influencing cellulose digestion by rumen microorganisms. *J. Nutr.* 40: 9-24.

39. **Burroughs, W., H. G. Headley, R. M. Bethke, and P. Gerlaugh.** 1950. Cellulose digestion in good and poor quality roughages using an artificial rumen. *J. Anim. Sci.* 9: 513–522. — This is an early use of an *in vitro* system to evaluate roughages commonly fed to cattle. Four each of good- and poor-quality roughages containing from 1.4% to 20% crude protein were fermented. After a given sample had fermented for 36-hr, the apparatus was dismantled, and the fermentation residue was divided into two equal portions. One was saved for chemical analysis and bacteriological examination. The other portion served as inoculum for a second 36-hr fermentation with a new supply of roughage. This procedure was repeated during each successive 36-hr fermentation. Cellulose in the poor-quality roughages was not digested efficiently without supplementation. Nitrogen alone had no effect, but available nitrogen combined with complex minerals greatly increased cellulose digestion. Addition of manure extract produced no further increase. Cellulose in the good-quality roughages was digested as efficiently without supplements as with them. Among the advantages of the artificial rumen system are a precision which can be exercised over laboratory conditions, the number of animals that can be compared, and the efficiency (in terms of expense) of experimentation, especially in studies on synthetic diets.
40. **Burroughs, W., C. Arias, P. DePaul, P. Gerlaugh, and R. M. Bethke.** 1951. *In vitro* observations upon the nature of protein influences upon urea utilization by rumen microorganisms. *J. Anim. Sci.* 10: 672–682.
41. **Caballero, H., I. O. Galli, and J. E. Moore.** 1962. A comparison of forage evaluation methods. Paper presented at the 54th annual meeting of the American Society of Animal Science, Chicago. 5 p. — The evaluation of forages is considered today to be one of the most challenging problems in agricultural research. In recent years, both economists and animal nutritionists have taken keener interest in this matter and the urgent need has been recognized for more precise, rapid, and reliable methods for the evaluation of forages. In this study, various *in vivo* and *in vitro* procedures were utilized including a comparison of the method of Quicke *et al.* and that of Tilley *et al.* in addition to cellulose solubility in cupriethylene diamine. Bermuda and Pangola grass hays were utilized. *In vitro*, there were greater differences between hays at 24 and 48 hr than at 12 hr. This was a highly significant interaction of hay and time.
42. **Chalupa, W., and D. D. Lee, Jr.** 1966. Estimation of forage nutritive value from *in vitro* cellulose digestion. *J. Dairy Sci.* 49: 188–192. — They evaluated 25 forages in a system of 100-ml beakers with Bunsen valves, 25 ml nutrient solution, 23 mg urea and glucose, 20 ml of filtered rumen juice, an initial CO<sub>2</sub> flush only, and fermentation times of 6, 12, 18, 24, 30, 36, 48 hr. Digestion was almost complete at about 30 hr. There was a lag in digestion at 6, 12, and 18, and it differed for various forages. There was higher correlation to *in vivo* data at 30 hr or more, than at less than 30 hr. Adjustment for day-to-day variability by means of a control forage decreased variability among trials. There was no significant forage x trial interaction.
43. **Cheng, E. W., G. Hall, and W. Burroughs.** 1955. A method for the study of cellulose digestion by washed suspensions of rumen microorganisms. *J. Dairy Sci.* 38: 1225–1230. — This was one of the earlier studies using washed suspensions of rumen microorganisms in *in vitro* studies. A large quantity of rumen fluid from the fistulated animal was centrifuged at about 1,000 rpm for 1 minute, and the protozoa and partially-digested feed particles were discarded. The supernatant was centrifuged again at about 5,000 rpm for 20 minutes. The resulting sediment, principally rumen bacteria, was suspended in water or a phosphate buffer and centrifuged again for 20 minutes at 5,000 rpm. After another washing, the final sediment was suspended in a nutrient solution. This technique was used to study the digestion of purified cellulose, but could be utilized in evaluating feedstuffs.
44. **Christiansen, W. C., R. Kawashima, and W. Burroughs.** 1965. Influence of protozoa upon rumen acid production and liveweight gains in lambs. *J. Anim. Sci.* 24: 730–734. — Lambs were

defaunated by starvation and by drenching with copper sulfate. Protozoa-free lambs were inoculated with four types of protozoa and a combination of the four types. Faunated lambs gained more rapidly and efficiently than protozoa-free animals. Rumen pH was also lower, and fatty acid and ammonia levels were higher in the inoculated lambs. A narrower acetate-propionate ratio was observed in rumen ingesta from faunated as compared with protozoa-free lambs. Inoculation with single types of protozoa resulted in rumen changes approaching those observed when the inoculum included all four protozoal types. The implication of this study on *in vitro* evaluation of forages is that protozoa may improve estimates of forage digestibility compared to *in vivo* systems. Products of cellulose degradation by bacteria might tend to alter the rate or type of degradation which takes place. Utilization of these products by protozoa reduces this tendency. The overall rumen and feedlot results suggest that rumen protozoa can, under certain conditions, benefit the nutrition of the ruminant. The method by which protozoa exert this influence cannot be described accurately from these studies. In this ration, about 50% were concentrates and 50% roughages. One would expect greater benefits due to protozoa when rations are highly fermentable, such as high-concentrate rations.

45. **Christiansen, W. C., W. Woods, and W. Burroughs.** 1964. Ration characteristics influencing rumen protozoal populations. *J. Anim. Sci.* 23: 984-988.
46. **Church, D. C., and R. G. Petersen.** 1960. Effect of several variables on *in vitro* rumen fermentation. *J. Dairy Sci.* 43: 81-92. -- Effects of the following rumen fermentation variables were compared: (1) amount of substrate, rumen liquor, and mineral solution, (2) source of rumen liquor, (3) pH adjustment, and (4) particle size of roughage substrates. These workers utilized a second-order central composite design in three incomplete blocks. This allowed the simultaneous evaluation of the effect of these variables over a wide range of concentrations with considerably fewer treatments than would have been required had a full factorial set of combinations been used. Linear, quadratic, and first-order interaction effects of the three variables were studied. Increasing levels of dried grass added to fermentation flasks depressed percentage digestibility of dried matter and cellulose. Changes in the mineral medium concentration with the ranges used had only negligible effect on *in vitro* digestion of dry matter and cellulose. The optimum pH for cellulose digestion appeared to vary according to substrate and/or source of rumen liquor. In order to standardize evaluations and comparisons of *in vitro* feedstuff fermentations within and among laboratories, the following variables should be considered: rumen liquor source, pH adjustments, quantity of liquor and substrate, and particle size, which should be standard as far as possible within and among laboratories. Other variables, such as length of fermentation, addition of trace minerals, and effects of various techniques, are important also.
47. **Clark, K. W.** 1958. The adaptation of an artificial rumen technique to the estimation of gross digestible energy of forages. Ph.D. Thesis. Purdue Univ. 110 p. -- Twenty-six samples of 13 grass species were studied using an artificial rumen-pepsin-HCl procedure. Cellulose digestion depended greatly on the method of preparing the rumen inoculum. The most significant correlation between *in vitro* and *in vivo* digestible energy was obtained when the digestion with whole rumen fluid alone was used.
48. **Clark, K. W., and G. O. Mott.** 1960. The dry matter digestion *in vitro* of forage crops. *Can. J. Plant Sci.* 40: 123-129. -- The workers evaluated various forages and varieties of a given forage in *in vitro* systems. Large seasonal differences in digestibility were detected by the artificial rumen technique. Of special interest was that the method of drying the herbage for these *in vitro* digestion experiments was studied with the conclusion that there was a significant difference in favor of freeze-drying. The dry matter digestion coefficients of the freeze-dried herbage were comparable to those expected for herbage of similar quality digested *in vivo*. These workers recommended that in using the artificial rumen technique for plant breeding studies, all lines

should be screened in a single trial to insure maximum controls of the variables affected by technique. Freeze-dried samples were 10 to 15% higher in dry matter digestion than were samples oven-dried at 85°C.

49. **Clarke, R. T. J.** 1965. Quantitative studies of digestion in the reticulorumen: III. Fluctuations in the numbers of rumen protozoa and their possible role in bloat. *New Zealand Soc. Anim. Prod., Proc.* 25:96-105. — The concentration and total numbers of protozoa and of individual species were measured in cows fed red clover. Concentrations of protozoa varied widely. Total numbers of protozoa increased after feeding and then dropped to a minimum at about 5 hr. Concurrently, there was a gain in net rumen content from 0-2 hr before a linear decrease occurred. Total protozoa numbers were a maximum of about  $5.2 \times 10^9$  and a minimum of about  $2.8 \times 10^9$ . Protozoa concentrations varied from a maximum of about  $16 \times 10^6/100$  g of rumen content to a minimum of about  $6 \times 10^6/100$  g. The marked diurnal variance in total numbers of ciliate protozoa in the rumen was found to result mainly from changes in the holotrich population. The changes in this population depended, however, upon the type of feed given previously. The drop in numbers of one species was too great to be the result of passage of ingesta out of the rumen, and probably it is attributable to bursting of the organisms in the period of 2-5 hr after feeding. These rumen holotrich protozoa have a metabolic abnormality whereby they are unable to control the storage of reserve polysaccharide inside their bodies. In the presence of excess substrate, they may burst as a result of continued synthesis. This has been shown both *in vitro* and *in vivo*. The substances released when the protozoa burst have foam-stabilizing properties and may in part be responsible for triggering the imbalance which leads to bloating of animals. The fact that the rumen flora and fauna are not necessarily the same in various animals maintained on the same feed may be related to instance of bloat.
50. **Clarke, R. T. J., and R. E. Hungate.** 1966. Culture of the rumen holotrich ciliate *Dasytricha ruminatum* Schuberg. *Appl. Microbiol.* 14:340-345. — These cultures were established in a salts medium containing 30% clarified rumen fluid. Rumen fluid was essential. There was a positive correlation between formation of methane in the cultures and growth of ciliates.
51. **Claypool, D. W., and D. R. Jacobson.** 1959. Effect of minor changes in the ingredient compositions of the ration on rumen microbial activity. *J. Dairy Sci.* 42: 913-914.
52. **Cline, J. H., T. V. Hershberger, and O. G. Bentley.** 1958. Utilization and/or synthesis of valeric acid during the digestion of glucose, starch and cellulose by rumen micro-organisms *in vitro*. *J. Anim. Sci.* 17: 284-292.
53. **Davey, L. A., G. C. Cheeseman, and C. A. E. Briggs.** 1960. Evaluation of an improved artificial rumen designed for continuous control during prolonged operation. *J. Agr. Sci.* 55: 155-163. — Two basic designs of artificial rumens have been (1) impermeable systems in which rumen contents were incubated with specific substrates, and (2) permeable systems in which rumen contents were incubated with specific substrates, the fermentation being dialyzed against mineral solutions of varying composition. Generally, permeable systems are closer to the *in vivo* state than impermeable systems. These workers list a series of criteria they consider necessary for establishing the validity of an artificial rumen system. They describe the construction of an apparatus for continuous flow studies, but the system is not suitable for large-scale evaluation of forages.
54. **Dawson, R. M. C., P. F. V. Ward, and T. W. Scott.** 1964. A micro-artificial rumen for isotopic experiments. *Biochem. J.* 90: 9-12.
55. **Dehority, B. A.** 1963. Isolation and characterization of several cellulolytic bacteria from *in vitro* rumen fermentations. *J. Dairy Sci.* 46: 217-222. — Samples of rumen ingesta were obtained

- from a fistulated steer maintained on a diet of mixed hay. The bacteria were obtained from a phosphate buffer extract of pressed rumen ingesta. Differential centrifugation was used to obtain an inoculum consisting primarily of cellulolytic rumen bacteria. Various bacteria were cultured under anaerobic conditions. Roll tubes were utilized. Colonies were picked from the roll tubes and stabbed first into slants and then into tubes of liquid medium containing cellulose. Stock cultures were maintained by daily transfer to fresh media. Five strains were selected for detailed studies, including representatives of ruminal cellulolytic bacteria. Their nutritional requirements differed, however, and these requirements differed from those previously reported.
56. **Dehority, B. A.** 1965. Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. *J. Bacteriol.* 89:1515–1520. — Hemicelluloses from flax, corn hulls, alfalfa, oat hulls, and fescue grasses were digested by pure cultures of eight bacterial strains. Three strains of ruminococci were able to partially utilize different hemicelluloses. The extent of degradation in utilization varied markedly among the three strains and for the different substrates. Corn hull hemicellulose was most resistant to degradation and utilization.
  57. **Dehority, B. A., K. El-Shazly, and R. R. Johnson.** 1960. Studies with the cellulolytic fraction of rumen bacteria obtained by differential centrifugation. *J. Anim. Sci.* 19: 1098–1109. — Digestion of cellulose by rumen microorganisms *in vitro* was first studied in detail by Marston in 1948. Workers at the Ohio Station developed a technique which separated bacteria from rumen liquor by centrifugation in the Sharples super-centrifuge, resuspended the bacteria in buffer, and used this suspension as inoculum in a purified basal medium. This paper describes a differential centrifugation method whereby a fraction of rumen bacteria containing primarily cellulolytic bacteria can be obtained. A diagram is given of the centrifugation scheme for determining the rumen bacteria which are cellulolytic. For bovine and ovine rumen bacteria, the fraction of bacteria still in suspension at  $1500 \times G$  but sedimented at  $3000 \times G$  contained the majority of the cellulolytic activity. These organisms were also found in large numbers in the inoculum obtained from the rumen. These microorganisms required both acid and biotin.
  58. **Dent, J. W.** 1963. Applications of the two-stage *in vitro* digestibility method to variety testing. *J. Brit. Grassland Soc.* 18: 181–189. — The Tilley and Terry system was utilized for varieties of kale, rape, cabbage, clover, lucerne, and silage maize. Results with crops of this nature were somewhat less satisfactory than have been obtained in the past with grasses. Rumen liquors appeared to vary in their ability to deal with feeds containing appreciable amounts of starch. This could account for a lack of reproducibility in results from week to week. It has been observed by Warner that the rate of starch digestion depends upon the previous diet of the sheep. El Shazly indicated that cellulose digestion may be partially inhibited by the presence of starch.
  59. **Dent, J. W., and D. T. Aldrich.** 1966. The *in vitro* digestibility of herbage species and varieties and its relationship with cutting treatment, stage of growth and chemical composition. *Int. Grassl. Congr., Proc.* 10:419–424. — Their *in vitro* technique is sufficiently precise that in herbage trials over two years at two locations differences of 0.9 to 3.2% have been established between varieties.
  60. **Doetsch, R. N., R. Q. Robinson, R. E. Brown, and J. C. Shaw.** 1953. Catabolic reactions of mixed suspensions of bovine rumen bacteria. *J. Dairy Sci.* 36: 825–831.
  61. **Doetsch, R. N., R. Q. Robinson, and J. C. Shaw.** 1952. Techniques employed in cultural investigations of the bacteriology of bovine rumen contents. *J. Anim. Sci.* 11: 536–544.
  62. **Donefer, E., E. W. Crampton, and L. E. Lloyd.** 1960. Prediction of the nutritive value index of a forage from *in vitro* rumen fermentation data. *J. Anim. Sci.* 19: 545–552. — These workers utilized an *in vitro* system similar to that of Quicke *et al.* The inoculum was prepared by straining rumen juice, resuspending the pulp in a phosphate buffer, centrifuging the resultant buffer

extract, and resuspending that centrifuged material in a phosphate buffer. Samples were taken at 3, 6, 12, 24, and 48 hr to examine rates of digestion. A good time curve of cellulose digestion against fermentation time is given comparing different forages. The prerequisite of a useful *in vitro* technique must be its ability to measure the effect on cellulose digestion of early lag differences in fermentation between forages. It is necessary to include a standard in all runs to test repeatability and to compare techniques. They proposed a 12-hr *in vitro* cellulose digestion be used to predict a nutritive value index for a forage. They found a correlation of about 0.90 between *in vitro* cellulose digestion at 12 hr and nutritive value index for forages ranging in cellulose digestion from 25–55% and nutritive value indices ranging from 35 to 70.

63. Donefer, E., E. W. Crampton, and L. E. Lloyd. 1966. The prediction of digestible energy in potential (NVI) of forages using a simple *in vitro* technique. *Int. Grassl. Congr., Proc.* 10:442–445. — A pepsin solution dissolved in HCl was used to determine percent dry matter disappearance which was found to be highly correlated with forage digestible energy intake potential expressed as NVI. An equation was developed for 35 grasses and 14 legumes.
64. Donefer, E., L. E. Lloyd, and E. W. Crampton. 1962. Prediction of the nutritive value index of forages fed chopped or ground using an *in vitro* rumen fermentation method. *J. Anim. Sci.* 21: 815–818.
65. Donefer, E., P. J. Niemann, E. W. Crampton, and L. E. Lloyd. 1962. Use of an *in vitro* enzyme digestion technique to predict the nutritive value of forages. Paper presented at annual meeting of American Dairy Sci. Assoc., College Park, Maryland. (Mimeograph)
66. Drew, K. R. 1966. The *in vitro* prediction of herbage digestibility. *N. Zealand Soc. Anim. Prod., Proc.* 26:52–70. — Experiments were conducted to compare *in vitro* methods for predicting herbage digestibility. Incubation of the test forage with rumen fluid from a grass-fed fistulated animal for 72 hr followed by a further 24 hr in acid pepsin gave *in vitro* digestibilities very close to *in vivo* figures. A relationship of  $y = 1.014x$ , where  $y$  is *in vitro* organic matter digestibility, was established. Week to week variation was greatly reduced when this method was used rather than a 48-hr microorganism digestion followed by a 48-hr pepsin digestion.
67. El-Shazly, K., B. A. Dehority, and R. R. Johnson. 1960. A comparison of the all-glass, semi-permeable membrane and continuous flow types of apparatus for *in vitro* rumen fermentations. *J. Dairy Sci.* 43: 1445–1451. — All three systems were utilized for 30-hr *in vitro* fermentations. Results among the three differed little. The authors concluded that the continuous-flow system may be more useful for *in vitro* experiments carried out for longer periods, such as 48–96 hr. One limitation of the semi-permeable membrane is that it apparently is attacked by bacteria and becomes weakened after periods longer than 30 hr. These authors suggested the use of a strip of the rumen wall epithelium as the membrane because it may be involved in biochemical reactions. Microscopic examination showed the bacteria that had propagated in 30 hr varied little among the three systems.
68. El-Shazly, K., B. A. Dehority, and R. R. Johnson. 1961a. Effect of starch on the digestion of cellulose *in vitro* and *in vivo* by rumen microorganisms. *J. Anim. Sci.* 20: 268–273. — Workers compared cellulose digestion *in vitro* with cellulose *in vivo* in nylon bags. The *in vitro* system involved using the resuspended phosphate buffer extract of a rumen pulp, centrifuging this extract, and resuspending the solids in buffer. The rumen liquor for *in vitro* studies came from the same animals used for the *in vivo* studies. When hay alone served as a ration, the cellulose digestion rates *in vivo* and *in vitro* were practically identical for the time period studied: 12, 24, 30 and 48 hr. There was a sigmoid curve for the results on cellulose digestion. Corn added to the hay inhibited the *in vivo* cellulose digestion. This inhibition was lessened somewhat if the corn was

supplemented by urea. Increasing the corn-hay ratio to 2:1 almost completely inhibited cellulose digestion *in vivo*, although *in vitro* there was still considerable cellulose digestion. The data suggest there was strong competition for essential nutrients *in vitro* with the result that starch-digesting microorganisms proliferated preferentially. There is a competition between the cellulolytic and the amylolytic groups of bacteria for nutrients, the major nutrient probably being nitrogen.

69. **El-Shazly, K., R. R. Johnson, B. A. Dehority, and A. L. Moxon.** 1961b. Biochemical and microscopic comparison of *in vivo* and *in vitro* rumen fermentations. *J. Anim. Sci.* 20: 839-843. — Rumen-fistulated steers and sheep were used for nylon bag and artificial rumen studies. Sedimented, strained rumen fluid and whole rumen fluid were used as the inoculum. Basal medium and *in vitro* procedures were those essentially of Dehority *et al.*, 1957. Digestion was studied at 3, 6, 12, 24, 30 and 48 hr. Microscopic examination was made of the bacteria after incubation periods of 24-30 hr. With sheep maintained on a purified cellulose diet there was almost an identical time curve relationship between *in vitro* and *in vivo* cellulose digestibility, but after 24 hr *in vitro* digestibility was greatly increased over that of the sheep. The rate of digestion *in vitro* for the steer seemed somewhat slower than that of the sheep. For incubation periods of 24 to 30 hr the microorganisms cultured *in vitro* are representative of those in the rumen when hay is used as substrate both *in vivo* and *in vitro*. When the inoculum from the hay-fed steer was incubated *in vitro* with purified cellulose, there was an obvious proliferation of the Gram-negative micrococci and a difference in bacterial concentrations. There was no marked morphological change in the bacterial population up to 24 to 30 hr.
70. **Elsden, S. R.** 1945-1946. The fermentation of carbohydrates in the rumen of the sheep. *J. Exp. Biol.* 22: 51-62.
71. **Elsden, S. R.** 1946. The application of the silica gel partition chromatogram to the estimation of volatile fatty acids. *Biochem. J.* 40: 252-256.
72. **Elsden, S. R., and A. T. Phillipson.** 1949. Ruminant digestion. *Annu. Rev. Biochem.* 17: 705-726.
73. **Elsden, S. R., and A. K. Sypsteyn.** 1950. The decarboxylation of succinic acid by washed suspensions of rumen bacteria. *J. Gen. Microbiol.* 4: 11-18.
74. **Erwin, E. S., C. J. Elam, and I. A. Dwyer.** 1957. The influence of sodium bentonite *in vitro* and in the ration of steers. *J. Anim. Sci.* 16: 858-862. — Sodium bentonite bound pure carotene *in vitro*, and altering the pH did not influence the release of carotene. Probably sodium bentonite would have no deleterious effect on carotene use if the clay were incorporated into rations rich in the pigment.
75. **Fauconneau, G.** 1961. Etude *in vitro* de l'influence des extraits aqueux de fourrage sur la croissance des bactéries du rumen. *Ann. Biol. Anim. Bioch. Biophys.* 1: 10-15.
76. **Fina, L. R., C. L. Keith, E. E. Bartley, P. A. Hartman, and N. L. Jacobson.** 1962. Modified *in vivo* artificial rumen (Vivar) techniques. *J. Anim. Sci.* 21: 930-934.
77. **Frederiksen, K. R.** 1961. Comparative *in vitro* digestibility of some major constituents of the summer diet of range sheep. M.S. Thesis. Colorado State Univ. 42 p.
78. **Frederiksen, K. R., and L. E. Washburn.** 1961. Comparative *in vitro* digestibility of some major constituents of the summer diet of range sheep. *West. Sect. Amer. Soc. Anim. Sci., Proc.* XLV: 1-6.

79. **Gall, L. S., and W. L. Glaws.** 1957. The bacteriology of the artificial rumen. *Bact. Proc. (Amer. Soc. Microbiol.)* p. 20-21. -- A comparison was made of an all-glass impermeable apparatus and a permeable type employing a cellophane sack. Fermentations were carried out for 24 to 48 hr, and residual cellulose was determined. Bacteriological counts were made of Gram stains and anaerobic cultural series designed to culture rumen bacteria from the original rumen contents and the material in both artificial rumens after 24 hr. The bacteriological examination suggested that the types of bacteria isolated from the permeable membrane system compared favorably with those observed in their original rumen contents. This contrasts with results from the impermeable system. Although cellulose digestion was complete in both systems, an accumulation of cellulase in the impermeable rumen may account for this digestion rather than viable bacteria. It is clear that the permeable system more nearly resembles rumen digestion.
80. **Gilchrist, F. M. C., and A. Kistner.** 1962. Bacteria of the ovine rumen. I. The composition of the population on a diet of poor teff hay. *J. Agr. Sci.* 59: 77-83. -- I. Studies were made of the composition of the population of the rumen bacteria from sheep on a diet of poor teff hay (*Eragrostis tef*). In a study in which the diet of the sheep was reduced in quantity, but not changed in composition, there was little or no influence on either the numbers or the morphological types of bacteria of each functional group. Diet varied from 1200 to 300 g of hay per day. In a later experiment with alfalfa, rumen flora differed from those of teff hay not only in significantly higher counts of carbohydrate-fermented bacteria, but also in distinctive types of cellulolytic bacteria (Kistner *et al.*, 1962). Whereas different types of rod-shaped bacteria, mainly slender Gram-negative rods and tiny coccoid forms were the most frequent cellulose digesters in sheep on teff hay, cocci of approximately 1.0 $\mu$  diameter were invariably predominant in the alfalfa hay-fed sheep.
81. **Gouws, L., and A. Kistner.** 1965. Bacteria of the ovine rumen. IV. Effect of change of diet on the predominant type of cellulose-digesting bacteria. *J. Agr. Sci.* 64: 51-57. -- Sheep diets alternated between alfalfa hay and teff hay twice. The teff hay contained 2.8% crude protein and the alfalfa, 13.6% protein. Cellulolytic bacteria were examined. When the diet was changed from alfalfa to teff hay, the time required for certain species of bacteria to establish themselves as the most numerous cellulolytic organisms varied from two to five weeks among different animals. When the diet changed from teff to alfalfa, *Ruminococcus albus* became the predominant bacteria in all sheep within two weeks after the change. In periods immediately following the changes in diet, cocci giving rise to atypical colonies and zones of cellulolysis transiently occurred as the most abundant cellulose digesters in the rumina of all the sheep. These studies suggest that relatively long preliminary periods are required to establish a ruminal population characteristic of the diet. They suggest also that a longer time is required when changing from a good diet to a poor diet than from a poor diet to a good diet.
82. **Grainger, R. B., M. C. Bell, J. W. Stroud, and F. H. Baker.** 1961. Effect of various cations and corn oil on crude cellulose digestibility by sheep. *J. Anim. Sci.* 20: 319-322.
83. **Gray, F. V., R. A. Weller, and G. B. Jones.** 1965. The rates of production of volatile fatty acids in the rumen. *Australian J. Agr. Res.* 16: 145-157.
84. **Gray, F. V., R. A. Weller, A. F. Pilgrim, and G. B. Jones.** 1961. A stringent test for the artificial rumen. *Australian J. Agr. Res.* 13: 343-349.
85. **Grimes, R. C., B. R. Watkin, and J. R. Gallagher.** 1966. An evaluation of pasture quality with young grazing sheep: II. chemical composition, botanical composition, and *in vitro* digestibility of herbage selected by esophageal-fistulated sheep. *J. Agr. Sci.* 66:113-119. -- Herbage samples were collected at fortnightly intervals by sheep with esophageal fistulae, and digestibility

was measured *in vitro*. Pastures being grazed were orchard grass, perennial ryegrass, and tall fescue each with and without white clover. Samples were obtained under two management systems—“short” pastures maintained at two to four inches height, and “long” pastures with unrestricted growth and flower development. *In vitro* digestibility was made with a modification of the technique of Tilley and Terry (1963) in which the second pepsin digestion was omitted. This modification was made because much of the herbage organic matter was transferred to the liquor when squeezing the extrusa. Organic matter digestibility was estimated from the *in vitro* cellulose digestibility by a regression equation established for 28 samples dried at 80°C for 24 hr without preliminary squeezing. The original two-stage technique was used to determine organic matter digestibility. The source of rumen liquor is not given. Field studies were conducted in the 1963–1964 season in New South Wales. In the early part of the experiment, samples from the grass plus clover plots had significantly higher organic matter digestibility and percent protein, but significantly lower percent cellulose than herbage from grass plots. In the latter part of the experiment there were no significant differences. Herbage from the “short” pastures showed a significantly higher organic matter digestibility and percent protein and lower percent cellulose than the herbage from the “long” pastures throughout most of the experiment. Differences between these treatments were not evident for animal data.

86. Gutierrez, J., and R. E. Davis. 1962. Isolation of saponin-digesting bacteria from the rumen of bloating cattle on ladino clover pasture. *J. Anim. Sci.* 21: 819–823.
87. Hale, E. B., C. W. Duncan, and C. F. Huffman. 1947. Rumen digestion studies. *J. Nutr.* 34: 747–758.
88. Hall, O. G., H. D. Baxter, and C. S. Hobbs. 1961. Effect of phosphorus in different chemical forms on *in vitro* cellulose digestion by rumen microorganisms. *J. Anim. Sci.* 20: 817–819.
89. Hall, O. G., C. D. Gaddy, C. S. Hobbs. 1959. Response by rumen microbes to phosphorus from different supplements. *Tennessee Farm and Home Sci. Progress Rep.* 31. 4 p.
90. Halliwell, G. 1959. The enzymic decomposition of cellulose. *Nutr. Abstr. Rev.* 29: 747–759. — Cellulose is the world's most plentiful, naturally-occurring organic compound. Up to 40% of plant residues are composed of cellulose. The capacity to degrade cellulose may be present in protozoa, insect larvae, worms, wood-boring insects, snails, and limpets, but in many of them the enzyme in question appears to be a polyglucosidase, not a cellulase. This extensive article continues with sections on estimation of the amount of cellulose decomposed and distribution of cellulolytic activity in animal and microbial species. There is a good section on cellulases of rumen microorganisms in which early work is reviewed. Enzymic preparations which decompose cellulose and mechanisms of the enzymic breakdown of cellulose are considered.
91. Halliwell, G., and M. P. Bryant. 1963. The cellulolytic activity of pure strains of bacteria from the rumen of cattle. *J. Gen. Microbiol.* 32: 441–448. — *In vitro* breakdown of degraded and undegraded varieties of cellulose by pure strains of bacteria isolated from the rumen of cattle was examined. The capacity of an organism or a cell-free enzyme to attack any one particular form of cellulose is no criterion of its ability to attack less degraded or undegraded types of cellulose. It has frequently been suggested that fibrous forms of cellulose are unsuitable substrates for rumen microorganisms because of the relatively small surface area exposed to attack. An increased susceptibility to biological attack that accompanies the transition from fibrous to powdery cellulose is liable to be associated not only with increased surface area, but also with degradative changes in the cellulose molecules. These studies have indicated that possibly different mechanisms are responsible for the breakdown of degraded and undegraded cellulose.

92. Hanold, F. J., E. E. Bartley, and F. W. Atkeson. 1957. Effects of combinations of feedstuffs, with and without aureomycin, on *in vitro* digestion of cellulose by rumen microorganisms. *J. Dairy Sci.* 40: 369-376.
93. Harbers, L. H., J. M. Prescott, and C. E. Johnson. 1961. Activities of dried rumen microorganisms *in vitro*. *J. Anim. Sci.* 20: 6-9.
94. Harris, C. E. 1963. Comparison of *in vivo* and *in vitro* measurements of the digestibility of fodder crops. *J. Brit. Grassland Soc.* 18: 189. -- The agreement between *in vivo* and *in vitro* tests on silage maize is less satisfactory than for most crops. Results for brassica are generally successful, but some kale data are not.
95. Hastings, E. G. 1944. The significance of the bacteria and the protozoa of the rumen of the bovine. *Bacteriol. Rev.* 8: 235-254.
96. Head, H. H. 1959. The rate and magnitude of digestion of a roughage as influenced by ethyl alcohol. M.S. Thesis. Rutgers Univ. 60 p.
97. Hershberger, T. V., O. G. Bentley, and A. L. Moxon, 1959. Availability of the nitrogen in some ammoniated products to bovine rumen microorganisms. *J. Anim. Sci.* 18: 663-670.
98. Hershberger, T. V., O. G. Bentley, J. H. Cline, and W. J. Tyznik. 1965. Formation of short-chain fatty acids from cellulose, starch, and metabolic intermediates by bovine rumen microorganisms. *Agr. Food Chem.* 4: 952-956.
99. Hershberger, T. V., T. A. Long, E. W. Hartsook, and R. W. Swift. 1959. Use of the artificial rumen technique to estimate the nutritive value of forages. *J. Anim. Sci.* 18: 770-779. -- These authors indicate that Marston (1948) was probably the first to study the metabolism of rumen microorganisms by culturing the population of rumen microorganisms *in vitro* and determining the end products formed. These studies were followed by a series of investigations by Burroughs *et al.* in the early 1950's. This paper represents one of the early studies of the comparison of *in vitro* and *in vivo* digestibilities. These workers used an all glass *in vitro* fermentation system, a 24-hr digestion period, and pooled microorganisms from two sheep. The results were compared with *in vitro* fermentation. Correlations between 24-hr *in vitro* fermentation and *in vivo* cellulose digestion were 0.97. *In vivo* cellulose digestibilities ranged from approximately 55% to 90%. *In vitro* cellulose digestibilities ranged from approximately 30% to 80%.
100. Hidioglou, M., P. Dermine, H. A. Hamilton. 1966. Chemical composition and *in vitro* digestibility of forage as affected by season in Northern Ontario. *Can. J. Plant Sci.* 46: 101-109.
101. Hinders, R. G., R. E. Smith, D. K. Nelson, and G. M. Ward. 1961. Preliminary observations on the use of total gas production from the artificial rumen to estimate alfalfa hay quality. Paper presented at the Western Division Meetings of the American Dairy Science Association, Moscow, Idaho, July, 1961. -- The possible use of total gas production measurements from hay samples fermented *in vitro* was studied to determine if this simple and rapid method could be used to estimate the degree of utilization of hay by rumen microorganisms. The apparatus consisted of 50-ml Erlenmeyer flasks with airtight connections to 100-ml graduated burettes. The burettes were filled with water acidified to pH 2.4 to prevent CO<sub>2</sub> absorption. The flasks were maintained at 39°C. A phosphate buffer, 15 ml, was added to 0.5 g of alfalfa hay, and CO<sub>2</sub> was bubbled into the flasks for ten minutes. Rumen juice was obtained from a rumen-fistulated cow 5 hr after feeding on an alfalfa hay ration. As fermentation proceeded, the gas produced by the microorganisms displaced the liquid in the burettes. Readings were

made after an 18-hr fermentation. A standard average quality alfalfa hay was fermented with each run, and gas production measurements were determined as the percentage of the gas produced by the standard alfalfa hay. Nineteen alfalfa hay samples were fermented in triplicate. The correlations of gas production to various chemical constituents were all equal to or less than 0.50.

102. **Hobson, P. N.** 1961. Some aspects of rumen microbiology 1956–1960, p. 49–55. *In* Collected papers – summary and subject, Rowett Res. Sta. 17. (Rowett Research Institute, Bucksburn, England.)
103. **Howie, J. W., and F. Baker.** 1952. Rumen and caecal micro-organisms as symbionts. Royal Soc. London, Proc. 139B: 193–196.
104. **Hubbert, F., Jr., E. Cheng, and W. Burroughs.** 1958. Mineral requirement of rumen microorganisms for cellulose digestion *in vitro*. J. Anim. Sci. 17: 559–568. — Washed suspensions of rumen microorganisms were prepared. Rumen contents were collected from steers on high corn-cob rations. Various minerals were evaluated by removing them singly with a complex nutrient medium. Of the minerals studied, sulphur, magnesium, and calcium were found to be the inorganic nutrients most likely to be deficient in a prepared fermentation medium. Additions of manganese, iron, copper, cobalt, zinc, and boron to the fermentation medium did not result in increased cellulose digestion. Extremely low levels of copper, cobalt, zinc, and boron depressed cellulose digestion.
105. **Huhtanen, C. N., and R. F. Elliott.** 1956. Factors influencing *in vitro* rumen cellulose digestion. J. Anim. Sci. 15: 1180–1187. — The semi-permeable membrane technique was used to evaluate the digestion of purified cellulose sources and alfalfa cellulose subjected to various treatments. On certain diets, dialyzing sacks containing the rumen fluid filled with gas in a few hours and would practically disintegrate. No actual digestion of the sack could be detected although it was very greatly weakened. This phenomenon of sack disintegration has been noted only with antibiotic-fed ruminants or on very prolonged (48 hr) incubation of rumen fluid. Alfalfa meal was extracted with boiling solvents in an attempt to decrease digestibility of the cellulose by removal of postulated cellulolytic factors. Studies on the effect of varying substrate concentrations showed that the cellulose digestion of alfalfa meal was approximately constant regardless of the amount of substrate present in the semi-permeable bag. The dilution of the rumen fluid to one-fifth of the original liquid volume had no effect on cellulose digestion. Further dilution resulted in progressively less activity. Addition of valeric or isovaleric acids indicated that these acids are normally present in sufficient amounts in the rumen fluid or they can be readily formed by rumen microorganisms. This is important because the centrifuged cell technique could give misleading information concerning the requirements for cellulose digestion of the normal rumen populations within the animal. The use of various solvents in extracting the alfalfa meal suggests that the possible stimulatory factor may be an alcohol-soluble protein. High levels of available glucose decreased cellulose digestion probably because the organisms used it for a carbon source rather than the less available cellulose. There was no evidence in this study that rumen fluid quickly deteriorates on contact with air or upon dilution.
106. **Huhtanen, C. N., R. K. Saunders, and L. S. Gall.** 1954. Fiber digestion using the miniature artificial rumen. J. Dairy Sci. 37: 328–351. — This is a description of a miniature artificial rumen system. This system consists of a small cellophane sack suspended in a 4-oz screw-cap jar containing a solution similar in mineral composition to sheep saliva. The whole system is placed in an incubator. The jar must be opened after about 3 hr to relieve gas pressure. Usually incubation is from 16 to 24 hr. The rumen sample is placed inside the sack with the feed sample to be studied. At the end of fermentation the contents of the sack are washed into a centrifuge tube, the fermentation is stopped, and the tube is refrigerated until analysis. In a study with 20 replicates

of rumen fluid, average digestion of a feed sample was  $47\% \pm 4\%$  (s.d.). In another study in which samples were taken from the same animal at different times over a 2-month period, the average was  $46.2\% \pm 3.8\%$ .

107. **Hungate, R. E.** 1946. The symbiotic utilization of cellulose. *Elisha Mitchell Sci. Soc. J.* 62: 9–24. — Various groups in the animal kingdom harbor microorganisms which act in symbiotic ways to digest cellulose. These include termites and ruminants, the best known examples, as well as horses, rodents, and certain beetles. Cellulases are produced by fungi, actinomycetes, molds, bacteria, and protozoa. Also, cellulose-splitting enzymes were discovered in barley seeds, in the snail, and in a fungus found in the rumen. Rumen bacteria vary in that some have an extracellular cellulase where others have none. In the termite, there is little multiplication of protozoa between molts of the insect. In the rumen, in contrast, there is a continuous growth of bacteria in protozoa with the consequence that large numbers of them pass into the adjacent portions of the stomach and are digested. The microorganisms associated with cellulose decomposition in horses, rodents, and insects other than termites have not been investigated in great detail. It would appear, however, that an initial anaerobic cellulose fermentation occurs as a mechanism of the symbiotic utilization of cellulose. The validity of this hypothesis can be determined only by further experimental studies.
108. **Hungate, R. E.** 1960. Symposium: selected topics in microbial ecology. I. Microbial ecology of the rumen. *Bacteriol. Rev.* 24: 353–364. — The rumen is an exceptional habitat in providing constant conditions of moisture, pH, temperature, anaerobiosis, and food, and in being an open system in which no stringently restrictive factors such as humoral defense mechanisms limit to a few the number of kinds of organisms which can survive. Among the factors influencing variation in counts of samples are variation in sampling procedures, counting method, time of sampling after feeding, culture medium, exposure to oxygen during dilution, and other factors. Many of the bacteria in the rumen are dead. This may also vary with the diet. It is possible that bacteria in cattle on a hay ration die more readily than those animals fed much concentrate, or that they are sequestered in the fibrous materials in a fashion not easily disrupted by mixing.
109. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press Inc. New York. 533 p. — This book is a complete review of rumen microbiology with related discussion of ruminant nutrition and physiology. It is based on more than 25 years experience by the author and more than 2000 references. Unfortunately, titles are not given for the references although inclusive pages of articles are given. There is good historical treatment of most aspects discussed. Chapters in the book especially pertinent to *in vitro* evaluation of roughages include those on bacteria and protozoa of the rumen, the rumen as a continuous fermentation system, and a section on *in vitro* evaluation of feedstuffs in a chapter on possible modifications in ruminant feeding practices. Of special interest is a comparison and discussion of fermentation curves giving some of their mathematical forms and properties.
110. **Hungate, R. E., G. D. Phillips, D. P. Hungate, and A. MacGregor.** 1960. A comparison of the rumen fermentation in European and zebu cattle. *J. Agr. Sci.* 54: 196–201. — A comparison was made of rumen fermentation by manometric methods of contents from zebu and mixed-breed European cattle. These animals were fed poor-quality grass hay, and another group was fed good-quality alfalfa hay. Hay was fed at 800 and 1600. Fermentation rates of rumen contents varied from zebu to European cattle due to time of feeding, individual animals, etc. The fermentation rate per gram of dry weight per hour was greater in the zebu in most instances.
111. **Hungate, R. E., G. D. Phillips, A. MacGregor, D. P. Hungate, H. K. Buechner.** 1959. Microbial fermentation in certain mammals. *Science* 130: 1192–1194.

112. Ifkovits, R. W., H. S. Ragheb, R. F. Barnes, and L. B. Packett. 1965. A pure-culture inoculum method for evaluation of forage cellulose digestibility. *J. Anim. Sci.* 24: 1092–1099. — A stab culture of *Bacteriodes succinogenes* S85 was utilized and was maintained on a rumen-glucose-cellobiose agar with weekly transfers. The time fermentation curve for this species showed that the percent cellulose digested of a grass followed a sigmoid relationship, whereas the cellulose digestion of an alfalfa sample had relatively small lag effect during initial fermentation. After 48 hr, for all practical purposes, the fermentation curves leveled off. The authors have outlined a proposed technique for evaluating cellulose digestibility by the pure culture method. This requires sterilizing the forage and nutrient medium. The advantage of this pure culture method is that there may be smaller within- and between-trial variations in the inoculum because they are maintained as a pure culture. However, it does not appear this technique will replace use of the whole rumen fluid and the mixture of species. Furthermore, it will be necessary to test the effect of a cellulase from a single species on a wide variety of forages. The system did, however, give good correlations with digestibilities obtained by other techniques.
113. Jamison, H. M., and O. G. Hall. 1964. The effect of Thimet on *in vitro* cellulose digestion by rumen microorganisms. *Tenn. Farm and Home Sci. Progr. Rept.* 51: 4–6. — *In vitro* rumen fermentation techniques were used to evaluate the influence of Thimet, an insecticide, on rumen microorganisms. It was postulated there might be some stimulation of rumen microbial activity due to Thimet. Whole rumen liquor was compared with washed-cell suspensions. Thimet was added at 20, 50, 70, and 100 ppm in the fermentation flask, as well as at lower concentrations. Beyond 10 ppm, Thimet decreased ruminal microbial activity. The rate of decrease was similar for washed-cell suspensions and whole rumen liquor. Overall, whole rumen liquor gave higher cellulose digestibility values than did washed cell-suspensions. Rumen liquor contains factors which increase cellulose breakdown by bacteria under laboratory conditions. The nutrient medium, however, was complete and included both inorganic and organic materials.
114. Jayasinghe, J. B. 1963. The limitations of the technique of suspending foodstuffs in the rumen for determination of the particular microorganisms attacking them. *Ceylon Veter. J.* 11: 9–12.
115. Johns, A. T. 1951. Isolation of a bacterium, producing propionic acid, from the rumen of sheep. *J. Gen. Microbiol.* 5: 317–325.
116. Johns, A. T. 1951. The mechanism of propionic acid formation by *Veillonella gazogenes*. *J. Gen. Microbiol.* 5: 326–336.
117. Johns, A. T. 1951. The mechanism of propionic acid formation by propionibacteria. *J. Gen. Microbiol.* 5: 337–345.
118. Johnson, R. R. 1963. Symposium on microbial digestion in ruminants: *in vitro* rumen fermentation techniques. *J. Anim. Sci.* 22: 792–800. — A good historical review of the development of *in vitro* systems is presented. A listing of earlier review articles includes such as Baker and Harriss (1947), McNaught and Smith (1947), Phillipson (1947), and Marston (1948). More recent reviews include those of Moxon and Bentley (1955), and Bentley (1959). Historical treatment begins with studies and suggestions of microbial importance as early as 1874–1882 by Wildt, Zuntz, and Ducleaux (cited by Marston, 1948). Then the work of Tappeiner between 1882–1888 was cited. The work by Zuntz in 1891 is the last cited before some in 1922 by Henneberg. Later studies by Hungate on individual bacteria are also cited. This review is confined primarily to the study of rumen microbial activity in mixed culture rather than in pure cultures. Historical development of systems *per se* included that of Woodman and Evans (1938), Quin (1943), Pearson and Smith (1943), Wagner *et al.* (1940), and Marston (1948). The publication of McDougall's mineral composition of sheep saliva was important because it formed the

basis of most rumen fermentation mineral mixtures. The basic types of *in vitro* systems include those in which, after 36-hour fermentations, half of the previous fermentation serves as inoculum for the next fermentation; semi-permeable membranes used in a dialysis mixture; all-glass systems; and continuous flow systems. In all-glass systems Johnson *et al.* 1958 showed that cellulolytic activity *in vitro* was not greatly inhibited by concentrations of end products normally reached in the rumen fermentation media. This suggests that perhaps the semi-permeable membrane is of extra work for little value. The author reiterates Warner's criteria for assessing the validity of *in vitro* systems but later defends the use of simpler systems. Various techniques for preparing the inoculum are outlined. These include (1) whole rumen fluid strained through cheesecloth and incubated with carbohydrate or nitrogen substrates in the presence of certain minerals, (2) whole rumen fluid diluted to a greater extent with mineral solutions, (3) rumen fluid from which coarse debris has been separated by slow-speed centrifugation, (4) rumen fluid from which bacterial cells were separated by high speed centrifugation, (5) resuspended bacteria using a phosphate buffer to re-extract fibrous pulp, and (6) washed cell suspensions made from resuspended pulp. To study nutrient requirements of rumen microflora, it is necessary to use high-speed centrifugation to remove all of the cofactors that might occur in the rumen fluid. Furthermore, by re-extracting these, the various types of bacteria can be partially separated by high-speed centrifugation. These resuspended or washed cell suspensions are used to study nutrient requirements of microorganisms. These techniques were used to show that valeric acid and biotin are growth stimulants for cellulolytic cultures. The author agrees that it is best to have the animal on the same ration as is being studied *in vitro*, especially if there is a wide variation in diet and substrate. The problem is somewhat complicated because the rumen is continually being reinoculated with bacteria clinging to the forage. Care should be taken in maintaining the source animal on a ration similar to that being tested or at least on a standard control type. The author lists various measures of bacterial activity including (1) bacterial growth, (2) cellulose digestion and protein synthesis, (3) production of volatile fatty acids, (4) production of gas as measured manometrically, and (5) disappearance of starch. Use of production of a gas is cautioned because of the lack of specificity and also because a lag period is usually required before high rates of gas production in an *in vitro* fermentation. Starch digestion is a poor criterion because, among other things, in contrast to cellulose digestion, if starch digestion is incomplete in the rumen, it may proceed to completion in the lower digestive tract. Second, there are more and different organisms for digesting starch than there are for digesting cellulose, and these organisms are highly variable from day to day even on the same ration. Vitamin synthesis has been studied from rumen bacteria, but generally is not a criterion of fermentation rate or extent. The author indicates that "certainly, one of the most striking applications of the *in vitro* rumen fermentation technique has been the recent use in forage nutritive value determinations. No attempt will be made to review this work here but the reader will be referred to such references as . . .". He then refers to nine different papers. In answer to one of Warner's criticisms on the validity of artificial rumen techniques, the author agrees that they are in no way "artificial rumens" and should not be called such. They are merely an *in vitro* fermentation by rumen microorganisms. He indicates if the researcher's objective is not to attempt to simulate the *in vivo* situation exactly, then various *in vitro* procedures that make no attempt to simulate the intact rumen can be used to good advantage. Furthermore, considerably greater numbers of fermentations can usually be run using simpler techniques. The more recent all glass *in vitro* rumen fermentation system, such as used in the Ohio laboratory, cultures true rumen cellulolytic species and the data obtained from these mixed cultures are valid. He agrees with Warner's criticism that rate phenomena associated with cellulose digestion may not be similar to the rates in the rumen itself. This is true because many factors affect the lag phase in measuring rates such as the media, buffer system, particle size of the cellulose substrate, and inoculum preparation. He indicates it is only logical that rate phenomena studied *in vitro* should not be measured until after the lag phase. He furthermore indicates that rate phenomena of this type can not legitimately be studied unless

substrates are non-limiting. He concludes with suggesting a few areas in which the technique has been especially vital in studying nutritional problems or in which it will undoubtedly be used in the future. He includes as his last and major item the *in vitro* forage evaluation studies. He states "this not only involves use of the technique in practical forage evaluation services but its use as a means of studying biochemical differences in plant tissues. We have found it to be a very valuable tool in studying the nature of plant carbohydrates, their digestibility and their interrelationships. In this case, rumen bacteria serve as an 'analytical reagent'."

119. **Johnson, R. R., B. A. Dehority, and O. G. Bentley.** 1958. Studies on the *in vitro* rumen procedure: improved inoculum preparation and the effects of volatile fatty acids on cellulose digestion. *J. Anim. Sci.* 17: 841-850. — A comparison was made of several methods of treating the inoculum. The first involved expressing rumen liquor through cheesecloth, centrifuging in a Sharples centrifuge, resuspending the sediment in a phosphate buffer at pH 7, and using this suspension to inoculate the *in vitro* fermentation flask. A second improved inoculum involved discarding the liquor which was passed through the cheesecloth in the former case, resuspending the pressed pulp with phosphate buffer, resqueezing this material through a press, filtering the filtrate through cheesecloth, centrifuging in a supercentrifuge, and resuspending and using this material as the inoculum. A third study involved obtaining washed cell preparations by recentrifuging the final inoculum described above and resuspending it in fresh buffer. In these studies valeric acid was used as an additive or omitted. Further studies involved aerating the inoculum for varying periods up to 30 minutes at room temperature with and without preincubation for varying medium. The second extraction (from the pulp) gave approximately 60% more cellulose digestion than did the first extraction. Washing the cells once reduced the cellulolytic activity to about one-third the original, and a second washing destroyed the cellulolytic activity entirely. Aerating the inoculum prior to fermentation decreased its activity, but 60% of the activity still remained after 15 minutes of aeration. Incubation up to 2 hr caused a slight decrease, but after 2 hr considerably decreased activity. Use of the pulp-extracted inoculum gave less variation between experiments than did the other types of inoculum preparations. In the extraction of the pressed pulp, probably more cellulolytic bacteria were separated from the fibrous mass than in the first extraction. These studies also show that elaborate practices for maintaining anaerobic conditions prior to inoculation of the flasks are unnecessary for measurement of cellulose digestion.
120. **Johnson, R. R., O. G. Bentley, J. W. Hibbs, and H. R. Conrad.** 1956. *In vivo* and *in vitro* nutritional requirements of rumen microorganisms. *Agr. Food Chem.* 4: 627-631. — The importance of the rumen microflora population in the nutrition of the ruminant animal is stressed. Consequently, many studies on ruminant nutrition now emphasize the nutrition of the rumen bacteria themselves. The development of *in vitro* techniques for studying their nutrition has assisted these investigations greatly. Among the numerous studies affecting the concentration of the bacteria in rumen is that of the concentration of cobalt in the diet. A cobalt deficiency is usually characterized by a severe loss of appetite, drop in numbers of bacteria in rumen, and subsequent anemia and general emaciation, leading to the death of an animal. This is mitigated primarily through a production of vitamin B<sub>12</sub> by the rumen microorganisms. Cobalt promotes vitamin B<sub>12</sub> synthesis by the microorganism. Cobalt deficiencies have been cured by either intravenous or oral administration of vitamin B<sub>12</sub>, providing sufficient quantities are given. It is not yet fully determined whether the vitamin B<sub>12</sub> is more important for the host animal or for the rumen microflora. Techniques of growing rumen bacteria in laboratory flasks are valuable because one is able to control growing conditions and the nutrient medium. Bacterial activity then can be measured by such standards as cellulose digestion, nitrogen utilization, vitamin synthesis, and volatile fatty acid production.
121. **Johnson, R. R., B. A. Dehority, H. R. Conrad, and R. R. Davis.** 1962a. The relationship of *in vitro* cellulose digestibility of undried and dried mixed forages to their *in vivo* dry matter digestibility. *J. Dairy Sci.* 45: 250-252. — A comparison was made of *in vitro* digestibility of

dried and undried samples of mixed alfalfa-bromegrass hay, which was green chopped and cut at five periods during the year. Part of the samples were placed in a dry ice chamber and stored prior to analysis, and part were dried in a forced draft oven at 65°C. Significant difference existed between the digestibility of the undried and dried samples at 12 hr but at no other time periods (6, 24, 30, and 48). The inoculum used in these studies was a supernatant of a centrifuged whole bacteria suspension. At 12 hr, the undried samples were about 7% more digestible than the dry samples. The correlation between dry matter digestibility and 12 hr *in vitro* digestibility was not significantly different for undried and dried samples, although the former was slightly higher. No explanation was given for the difference in *in vitro* digestibility of the undried and dried forages.

122. Johnson, R. R., B. A. Dehority, K. E. McClure, and J. L. Parsons. 1964. A comparison of *in vitro* fermentation and chemical solubility methods in estimating forage nutritive value. *J. Anim. Sci.* 23: 1124-1128.
123. Johnson, R. R., B. A. Dehority, J. L. Parsons, and H. W. Scott. 1962b. Discrepancies between grasses and alfalfa when estimating nutritive value from *in vitro* cellulose digestibility by rumen microorganisms. *J. Anim. Sci.* 32: 892-896. — Studies were made using the *in vitro* technique of Dehority and phosphate buffer extract microorganisms taken from a ruminal fistulated steer maintained on a high-quality mixed hay with a high proportion of alfalfa. A variety of grass and alfalfa forages, 9-24% crude protein, were fermented. There was good correlation between *in vitro* and *in vivo* cellulose digestibility only when the grasses and alfalfa were evaluated in separate correlations. This was true whether the correlation was for a 12-, 24-, or 49-hr fermentation period. Other work from the Ohio laboratory indicated that lignification affects cellulose digestibility differently as grass matures compared to alfalfa.
124. Julen, G., and A. Lager. 1966. Use of the *in vitro* digestibility test in plant breeding. *Int. Grassl. Congr., Proc.* 10:652-657. — There were differences among plants in the digestibility of both the leaves and the straw. Plants that were late-heading tended to have lower digestibility than species that were early-heading. Plants that were highly digestible at a certain stage of development generally had a relatively high digestibility at other stages.
125. Kamstra, L. D., and N. T. Miller. 1960. Ruminal variation in pH, temperature and *in vitro* activity as affected by ration change and season. *South Dakota Acad. Sci., Proc.* 39: 84-88. — Variations in pH and temperature of the rumen and *in vitro* cellulose digestion activity were studied over a 28-week period. Some studies were made on the bacterial flora as related to seasonal and dietary changes. Changes in the ration had much greater effect on all factors studied than did seasonal changes. Changing the ration from a high roughage to a low roughage invariably produced a decrease in cellulose digestion, lower pH, and somewhat higher temperature. Weekly variations in many factors were high and tended to mask any effect due to seasonal changes. Total counts of bacteria generally were about  $1 \times 10^9$  per ml. Types of bacteria and protozoa, however, changed markedly with ration changes.
126. Kamstra, L. D., and M. M. Thurston. 1965. Determination of forage holocellulose digestibility by *in vitro* fermentations. *S. Dak. Acad. Sci., Proc.* 33:122-127. — The holocellulose fraction isolated from five forages and native prairie grass samples was digested *in vitro* in a 48-hr fermentation. The digestibility of the entire holocellulose fraction was greater than the cellulose portion in most instances. This indicates that the hemicelluloses are more digestible than cellulose. The hemicelluloses appeared to be digested almost completely within the 48-hr fermentation period. The digestibility of the holocellulose fraction decreased greatly as plants matured. This decrease, in general, was greater than the decrease in digestibility of cellulose with maturity.

127. Kamstra, L. D., A. L. Moxon, and O. G. Bentley. 1958. The effect of stage of maturity and lignification on the digestion of cellulose in forage plants by rumen microorganisms *in vitro*. *J. Anim. Sci.* 17: 199–208. — The suspended microorganisms were used in an *in vitro* system in which the basal medium was a complex mineral mixture with added biotin, para-aminobenzoic acid, and valeric acid. Cellulose digestion of the whole plant and various cellulose fractions such as isolated cellulose and holocellulose were studied *in vitro*. Orchard grass, alfalfa, and timothy at three stages of maturity were investigated. Separation of the cellulose from lignin greatly improved its digestibility *in vitro*. Cellulose in holocellulose was similar in digestibility to isolated cellulose. This indicates that lignin but not hemicellulose has an inhibitory effect on cellulose digestion. Cellulose digestion decreased as the plants matured. A good figure is given showing the percent cellulose digested with increasing fermentation times from about 4 to 30 hr for four different stages of maturity. They found the end point in cellulose digestion was near 24 hr.
128. Kistner, A. 1965. Possible factors influencing the balance of different species of cellulolytic bacteria in the rumen. p. 419–432 *in*: Dougherty, R. W. *et al.* Physiology of digestion in the ruminant. Butterworths, London. — Historical development of microbiological aspects of the rumen is considered. An excellent diagram summarizes factors affecting the physical and chemical nature of the diet eaten and the environmental conditions in the rumen. The author emphasizes that different bacteria exist differently in the rumen: some occur free in the rumen liquor, whereas others are fixed to solid food particles. Thus, the method of sampling could give quite biased results. All the common cellulolytic species of bacteria are normally present in the rumen at all times, but their levels at any given time depend on the extent of their adaptation to the conditions then prevailing. After a change-over from one diet to another, species which are normally of minor importance in animals on either diet may temporarily gain ascendancy. This means that care must be taken to ensure that the animals used are fully conditioned when a study is made of the ruminal flora of sheep or cattle on a given diet. The redox potential is known to be an important factor in limiting the growth of rumen bacteria, but because of the difficulty in poisoning media at different  $E_h$  values with a single redox system, not even the upper limit which will permit growth is well defined. It appears to lie in the region of  $-150$  to  $-200$  mv for the more common cellulose digesters of the rumen. The minimum steady-state values of  $pCO_2$  which will support optimum growth of common rumen bacteria are unknown. Most of the bacteria have simple nitrogen requirements, and ammonia can serve as a major source of nitrogen even in the presence of casein hydrolysate. Some strains, however, have complex nitrogen requirements, and most strains require one or more of the volatile fatty acids, n-valeric, isovaleric, isobutyric, and 2-methyl butyric for growth. There is also a wide variation in the requirement for B vitamins among strains of different bacteria.
129. Kistner, A., and L. Gouws. 1962. Effect of change of diet on the predominant type of cellulose-digesting bacteria in the rumen of sheep. XIII. *Inter. Cong. Microbiol.* B9. 2:41. (Abstract) — On a diet of 4.6% crude alfalfa protein, *Ruminococcus albus* composed 84% of the cellulolytic bacteria, but only 17% on a 4.6% crude protein grass diet. Within 24 to 48 hr, there was a drop in number of *Ruminococcus albus* when changing from alfalfa to the low protein diet.
130. Kistner, A., L. Gouws, and F. M. C. Gilchrist. 1962. Bacteria of the ovine rumen. II. The functional groups fermenting carbohydrates and lactate on a diet of lucerne (*Medicago sativa*) hay. *J. Agr. Sci.* 59: 85–91. — See Gilchrist and Kistner, 1962.
131. Kitts, W. D., and L. A. Underkofler. 1954. Hydrolytic products of cellulose and the cellulolytic enzymes. *Agr. Food Chem.* 2: 639–645. — In the microbiological degradation of cellulose, specific enzymes are elaborated which are able to split the large polysaccharide molecule into smaller water-soluble compounds. Many workers have observed that the cellulolytic microorganisms from the rumen act upon insoluble cellulose fibers only by direct contact with the fibers.

In this experiment, the cellulolytic enzymes were not cell-free but were tightly absorbed on the outer surface of the cell membrane. Poor results were obtained by using supersonic vibration to free the enzymes from the rumen microorganisms. Cell-free extracts were obtained, however, by a complex process of grinding, centrifugation, and resuspension.

132. **Knipfel, J. E., and J. E. Troelsen.** 1966. Interaction between inoculum donor diet and substrate in *in vitro* ruminant digestion studies. *Can. J. Anim. Sci.* 46:91-95. — A two-stage *in vitro* digestion technique was used for 12 and 48 hr with inoculum from six ruminally fistulated wethers fed alfalfa hay, wheat straw hay, and barley grain separately and in different combinations. There were significant interactions among inoculum donor diets, *in vitro* substrate mixtures, and fermentation periods. Inocula containing all feed ingredients gave similar digestibilities of each substrate, showing that the microflora more quickly adapted to the substrate when the donor diet contained all the substrate ingredients. The relative rank of quality of the six substrates estimated by *in vitro* digestibility depended upon the inoculum donor diet as the length of fermentation period. Forty-eight-hour fermentation periods with each of the inocula resulted in greater spreads in quality ranking of the substrates than did 12-hour fermentation periods. Increasing the proportion of alfalfa in the substrate increased the difference between *in vitro* digestibility of dry matter and organic matter, probably due to the higher concentration of soluble ash in the alfalfa.
133. **Lambert, M. R., and N. L. Jacobson.** 1956. The effect of chlortetracycline feeding on *in vitro* cellulose digestion by rumen microorganisms. *J. Anim. Sci.* 15: 509-514. — Chlortetracycline in the diet of cows at 240 mg/day decreased digestibility of a purified cellulose from 45% to 34% and decreased digestibility of cellulose in alfalfa from 59% to 45% as compared to animals on the same diet not receiving the chlorotetracycline.
134. **Lampila, M.** 1959. On the effect of pH *in vitro* upon the microbial processes in the rumen contents of the cow. *Suomen Maataloustieteellisen Seuran Julkaisuja* 94: 1-10. — A study was made of the effect of pH on the fermentation rate of whole rumen ingesta contents. The rumen contents were put in sealed flasks, incubated at 39°C, and shaken at 1-hr intervals. pH was measured at 2-hr intervals. Range in pH was from 5.0 to 6.7, and the rate of production of volatile fatty acids was highest at about pH 6.2.
135. **Leatherwood, J. M.** 1965. Cellulase from *Ruminococcus albus* and mixed rumen microorganisms. *Appl. Microbiol.* 13: 771-775. — Cellulase was extracted from mixed rumen organisms of steers on a grass-hay diet by centrifuging the rumen inoculum through a continuous flow system at 30,000 × g, discarding the supernatant, resuspending the remaining cells and liquid in a phosphate buffer, adding n-butanol, stirring the suspension for 18 hr, centrifuging again at 25,000 × g, and collecting the clear supernatant fluid. This extract was dialyzed about 18 hr and stored until used as a source of cellulase. Sources of cellulase were investigated with acid-swollen cellulose and carboxymethylcellulose as substrates. Cellulase was extracted from pure cultures of *Ruminococcus albus*. Maximum activity occurred at approximately pH 5.8 at 47°C. Approximately 20% of the cellulase of the mixed rumen microorganisms was immunologically similar to the cellulase from *Ruminococcus albus*. The mechanism of degradation indicated the two were similar. However, glucose is the main product of cellulose degradation by *Ruminococcus albus*. This article indicates "there are no reports in the literature concerning cellulolytic activity in cell-free preparations from pure cultures of rumen microorganisms."
136. **LeFevre, C. F., and L. D. Kamstra.** 1960. A comparison of cellulose digestion *in vitro* and *in vivo*. *J. Anim. Sci.* 19: 867-872. — A comparison was made between the digestive power of the inoculum from cattle and from sheep. These animals received rolled corn, soybean oil meal, and medium quality alfalfa hay twice daily. Inoculum was secured before the animals received

morning feed and water. *In vitro* cellulose digestion results (48 hr) were similar to those obtained *in vivo*. Correlations for all samples combined was only about 0.30; however, if six prairie hay rations were removed the correlation increased to about 0.84. This suggests that there is a basic difference in the types of bacteria in the animal on the high-quality diets as compared to those required to digest cellulose on the low-quality diets. There was very little difference in their ability to digest cellulose between the inoculum from sheep and cattle maintained on similar high-quality diets.

137. **Lewis, E. (Ed.).** 1961. Digestive physiology and nutrition of the ruminant. Butterworths Limited. London. 297 pp. — This is the proceedings of the University of Nottingham's 7th Easter School in Agricultural Science. Of special interest is part II of this book concerned with metabolism in the rumen. Chapters of interest are Descriptive Microbiology of the Rumen, Techniques of Counting Rumen Organisms, Carbohydrate Metabolism in the Rumen, The Fate of Nitrogenous Compounds in the Rumen, Influence of the Rumen on Digestion and Metabolism of Lipids, Mineral Relationships of the Ruminant, and Techniques for Studying Rumen Metabolism with Special Reference to the Use of Radioisotopes.
138. **Lloyd, L. E., H. F. M. Jeffers, E. Donefer, and E. W. Crampton.** 1961. Effect of four maturity stages of timothy hay on its chemical composition, nutrient digestibility and nutritive value index. *J. Anim. Sci.* 20: 468–473. — *In vitro* cellulose digestion decreased with advancing maturity stage of timothy hay. All four stages evaluated had a sigmoid relationship between fermentation time and *in vitro* cellulose digestion percent. Lag periods in the start of *in vitro* cellulose digestion appeared to be related to the nutritive value of the forage species studied. The *in vitro* system that was used was that of Donefer *et al.* (1960). Forages were evaluated at 3, 6, 12, 24, and 48 hr.
139. **Louw, J. G., H. H. Williams, and L. A. Maynard.** A new method for the study *in vitro* of rumen digestion. *Science* 110: 478–480. — This research was an early comparison of a semi-permeable bag vs. an all-glass system for *in vitro* cellulose digestion. In this study, acetic and propionic acids were formed in the same portions in both types of systems, but less total volatile fatty acids per grams of cellulose digested were formed in the semi-permeable bag. Acetic acid diffuses at a greater rate from the bag than propionic. There was no attack by microorganisms on the bag. Such attack usually begins at broken or torn ends, and no such surfaces were exposed in these experiments. Stirring of the contents of the inner bag of the flask increased the extent of cellulose digestion; however, earlier studies had shown that rapid frequent stirring caused decreased fermentation.
140. **MacLeod, R. A., and C. A. Brumwell.** 1954. *In vitro* cellulose digestion by rumen microorganisms and its stimulation by fishery by-products. *Appl. Microbiol.* 2: 130–135. — Cellulose digestion was measured by the loss in weight of a roll of vegetable parchment after fermentation. This fermentation was conducted in tubes with varying amounts of rumen liquor (strained) collected from cattle from a slaughter house. The cattle had previously been fed alfalfa or timothy hay. Varying amounts of nitrogen supplements were included (fishery products). The fermentation was aided by a salt mixture patterned after that used previously by Burroughs. Each tube was stoppered with a one-hole stopper from which an outlet tube ran. A small bulb attached to the end of the tube and open at the other end was placed in a test tube of water. This arrangement permitted escape of fermentation gases while maintaining anaerobic conditions. Fermentations were conducted from 70 to 120 hr, and were characterized by a lag period of approximately 36 hr during which microbial populations increased rapidly. Nitrogen added almost doubled the cellulose digestion. The extent to which nitrogen affected the digestion was affected by the ration of rumen liquor to water in the inoculum. Maximum fermentations were obtained with a 1.5:1 or 1.0:1 ration of rumen liquor to warm air-free water.

141. **Marquardt, R. R., and J. M. Asplund.** 1964a. The effects of water extracts of forages on *in vitro* cellulose digestion by rumen microorganisms. *Can. J. Anim. Sci.* 44: 16–23. — Water extracts were made of 14 forages ranging from leafy alfalfa to wheat straw. Most of the forages were legume and non-legume hays and silages. The extraction procedure took 25% of the dry matter, 40% of the protein, and 60% of the ash in most of the forages, but a much lower concentration in wheat straw. The water-extracted material was lyophilized, and 200 mg were added to each *in vitro* flask with 200 mg of sodium carbonate and 200 mg of purified cellulose. These fermentations were compared against a control medium outlined by Donefer. The control mediums supported the most rapid cellulose digestion. Of the forage extracts, on an equal dry-matter basis, the wheat straw extract supported the fastest cellulose digestion and the grass hay extract the slowest. The other four extracts supported intermediate rates. The grass hay extract had similar composition to that of wheat straw except that it had a much higher content of carbohydrates. Earlier work has shown that large amounts of readily available carbohydrates depress cellulose digestion. In all trials the increase in cellulose digestion when extracts were supplemented with phosphate-sulphate and urea was greater than the sum of the increases when these supplements were added singly. Because large differences were necessary to demonstrate significance between forages, it is felt that this technique of using aqueous extracts is not sufficiently precise to be used as a criterion of forage quality.
142. **Marquardt, R. R., and J. M. Asplund.** 1964b. The effects of variations in volume of inocula on the *in vitro* cellulose digestion by rumen microorganisms supported by nutritionally inadequate media. *Can. J. Anim. Sci.* 44: 24–28. — Washed and rewashed inocula were compared at various volumes per milligram of cellulose with and without added urea. In a second experiment the basal medium (Donefer) was diluted. In a third experiment autoclaved rewashed inoculum was compared with nonautoclaved inoculum. Cellulose digestion increased linearly ( $r^2_{xy} = .99$ ) as the volume of inoculum. However, volume of inoculum did not have an effect on fermentations in the basal medium. There was a curvilinear relationship between percent cellulose digestion and millimeters inoculum at low levels of the basal medium. Successive increments of both fresh and autoclaved inocula supported increased cellulose digestion. However, when the fresh inoculum was used in artificial rumen studies where different fermentation media are used, and especially where a constant fermentation time is used, great care must be taken to standardize the inocula or to control the variation by statistical means. The effect of the amount of inoculum on the maximum cellulose digestion supported by inadequate media was not determined. Time durations were too short to evaluate this.
143. **Marston, H. R.** 1948. The fermentation of cellulose *in vitro* by organisms from the rumen of sheep. *Biochem. J.* 42: 564–574. — This is one of the early detailed studies on *in vitro* fermentation. There is a good review of the very early literature on rumen fermentation going back to Wildt, 1874, who suggested that microorganisms are responsible for the decomposition of cellulose in the alimentary canal. Marston's apparatus consisted of a 3.5 l glass vessel with a stirrer, access ports for introduction of alkali, electrodes for pH and oxidation-reduction potential measurements, a thermo-regulator, an in-going stream of  $N_2$ , and an out-going stream of fermentation gases. Cellulose which was digested was "practically pure alpha cellulose from birchwood" in two experiments and cellulose prepared from filter paper which had been suspended in sodium hydroxide and then washed with dilute hydrochloric acid in the other two. He provided a rather complex mixture of inorganic constituents including ammonium sulfate, potassium acid phosphate, magnesium sulfate, and calcium chloride along with traces of iron, copper, zinc, and cobalt. Inoculum was obtained from slaughtered sheep which had been fed on cereal hays. The rumen ingesta was strained, the liquid portion was centrifuged (to remove contaminants), the supernatant was separated in a centrifuge and the residual sludge was taken up in a phosphate buffer and made up to volume. This sludge material was used to inoculate the flasks. Upon inoculation,  $N_2$  was passed through the system and then sodium carbonate buffer

- was used to bring the pH to 6.8. Reaction was adjusted throughout the run. Rapid stirring of the flask contents reduced the rate of fermentation. Marston found ruminal fermentation yielded acetic acid, propionic acid, carbon dioxide, and methane as main products and smaller quantities of formic and buteric acid and traces of acetaldehyde, pyruvic acid, and lactic acid.
144. **McAnally, R. A., and A. T. Phillipson.** 1944. Digestion in the ruminant. Cambridge Phil. Soc. Biol. Rev. 19: 41–54.
145. **McBee, R. H.** 1953. Manometric method for the evaluation of microbial activity of rumen with application to utilization of cellulose and hemicelluloses. *Appl. Microbiol.* 1: 106–110.  
 — Any major dietary change will be reflected upon the microorganisms long before a change in the nutritional state of the animal could be detected by other means. This author used a Warburg respirometer to study fermentation rates of rumen microorganisms. He stressed the importance of using a fistula for sampling the rumen microorganisms because replicate samples taken from steers by means of a stomach tube gave differences in results of over 100%. There were large differences between whole rumen fluid, the solid fraction, and the liquid fraction of the same specimen. Digestive activity of fluid taken from a sheep on alfalfa hay was compared to fluid from the same animal after the hay diet had been supplemented with cellulose and hemicellulose. There was an increase in the rate of fermentation of hemicellulose when the diet was supplemented with cellulose over that found while the animal was on an exclusive hay diet, and the lag in measurable fermentation of cellulose was reduced from a period of several hours to about 15 minutes. Addition of hemicellulose to the diet gave a higher rate of fermentation than did the basal hay diet but did not decrease the lag in fermentation of cellulose. All of the hemicellulose fermenters could not ferment cellulose. The rate of fermentation of various substrates is not constant, but is subject to wide fluctuations following changes in the diet of the ruminant.
146. **McDougall, E. I.** 1948. The composition and output of sheep's saliva. *Biochem. J.* 43: 99–109.  
 — An early relatively complete description of salivary composition. His suggested "artificial saliva" became the standard for many *in vitro* buffer systems.
147. **McNaught, M. L., E. C. Owen, and J. A. B. Smith.** 1950. The utilization of non-protein nitrogen in the bovine rumen: 6. The effect of metals on the activity of the rumen bacteria. *Biochem. J.* 46: 36–43. — These authors note "there are grave doubts as to whether results obtained with pure cultures would help much in elucidating the processes occurring in the normal rumen where a mixed population of bacteria and protozoa is always found . . . and where symbiotic relationships of one species of micro-organisms to another almost certainly obtain . . ." The *in vitro* procedure in this study was to obtain rumen ingesta from a permanently ruminal-fistulated cow, strain the material through muslin, centrifuge to remove the debris, strain the centrifuged rumen liquid, and divide the liquid into various portions to which urea was added to provide non-protein nitrogen. Various metals or chelating agents were added. Samples were obtained from the flasks before and after a 4-hr fermentation, and their non-protein nitrogen content was determined. The decrease in non-protein nitrogen occurring during incubation is correlated with an increase in bacterial protein and was the measure of activity (mg nitrogen/100 g rumen liquid).
148. **Meißeke, J. C., R. L. Salsbury, J. A. Hoefler, and R. W. Luecke.** 1958. The effect of starvation and subsequent refeeding on some activities of rumen microorganisms *in vitro*. *J. Anim. Sci.* 17: 774–781. — The inoculum used was strained rumen fluid obtained from a fistulated steer. The *in vitro* system was a semi-permeable membrane system with incubation periods of 3, 6, 9, and 24 hr. The measure of digestive power of the inoculum was the decrease in viscosity of a stable suspension evaluated by the time required for a definite volume of the solution to flow through a calibrated capillary tube. The substrate was a 0.3% solution of carboxymethylcellulose. Comparison was made of the strained rumen fluid, a fraction centrifuged at 1500 rpm

for 5 minutes (to remove protozoa and plant material), and a fraction prepared by centrifuging the material further at 12,000 rpm for 110 minutes in a refrigerated centrifuge. This procedure removed the bacteria and resulted in a cell-free preparation of rumen fluid. Various combinations of timothy and alfalfa hay were utilized before and after the steer had fasted for periods of 48–72 hr. The diet was supplemented with a complex ration. The pH of the rumen fluid rose continuously during fasting to a high between 7.6 and 7.9. The ability of rumen fluid to digest cellulose *in vitro* decreased greatly when the steer fasted for three days. All fractions of rumen fluid also decreased in their activity upon carboxymethylcellulose. The ability to digest cellulose *in vitro* was normal 3–4 days after resumption of feeding following fasting. There were no major differences in activities of rumen microorganisms due to the type of hay and diet received by the steer before and after fasting. On a normal diet there was relatively little cellulose digesting activity of the cell-free preparation. However, during early refeeding following fasting the activities of the three fractions were approximately equal indicating that the enzymes were free of the cell. Shortly following refeeding all portions of rumen fluid showed a higher than normal, but approximately equal activity on carboxymethylcellulose suspensions for at least a short time. The ability to reduce the viscosity of carboxymethylcellulose and the ability to hydrolyze cellulose are not necessarily related and may not be accomplished by the same enzyme.

149. Miles, D. G., G. Griffith, and R. J. K. Walters. 1964. The effect of "winter burn" on the chemical composition and *in vitro* dry-matter digestibility of eight grasses. *J. Brit. Grassland Soc.* 19: 75–76.
150. Mohammed, A. S. 1966. A comparison of different methods which estimate nutritive value of forages. Ph.D. Thesis. Univ. Tennessee. 91 p. — The washed-cell suspension method and whole rumen fluids were used. Inocula were obtained from a fistulated steer fed alfalfa hay. Fermentation periods were 8, 12, and 24 hr to study 11 forages. Dry matter and cellulose digestion were evaluated. A sodium bicarbonate buffer but no nutrient medium was used with whole rumen fluid. *In vivo* digestibility was not well-correlated with *in vitro* cellulose digestion. Samples were 0.4 g of forages ground through a 40-mesh screen. Cottonseed meal and/or concentrates were added to the mixture for *in vivo* studies. Each sample was run in duplicate in three trials.
151. Moore, J. E., R. R. Johnson, and B. A. Dehority. 1962. Adaptation of an *in vitro* system to the study of starch fermentation by rumen bacteria. *J. Nutr.* 76: 414–422. — Microscopic examination of the bacteria of different sheep suggested there would be differences in their microbial activity. Experiments were conducted in which the grams of starch fermented were regressed with the milliequivalents of total volatile fatty acids produced. Graphs of these data suggest the relationship between the two variables are similar for the three sheep. This suggested that, although slight differences in microscopic appearance of the mixed cultures were observed, these 18 mixed cultures may have had the same gross metabolic activities. In another study there were no consistent differences between the inocula from four sheep in their ability to ferment starch. However, in all instances the sheep were fed a constant diet of ground shelled corn, chopped mixed hay and soy bean oil meal. Additionally a small amount of long hay was fed to prevent loss of appetite, and trace mineralized salt and water were offered *ad libitum*. A good description is given of the system for inoculum preparation by stepwise centrifugation.
152. Morris, J. G., L. E. Harris, J. E. Butcher, and C. W. Cook. 1965. Indices of efficiency of rumen fermentation of sheep grazing desert range forage as influenced by supplements of nitrogen and phosphorus. *J. Anim. Sci.* 24: 1152–1158.
153. Munch-Petersen, E., and A. A. James. 1964. A method of assessing "free-living" and "attached" bacteria in rumen content. *J. Zentralblatte fur Bakteriologie Parasitenkunde, Infektionskrankheiten und Hygiene.* 194:358–364. — A spray unit was designed to separate

rumen fluid into a filtrate and other material. Isotonic culture medium in rolled tubes was used to determine colony counts for these two sources. The number of fertile tubes sown with a filtrate was higher than those of the untreated controls.

154. **Naga, M. M. A., and K. L. El-Shazly.** 1963. The use of the *in vitro* fermentation technique to estimate the digestible energy content of some Egyptian forages: I. The *in vitro* digestion of cellulose as a criterion of energy content. *J. Agr. Sci.* 61:73-79. — A continuously gassed, 24-hr *in vitro* system with whole rumen fluid was used to measure percentage cellulose digestion. Percent cellulose digestion was correlated with *in vivo* cellulose digestion and digestible energy. Digestible energy of the legumes was less well-correlated with *in vitro* cellulose digestion than was digestible energy of non-legumes.
155. **Nagy, J. G., H. W. Steinhoff, G. M. Ward.** 1964. Effects of essential oils of sagebrush on deer rumen microbial function. *J. Wild. Manage.* 28: 785-789. — Extracts of essential oils from big sagebrush (*Artemisia tridentata*) were evaluated as they influenced fermentation ability of microbes from deer rumens and the effect on digestive action in a steer. To obtain rumen fluid from deer, the deer were killed by rifle shot. Minutes after death the rumen wall was opened and contents were placed into sterile containers. The elapsed time between killing the deer and arrival of the liquid in the laboratory was about two hours. Preliminary studies showed that keeping rumen fluid at lower temperatures for short periods of time did not affect bacterial fermentation. Rumen microorganisms occurred at about  $4 \times 10^9$ /g of rumen contents in the deer. Increasing amounts of essential oils from big sagebrush showed definitely decreased amounts of cellulose digestion by these deer rumen microorganisms. Similar results were obtained by manometric devices for evaluating gas production. A steer fed sagebrush, per fistula, at about 3 kg/day soon had a failure of appetite and rumen movement was stopped. Replacing the solid rumen contents with only the liquid portion failed to stimulate appetite. Replacement of the rumen contents with that from alfalfa-fed cows reinitiated appetite. Deer rumens contain about 10-20% solid material. Thus, if the diet contained 15-30% of big sagebrush, there would be only a slight decrease in cellulose digestion. Greater levels of sagebrush in the diet could appreciably decrease cellulose digestion rates in the deer. However, adaptation of the rumen bacteria while the animals are continuously digesting sagebrush may be an important factor and should be evaluated.
156. **Nicols, R. E.** 1955. A sampling tube for rumen fluid. *Am. J. Vet. Res.* 16: 410.
157. **Ochi, Y., M. Ogata, T. Mitsuoka, C. Kaneuchi, and H. Kawaguchi.** 1960a. Studies on microflora in the alimentary tract of livestock: I. Method for the culture of the bacteria in rumen. *Japanese J. Vet. Sci.* 22: 167-174.
158. **Ochi, Y., M. Ogata, T. Mitsuoka, C. Kaneuchi, and H. Kawaguchi.** 1960b. Studies on microflora in the alimentary tract of livestock: II. Bacterial flora in the rumen of cows fed different rations. *Japanese J. Vet. Sci.* 22: 233-239. — No marked variations of flora occurred except in cows fed roughage (*Lactococcus* predominated) or concentrate (*Bifidobacterium* predominated). Counts were about  $10^7$ - $10^8$ /ml, and counts were higher on concentrate diets.
159. **Oh, H. K., B. R. Baumgardt, and J. M. Scholl.** 1966. Evaluation of forages in the laboratory. V. Comparison of chemical analyses, solubility tests, and *in vitro* fermentation. *J. Dairy Sci.* 49:850-855. — Prediction of digestibility of crude protein, acid-detergent fiber, acid-detergent lignin, cell-wall content, cellulose solubility, dry matter solubility, cellulose digestion *in vitro*, and dry matter digestion *in vitro* were related to *in vivo* dry matter digestibility. None of the chemical components or solubility methods studied should be used to compare forages of different species. Best results could be expected from a two-stage *in vitro* digestion.

160. Oellermann, R. A. 1964. Nutritive value of *Themedia triandra*. I. The influence of variables on the determination of the different protein fractions in forages and on the *in vitro* fermentation procedure employed. S. Afr. J. Agr. Sci. 7: 633-648. -- Samples of rumen fluid were obtained from 14 grazing wethers by means of a stomach tube. Approximately 250 ml of rumen fluid was taken from each sheep. Samples were pooled and transported to the laboratory, where the material was pressed and the ingesta was resuspended in a phosphate buffer. The resuspended phosphate buffer extract was employed as the inoculum for the *in vitro* fermentations. The *in vitro* system of Quicke *et al.* was utilized. Cellulose digestibility was evaluated for 12 to 72 hr of fermentation. Maximum cellulose digestibility was reached near 48 hr, but maximum time required for protein synthesis on a grass substrate was near 24 hr. The rate of cellulose digestion *in vitro* was enhanced by addition of urea to the fermentation tubes. The maximum level of cellulose digestibility was about 3% higher with urea than without urea. The conclusion was that urea supplementation did not improve cellulose digestibility with leaf sample as a substrate but it did with the stem sample and with a purified cellulose sample (filter paper). Optimal cellulose digestion *in vitro* could not be obtained without nitrogen supplementation for the stem sample and purified cellulose.
161. Pant, H. C., J. S. Ruwat, and A. Roy. 1962. Studies on rumen physiology: I. Growth of fistulated animals and standardization of methods. Indian J. Dairy Sci. 15: 167-185. -- A variety of studies were made with buffalo, cattle, sheep, and goats to evaluate rumen fermentation techniques. The comparison was made between rumen liquor obtained through a fistula by suction from live animals and from slaughtered goats on the same diet. There was no significant difference in the total volatile fatty acids, ammonia concentration, or the percentage cellulose digestion in liquor samples obtained by the two techniques. Percent cellulose digestion in strained rumen liquor from goats fasted for 24 hr was about 25% as compared to about 57% for fed goats. Fasting apparently affects the inoculum processed by different methods in different ways. A comparison was made of the ability to digest pure cellulose powder by goats and sheep. These studies, spread over eight months, showed goat rumen liquor could digest more cellulose than sheep. These animals were allowed to graze throughout the year and there was considerable change in pasture quality. Some dietary habit differences did occur; goats grazed leaves of Jherberi, but sheep did not.
162. Pearson, R. M., and J. A. B. Smith. 1943. The utilization of urea in the bovine rumen. 2. The conversion of urea to ammonia. Biochem. J. 37: 148-153. -- The effects of passing air, carbon dioxide, or nitrogen through fermentations of strained rumen liquor did not differ greatly among the three gases. This was measured as urea nitrogen per 100 g of rumen fluid disappearance. Fermentation mixtures containing a phosphate buffer, starch, and rumen fluid over a range 4-89°C showed a maximum rate of urea nitrogen conversion to ammonia at 49°C. pH was investigated over a range of 3-11. The optimum was between 7 and 8. Rumen liquor samples, obtained 1 hr post-feeding of the host animal, converted urea to ammonia at a slightly, but not significantly, faster rate than a sample collected at 16 hr post-feeding. The data suggest that 40-80 g of urea could be converted in the rumen (75 kg) in one hour.
163. Pearson, R. M., and J. A. B. Smith. 1943. The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of protein in rumen ingesta. Biochem. J. 37: 153-164. -- In this early *in vitro* fermentation study 900 ml of rumen liquor was incubated for 8 days with 2.5 g of urea, 12 g of potassium acid phosphate buffer, 10 g of glucose, and 0.1 g of ferric sulfate. Microscopic examinations of the microflora and fauna indicated that even after one day's incubation, great changes had taken place so that the "microbial picture" bore little resemblance to that of the initial sample. Based on microscopic evidence, there was no significant change in the "microbial picture" during initial 2-4 hr incubation period.

164. Pettyjohn, J. D., J. M. Leatherwood, and R. D. Mochrie. 1964. Simplified technique for *in vitro* comparison and dry matter digestibilities of forages. *J. Dairy Sci.* 47: 1102-1104.
165. Preston, R. L., and W. H. Pfander. 1961. Cellulose, starch and nitrogen levels for maximum cellulose digestion by rumen organisms *in vitro*. *Fed. Proc.* 20:372 (Abstr.). — Both small amounts of urea and cornstarch were required for maximum cellulose digestion in Solka-floc. Their maximum rates of digestion were found in the 16-20 hr period.
166. Pritchard, G. I., L. P. Folkins, and W. J. Pigden. 1963. The *in vitro* digestibility of whole grasses and their parts at progressive stages of maturity. *Can. J. Plant Sci.* 43: 79-87. — *In vitro* techniques were used to investigate digestibility of heads, leaves, and upper and lower stems of several domestic forages. The most rapid decline in *in vitro* digestibility began with head emergence. The rate of decline for the heads and stems was greater than for the leaves. The upper segments of the stems tended to have a lower *in vitro* digestibility than the basal segments.
167. Pritchard, G. I., W. J. Pigden, and D. J. Minson. 1962. Effect of gamma radiation on the utilization of wheat straw by rumen microorganisms. *Can. J. Anim. Sci.* 42: 215-217. — The effects of gamma radiation (from  $^{60}\text{Co}$ ) upon the feeding value of wheat straw were determined by *in vitro* fermentations with rumen microorganisms. Exposure of straw to dosages of  $1 \times 10^7$  rads caused a slight increase in dry matter digestion, whereas higher exposures caused marked increases. The solubility of straw was also increased with dosages of  $10^8$  rads or more. Above  $2.5 \times 10^8$  rads there was no increase in volatile acid production from the fermentations. This suggests that above this level of radiation the carbohydrates are disintegrated to such a degree that they are no longer suitable substrates for rumen microorganisms. These studies confirmed that there are nutrients available in wheat straw, but that they are entrapped or encrusted by non-digestible substances. This occurred even when the wheat straw was ground through a hammer mill equipped with a 0.02-cm screen.
168. Purser, D. B., and R. J. Moir. 1959. Ruminal flora studies in the sheep. IX. The effect of pH on the ciliate population of the rumen *in vivo*. *Australian J. Agr. Res.* 10: 555-564. — No consistently significant differences were found in the concentration of ciliate protozoa at four different sites in the rumen, although there were significant differences among sheep. Sampling at various times after feeding showed there was a marked diurnal fluctuation in the ciliate population, the concentration after feeding falling to as low as one-third of the pre-feeding levels. The ability of the protozoa to divide was strongly inhibited by low pH, which was experienced 2-4 hr postfeedings.
169. Purser, D. B., and R. J. Moir. 1966. Rumen volume as a factor involved in individual sheep differences. *J. Anim. Sci.* 25: 509-515. — Rumen volume may have a double influence on microbial substrate relationships within the rumen when animals with different rumen volumes are fed at the same level of feed intake. First, competition for food would be great and extended over a relatively long period of time in small rumens. In large rumens there would be a relatively large ratio of substrate to microorganisms for at least a short period of time. These two systems present vastly different ecological environments, and it is likely that this has some effect on population composition and possibly also on metabolic relationships. The second effect of a variable rumen volume on microbial relationships follows from the fact that different percent dry matter of rumen content may occur. This also may affect the substrate-microorganisms relationship. These authors concluded that for comparative studies different animals should be fed at the same proportions of their *ad libitum* intakes. Animals with small rumen volumes exhibit extended feeding habits and possibly these animals may be more efficient when feed intake is restricted because they provide a more continuous and relatively active type of metabolism. This may be especially important for differences among animals in ability to

digest feed when under grazing conditions. The authors further suggest it may be advantageous to use groups of animals with similar rumen volumes for microbiological studies and possibly also for rate of passage studies. For 11 of their sheep of 62–76 kg body weight, rumen volumes were 2.5–7.6 l (rumen physiological volumes). There was a positive correlation between *ad libitum* intakes of material low in nitrogen and physiological rumen volume.

170. Purser, D. B., and R. J. Moir. 1966. Variations in rumen volume and associated effects as factors influencing metabolism and protozoa concentrations in the rumen of sheep. *J. Anim. Sci.* 25: 516–520. — This study analyzes the effects of rumen environment variations among animals on forage evaluation. Animals exhibiting below-average rumen pH had a greater concentration of microorganisms. Individual sheep differences in nitrogen utilization were the result of variations in quantitative conversion of the food protein to microbial protein and ammonia and of variations in the quality of the microbial protein. These studies indicate that the utilization differences among animals were related to microbial activity. Generally animals with small rumen volume utilize added nitrogen more efficiently than animals with large rumen volume. The 11 sheep used in this study had variations in concentration of bacteria prior to feeding of  $7.7\text{--}12.7 \times 10^9/\text{ml}$ .
171. Quicke, G. V., O. G. Bentley, H. W. Scott, and A. L. Moxon. 1959. Cellulose digestion *in vitro* as a measure of the digestibility of forage cellulose in ruminants. *J. Anim. Sci.* 18: 275–287. — In this study ruminal microbes were obtained from a steer maintained on good quality hay. The three types of inoculum preparation compared were strained rumen juice, a phosphate buffer extract of the rumen pulp, and centrifuged and resuspended ruminal microorganisms. Studies were run with 0.2 g cellulose from different sources which had been ground through a 40-mesh screen. Total sample volume was 50 ml, temperature was 39°C, CO<sub>2</sub> was passed through the solutions continuously, 5 ml of resuspended rumen inoculum fluid was utilized, pH was adjusted at various intervals to 6.9, and 30–72 fermentation periods were used for different experiments. Details are given on the basal medium and the mineral mixture utilized in these studies. Eight different grass and alfalfa hays were utilized. Preliminary study showed that 0.2 g cellulose per tube gave highest digestibility in 48 hr. Utilizing the same technique and samples, but with inocula obtained from an animal on the same diet but at different dates over a several-month period, the variance in *in vitro* data was less than that found in sheep-trial data in most instances, and in no instance was it greater. Comparison was made of inoculum obtained from animals on brome-grass, alfalfa, and timothy hay. Generally the digestibility of cellulose *in vitro* of these forages with the different inocula did not vary greatly. The same steer was used in producing all three inocula. For a 30-hr fermentation there was a significant difference in the digestibility of alfalfa, and the inoculum prepared when timothy was fed was significantly better than that obtained when brome-grass was fed. Chemical composition of these hays was not given. Comparison of three methods of preparing the inoculum showed that slightly higher results were given with resuspended rumen microorganisms than for phosphate buffer extract, and both of these were higher than for strained rumen juice alone, especially in the case of first-stage brome-grass and second stage orchard grass hays. In discussion of their work these authors suggested that it would be desirable to get a comparison of inocula from sheep and cattle under similar conditions. Also they indicated that at least one standard sample of known cellulose digestibility should be included in each run. Furthermore, the standard sample should consist of a forage rather than a purified cellulose, particularly if a simple medium and inoculum are to be used.
172. Raun, N. S., W. Burrough, and W. Woods. 1962. Dietary factors affecting volatile fatty acid production in the rumen. *J. Anim. Sci.* 21: 838–843.
173. Reid, C. S. W. 1965. Quantitative studies of digestion in the reticulorumen. I. Total removal and return of digesta for quantitative sampling in studies of digestion in the reticulo-rumen of cattle. *New Zealand Soc. Anim. Prod., Proc.* 25: 65–84.

174. Reid, R. L., G. A. Jung, and S. Murray. 1964. The measurement of nutritive quality in a blue-grass pasture using *in vivo* and *in vitro* techniques. *J. Anim. Sci.* 23: 700-710. — Two fistulated yearling wether sheep were utilized. One was fed a standard diet of regrowth timothy hay and the other a grass hay from various dates. The *in vitro* technique, that of Barnett and Reid, utilized samples either freeze-dried and ground through a 40-mesh sieve or else dried under a forced draft at 65°C and then ground through the 40-mesh sieve. The rumen fluid obtained from one of the above two sheep was strained whole fluid. Each tube received 0.6 g of forage dry matter which was fermented for 35 hr with 20 ml of McDougall's nutrient medium plus 10 ml of the strained rumen fluid. Anaerobic conditions were maintained by slow and continuous passage of CO<sub>2</sub> through the tubes. Dry matter and protein digestibility *in vitro* were determined by filtering the fermentation mixture through a 140-mesh metal sieve and washing the residue thoroughly with water. There was a consistent and significant increase in the *in vitro* digestibility of both dry matter and cellulose in the freeze-dried grasses as compared to the oven-dried preparation. The results with the fresh grass tended to be more variable, both between replicated samples and between trials. This was expected because of the poor techniques for chopping or mincing the fresh material. The *in vitro* digestibility of cellulose was consistently higher for the three grass preparations when the tubes were inoculated with rumen fluid from hay-fed sheep than when they were inoculated with fluid from animals fed fresh grass in the collection trials. Thus, in this instance, the cellulose digestibility was higher with ruminal microbes adapted to a sheep on a diet different from the one which was being evaluated. Although all regression relating *in vitro* to *in vivo* digestibility were comparable, the highest R<sup>2</sup> values were derived from the *in vitro* cellulose digestibility of freeze-dried grass using either a grass or hay inoculum. By means of multiple regression analysis the effects of (a) growth phase, (b) stage of maturity, (c) level of nitrogen fertilization, (d) cutting management, (e) sample preparation, and (f) nature of rumen inoculum were evaluated on the *in vivo-in vitro* relation. All of these factors had a significant effect on the *in vivo-in vitro* digestibility ratios. This suggests that individual regression equations may be necessary for individual species of forage and for specific *in vitro* situations. In later unpublished work the author indicated that a stage of maturity factor added significantly to the accuracy of the *in vitro* prediction equation.
175. Reis, P. J., and R. L. Reid. 1959. *In vitro* studies on the effect of pH and of glucose on ammonia accumulation in the rumen of sheep. *Australian. J. Agr. Res.* 10: 71-80.
176. Rice, R. W., R. L. Salsbury, J. A. Hoefler, and R. W. Luecke. 1962. Relation of certain end products of rumen fermentation to forage feeding value. *J. Anim. Sci.* 21: 418-425. A comparison was made of the digestibility of an alfalfa bromegrass hay vs. an oat straw when the inoculum was taken from steers on the alfalfa-bromegrass hay vs. steers on an oat straw plus about 1 kg of a 36% protein supplement fed daily. The *in vitro* system was a semipermeable membrane system described previously by Huhtanen *et al.* 1954. The fermentation was evaluated at 2, 4, 8, 12, and 24 hr. After 24 hr there was no significant difference in the percent cellulose digestion. Oat straw appeared to contain cellulose which was more resistant initially to attack than the alfalfa cellulose. Over 24 hr the oat straw inoculum was able to degrade a slightly greater proportion of cellulose than the alfalfa inoculum. There was a lag in cellulose digestion in these experiments, especially on the oat straw inoculum. These authors indicated that important differences exist in the types of microflora and the fermentation products produced from animals fed different kinds of forage. Thus, *in vitro* comparisons of various forages were most likely to show significant differences when the inoculum for *in vitro* fermentation is obtained from an animal which has been maintained on that particular forage. Their differences probably would have been much greater had not the oat straw diet been supplemented.

177. **Rogers, H. H., and E. T. Whitmore.** 1966. A modified method for the *in vitro* determination of herbage digestibility in plant-breeding studies. *J. Brit. Grassl. Soc.* 21:150-152. — An apparatus is described and illustrated for *in vitro* dry matter determinations. A filter tube equipped with a sintered filter is used both for fermentation by rumen microbes and for pepsin digestion. The entire operation is carried out within a single vessel. This prevents transfer errors and is highly useful for dry matter determinations, but is not used in organic matter analyses. In their work two blanks and three samples of a standard grass of known digestibility are included in each batch. The filter is a No. 3 sintered glass filter. About 0.7 g of Selite 945 also is used for each 0.5 g sample. To this is added an aliquot of 50 ml of a rumen-buffer liquor (10:40) for the 48 h digestion. With this device 85 tubes may be filtered in 1 hr by 2 people.
178. **Rojas, S. W., G. M. Ward, and R. G. Hinders.** 1962. Effect of pelleting alfalfa on *in vitro* gas production, cellulose digestion and volatile fatty acid production. Presented at the annual meeting of the Amer. Dairy Sci. Assoc.
179. **Salsbury, R. L., J. A. Hoefler, and R. W. Luecke.** 1961. Effect of heating starch on its digestion by rumen microorganisms. *J. Anim. Sci.* 20: 569-572.
180. **Salsbury, R. L., A. L. VanderKilk, Betty V. Baltzer, and R. W. Luecke.** 1958. The rates of digestion of the cellulose of some plant fractions by rumen microorganisms *in vitro*. *J. Anim. Sci.* 17: 293-297. — Ruminal fistulated steers maintained on either timothy hay or alfalfa hay were the source of inocula. The rate of fermentation of Solka-Floc was not measurably affected by change in diets. The *in vitro* system was a semi-permeable membrane into which the inoculum-substrate suspension was added and which was suspended in a mineral solution. Plant materials examined were Solka-Floc, cotton linters, dehydrated alfalfa meal, wheat straw, and corn cobs. The holocellulose and alpha-cellulose were extracted from these materials as well as using the untreated plant materials. Hemicellulose was extracted from some of the material additionally. Fermentations were 3-24 hr. Holocelluloses and alpha-celluloses prepared from the roughages showed more rapid and complete cellulose digestion than did the original plant materials.
181. **Sapiro, M. L., S. Hoflund, R. Clark, and J. I. Quin.** 1949. Studies on the alimentary tract of the Merino sheep in South Africa. XVI. The fate of nitrate in ruminal ingesta as studied *in vitro*. *Onderstepoort J. Vet. Sci. Anim. Ind.* 22: 357-372.
182. **Schillinger, J. A. Jr., and F. C. Elliott.** 1966. Bioassays for nutritive value of individual alfalfa plants. *Michigan Agr. Exp. Sta. Quart. Bull.* 48:580-590. — Six- and 36-hour *in vitro* rumen fermentations were carried out to determine dry matter and cellulose digestibility of individual alfalfa plants. The standard error of the mean of triplicate samples using one rumen fluid sample was 0.79 and 0.64 digestibility units for plants grown in the field and greenhouse respectively. Samples from one clone reduced the capacity of rumen inoculum to degrade cellulose. This effect was probably caused by a bacteriostatic agent selective for certain cellulolytic bacteria. Available evidence suggests that cellulose enzymes are closely associated with live bacteria only and are highly labile when free of the bacterial cell.
183. **Siu, R. G. H.** 1951. Microbial decomposition of cellulose—with special reference to cotton textiles. Reinhold Publishing Corporation, New York, New York. 531 pages. — Of special interest is the first chapter in which cellulose decomposition in nature is discussed. In that chapter there are sections on the carbon cycle, decomposition of cellulose by microorganisms, commercial uses of cellulolytic microorganisms, and economic aspects of microbial deterioration of cellulose. The remainder of the text is detailed discussion of cellulose in textiles,

- breakdown of cellulose in textiles, and methods of its prevention. Good references are given to cellulose breakdown by organisms, primarily bacteria but also by insects. Insects such as the rose beetle and woodboring beetles can degrade cellulose. In some cases the cellulolytic action is due to microbiological symbionts in the insect gut, but in other cases the insects themselves are capable of digesting cellulose. The latter is true also for certain snails.
184. **Slyter, L. L., M. P. Bryant, and M. J. Wolin.** 1966. Effect of pH on population and fermentation in a continuously cultured rumen ecosystem. *Appl. Microbiol.* 14:573-578. — Maintaining pH values below 6.0 caused a decrease in production of volatile fatty acids and methane. Decrease in acetate and methane production was greater than that for propionate. Cultures maintained at 6.7 contained types of bacteria normally found in the rumen, but cultures maintained at 5.0 had many bacteria which could not be identified as normal rumen bacteria. A carbonate-phosphate buffer and a phosphate buffer alone at the same pH gave similar fermentation products.
185. **Slyter, L. L., W. O. Nelson, and M. J. Wolin.** 1964. Modifications of a device for maintenance of the rumen microbial population in continuous culture. *Appl. Microbiol.* 12:374-377. — The performance of this continuous culture device was judged on the maintenance in the cultures of a relatively constant DNA content and protozoal concentrations which reached a steady state of  $2 \times 10^3$  per ml in 4 days. Steady states were held between 12 and 21 days for these criteria, as well as for fermentation patterns.
186. **Smith, H. W.** 1965. Observations on the flora of the alimentary tract of animals and factors affecting its composition. *J. Path. Bact.* 89:95-122. — Adult healthy animals fed on diets considered normal for the species were utilized. The 15 homeothermic species were monkey, dog, cat, horse, ox, sheep, pig, guinea pig, rat, gerbil, hamster, mouse, fowl, and duck. The poikilothermic animals were cockroach, frog, tortoise, roach, and whiting. The animals were sacrificed, the abdomen was opened quickly, and ligatures were applied to various parts of the digestive tract. Contents of the tract were removed under sterile conditions, their character and approximate volumes were noted, and their pH was measured. The numbers of different kinds of bacteria and yeasts were estimated by making colony counts on plate cultures incubated aerobically and anaerobically. Qualitatively, the organisms were similar among homeotherms, but as a group they differed from those in the poikilotherms. In homeotherms the numbers of organisms increased progressively from duodenum to ileum and highest numbers were found in the large intestine. Comparable differences in the flora of the digestive tract were reproduced in rats fed on different diets. The anatomy and physiology of the stomach and the feeding habits of the different species of animals also had a profound influence on determining the composition of the alimentary flora.
187. **Smith, J. A. B., and F. Baker.** 1944. The utilization of urea in the bovine rumen. 4. The isolation of the synthesized material and the correlation between protein synthesis and microbial activities. *Biochem. J.* 38: 496-505. — In studies made by incubating rumen liquor, it was found that the non-protein nitrogen content of the mixture decreases while the total nitrogen content remains constant. The decrease in non-protein nitrogen is accompanied by the accumulation of a bulky sediment which gives an intense blue color with iodine. This sediment consists almost entirely of bacteria. It contains a starch-like polysaccharide and at least the main proportion of the synthesized protein. Protein synthesis was shown to occur in the absence of protozoa, and the chief contributors were found to be the iodophile bacteria. Various species were found, but it was assumed that the bulk of the synthesized polysaccharide and protein is incorporated in the small rods, cocci, and vibrios of the microiodophile population. The greater part of the synthesis occurs within the first two hours of incubation while microbiological normality is maintained in the samples. In the rumen, bacteria readily convert significant amounts of nonprotein nitrogen to protein and sugars to polysaccharide, thus synthesizing protein which is of use to the ruminant.

188. **Smith, P. H., and R. E. Hungate.** 1958. Isolation and characterization of *Methanobacterium ruminantium* N. sp. J. Bacteriol. 75: 713-718.
189. **Smith, P. H., H. C. Sweeney, J. R. Rooney, K. W. King, and W. E. C. Moore.** 1956. Stratifications and kinetic changes in the ingesta of the bovine rumen. J. Dairy Sci. 39: 598-609. — Experiments were made with ruminal fistulated steers, and by rumenotomy, to sample ingesta from the top and bottom of the rumen. Samples were analyzed for various constituents over a 12-hr period that included two feedings, as well as for cellulose digestion *in vitro*. Cellulose digestion was measured by the loss in weight of a 500-mg square of Patapar (Patterson Parchment Paper Company, Bristol, Pennsylvania). The Patapar was placed in a tube nearly filled with strained rumen juice. The contents were layered with mineral oil and incubated at 39°C for 24 hr. Concentrations of almost all factors measured, except ether extract, were significantly higher in samples from the top of the rumen. The data also suggested there was a greater concentration of microorganisms in the top ingesta. There was more rapid *in vitro* digestion of cellulose by bottom samples of ingesta, apparently due to a higher initial pH and buffering capacity in the inoculum and to a lower concentration of competing substrates.
190. **Smith, R. E., R. G. Hinders, and G. M. Ward.** 1962. An *in vitro* artificial rumen technique, with studies on the relative value of different ration constituents. Paper presented at the annual meeting of the Amer. Dairy Sci. Assoc. — A manometric system was developed to measure gas production by fermenting 0.5 g of substrate, 15 ml of phosphate buffer, and 25 ml of strained rumen fluid. The mixture was added to fermentation flasks which were flushed with CO<sub>2</sub> to exhaust air, stoppered, and connected to the manometers which are burettes connected to one-l wells. A partial vacuum allows the direct measurement of gas production. This system was utilized in studying gaseous production by three different inocula with a variety of substrates varying from barley to sagebrush. Standard hay samples were included in each run and their results of net gas production (gross-blank) were corrected to a percentage of the net of the standard. Fermentation was measured through 24 hr. Rate of gas production was greatest in 0-8 hr and least in 16-24 hr. For periods of 4-6 hr there was an increase in gas production with the amount of rumen fluid in the system. The rate of gas production of a standard sample decreased with time.
191. **Stewart, D. G., R. G. Warner, and H. W. Seely.** 1961. Continuous culture as a method for studying rumen fermentation. Appl. Microbiol. 9: 150-156. — A complex continuous flow system is described and evaluated. A 5.5-l culture was maintained in an 8-l glass vessel at 39°C. The device was covered to exclude most light, and was stirred by paddles at 33.3 rpm. The apparatus was fitted with a rubber gasket to make it airtight except for the stirring shaft opening. A suction flask with 0.5-inch i.d. glass tubing was used for sampling. The substrate was stored, stirred magnetically, and refrigerated. A CO<sub>2</sub> pressure of 0.5 psi was maintained to minimize effect of depth on substrate flow pressure. The periodic substrate flow into the culture vessel was controlled by a solenoid valve. Culture flowed from the system through a solenoid-controlled outflow tube near the bottom of the vessel. The operation of this solenoid was regulated by a float on top of the culture to maintain a given level or volume in the system. Flow rates were about 9% of the culture volume per hour. Conditions in this system were comparable to those *in vivo*.
192. **Terry, R. A., and J. M. A. Tilley.** 1964. The digestibility of the leaves and stems of perennial ryegrass, cocksfoot, timothy, tall fescue, lucerne and sainfoin, as measured by an *in vitro* procedure. J. Brit. Grassland Soc. 19: 363-372. — Determinations were made of *in vitro* digestibilities of various plant parts of important forage grasses and legumes. All plant parts had a higher digestibility at early stages of growth than later. With maturity the digestibility of the stem fell off at a much faster rate than that of the leaf. Leaf sheaths of grasses declined in digestibility at an intermediate rate. A comparison was made of *in vitro* dry matter digestibility and that of pepsin digestion.

193. **Thomas, G. J.** 1960. Metabolism of the soluble carbohydrates of grasses in the rumen of sheep. *J. Agr. Sci.* 54: 360–372. — Rumen contents from grass-fed sheep were twice as active as those from hay-fed sheep towards grass carbohydrates. This was found in studies using a semi-permeable membrane.
194. **Tilley, J. M. A., and R. A. Terry.** 1963. A two-stage technique for the *in vitro* digestion of forage crops. *J. Brit. Grassland Soc.* 18: 104–111. — This widely used two-stage technique for *in vitro* forage evaluation was evolved after a comparison of several published methods. After preliminary studies it was found preferable to use glass tubes rather than dialysis sacks and to rely on gas production during digestion to maintain anaerobic conditions rather than to gas the samples continuously. The relatively large volume of buffer solution maintains adequate nutrients and usually adds accessory factors, such as valeric acid and trace elements, which are required for bacterial growth. The system has the advantages of a simple apparatus: reproducibility and many samples can be run. It is therefore useful for routine evaluation of forage samples. Samples are ground to pass through a 0.8 mm screen, and 0.5 g are weighed into 80–90 ml glass tubes. Inoculum is obtained from sheep maintained on a diet of hay. The liquor is strained through two layers of muslin, CO<sub>2</sub> is passed into the flask to displace air, and the inoculum is kept at 39°C until required. Buffer solution is similar to that of McDougall with calcium chloride added last. The solution is saturated with CO<sub>2</sub> and kept at 38°C until required. Each tube contains 40 ml of buffer solution, 10 ml of strained rumen liquor, and 0.4 g of forage sample. This is gassed with CO<sub>2</sub>, stirred, and sealed by a rubber cork with a Bunsen gas valve. The tubes are incubated in the dark at 38°C and are shaken 3–4 times a day by hand. Usually pH remains between 6.7 and 6.9, but, if necessary, adjustment is made with normal sodium bicarbonate. After 48 hr the microbial digestion is halted by adding 1 ml of 5% mercuric chloride solution. Normal sodium bicarbonate also is added to improve sedimentation. The tubes are centrifuged for 15 minutes at 1800 × g. After discharging the supernatant, 50 ml of the freshly made pepsin solution are added to the residue in each tube. The tubes then are incubated at 38°C for 48 hr with occasional shaking, but anaerobic conditions are not maintained during this stage. At the end of incubation, supernatants are discarded, and insoluble residues are washed with water in the centrifuge. Residues are transferred with little water to a tared glass basin or a beaker, and these are dried at 100°C to constant weight. The measure of digestion is the dry matter digestion in the 0.5 g of herbage after correction for the blank (which represents undigested food particles and microorganisms from the rumen liquor). In each trial two standard samples are selected, one of high and one of low digestibility relative to the unknown materials being analyzed. These data are used to correct the values for any given run. These workers note that some variation in digestive efficiency between experiments is unavoidable and similar herbages should be compared within the same experiment if possible. They find it advisable to leave the donor animal on *ad libitum* intake and to use large volumes of rumen liquor. Separate experiments showed that freeze-dried samples of fresh herbage had the same *in vitro* digestibility as samples heated and dried at either 40 or 100°C. Drying at 100°C had a marked effect on the digestibility only if continued for longer than 4 days. Size of grind, within usual ranges, had no influence on digestibility. However ball milling the samples (very fine grinding) greatly increases *in vitro* digestibility probably due to the destruction of cell walls. *In vivo* digestibilities are not constant characteristics of herbages. They vary according to type of animal (cattle or sheep), age and health of animals, level of feed intake, and manner in which the feed is prepared.
195. **Tisserand, J. L., and S. Z. Zelter.** 1965. An attempt to standardize the technique of measuring the digestion of fodders *in vitro* (artificial rumen). *Ann. Biol. Anim. Bioch. Biophys.* 5:101–111. (in French). — A continuously and individually gassed system is fermented for 24-hr at 39°C. Sheep rumen fluid (20 ml) filtered through gauze is used with an artificial saliva (20 ml). Up to 2-hr of exposure to air at room temperature does not decrease the cellulolytic activity of the rumen fluid. For each 100 ml of fluid, 2.5 g of dry matter of sample are used.

196. Tomlin, D. C. 1960. Crystallinity of cellulose and digestibility of feedstuff cellulose in the bovine rumen. Ph.D. Thesis. Univ. Florida. 86 p. — The purpose of this study was to determine whether the relative amount of crystalline order in cellulose of natural fibrous feedstuffs had an effect on the rate of digestion of cellulose from these feeds in the rumen. Cellulose in citrus pulp, Solka-Floc, and cotton linters was digested in inverse proportion to the crystalline indices for these feeds. Cellulose digestion from hays and bagasse showed no apparent relation between crystallinity and digestibility. Part of this effect was due to associated materials, and it was concluded that the rate of digestion of a relatively pure cellulose by rumen micro-organisms is inversely related to the relative crystallinity of the cellulose. The difficulty is in isolating cellulose from natural feedstuffs in a way that permits estimation of its relative crystallinity.
197. Tomlin, D. C., R. R. Johnson, and B. A. Dehority. 1965. Relationship of lignification to *in vitro* cellulose digestibility of grasses and legumes. *J. Anim. Sci.* 24: 161–165.
198. Troelsen, J. E., and Donna J. Hanel. 1966. Ruminant digestion *in vitro* as affected by inoculum donor, collection day, and fermentation time. *Can. J. Anim. Sci.* 46:149–156. — Nine sheep were compared on three different days as inoculum donors for a 2-stage *in vitro* digestibility study. Various fermentation times and sources of cellulose and non-cellulosic organic matter were studied. Variation among days was greater than variations among animals. Still, these variations were smaller than those obtained in *in vivo* digestibility determinations with sheep. Interactions of sheep and days resulted from variations in water consumption, which caused dilution of the inoculum. A practice was adopted of not allowing the animals to drink for 16 hours prior to the collection of rumen liquor. The fermentation curves for straw and alfalfa were drawn from data taken at 3, 6, 12, 24, 48, and 96 hrs. Alfalfa reached maximum digestion values at 48 hr, but wheat straw took 96 hr to reach a corresponding plateau.
199. Van Dyne, G. M. 1962. Micro-methods for nutritive evaluation of range forages. *J. Range Manage.* 15: 303–314. — Studies with both cattle and sheep under corral-feeding and range-grazing conditions were conducted. Forage sources included hand-clipped range plants and forage samples taken from esophageal-fistulated steers and wethers grazing the same range as the rumen-fistulated animals. *In vitro* cellulose digestion values varied significantly at all time periods. From 24–48 hr all samples of forage maintained their relative digestion values. Inocula prepared by simply straining rumen contents through several layers of cheesecloth gave as high and as uniform cellulose digestion values as did more elaborate procedures of processing the inocula. The diet of the fistulated animal influenced the estimates of cellulose digestion. Cellulose digestion values of range forage and pure cellulose samples were considerably higher when the base diet was alfalfa hay than when the base diet was oat hay. Estimates of cellulose digestibility were similar for artificial rumen and nylon bag techniques. There were closer agreements between artificial rumen duplicate tubes than between duplicate nylon bags.
200. Van Dyne, G. M. 1963. An artificial rumen system for range nutrition studies. *J. Range Manage.* 16: 146–147. — An artificial rumen system is described which has large capacity, simplicity, and portability. The system accommodates 170 round-bottom Pyrex centrifuge tubes of 100 ml capacity. Major components of the system, gas control, pH control, and details of the operating sequence are explained. Approximately 10, 20, and 20 ml, respectively, of buffer, nutrient medium, and rumen fluid are added to each tube. CO<sub>2</sub> is bubbled through the tubes continuously during the fermentation, and the pH remains near 6.9–7.0. The nutrient media and buffer are those of Quicke *et al.*, 1959, to which 2.5 ml per tube of a 20 mg/ml solution of enzymatic casein hydrolysate is added. Cellulose analyses are carried out within the fermentation tube.
201. Van Dyne, G. M., and J. H. Meyer. 1964. A method for measurement of forage intake of grazing livestock using microdigestion techniques. *J. Range Manage.* 17:204–208. — A new

procedure for determining forage intake by grazing animals is described. This procedure involves determination of the digestion value of range forage and standard forage samples using micromethods with inocula from grazing animals. Then prediction of macrodigestion from microdigestion of range forage is made by use of a regression equation. The microdigestion of range forage is adjusted to microdigestion of a standard sample. The predicted macrodigestion estimate, the composition of the range forage, and the composition and amount of the feces are used to calculate forage intake. This procedure of estimating forage intake eliminates the necessity of assuming indigestibility of naturally occurring indicators. This new procedure also obviates harvesting range herbage for dry-lot digestion trials.

202. **Van Dyne, G. M., and W. C. Weir.** 1964. Variations among cattle and sheep in digestive power measured by microdigestion techniques. *J. Anim. Sci.* 23: 1116--1123. — Eighteen ruminal fistulated steers and wethers provided inocula for individual microdigestion estimates in three range-grazing trials and one drylot feeding trial. Artificial rumen and nylon bag cellulose digestion and nylon bag dry matter digestion data were obtained. Between-class and within-class of stock differences were analyzed. When averaged over all procedures, there were no significant differences in digestive power between cattle and sheep. Differences among animals within a species were less in drylot. Averaged over all techniques and samples, there were 12 highly significant differences among 18 animals. There were more differences in digestive power among steers than among sheep. Averaged over all techniques, samples, and periods about 5 cattle and 4 sheep would be required as inocula sources to estimate microdigestion with 10% of the mean with 95% confidence. The numbers required varied from about 11 in early summer grazing to 6 in late summer as compared to 3 on drylot. More sheep than cattle would be required in drylot, but more cattle than sheep would be required on the range. More animals would be required for digesting purified cellulose sources than for range forages, and more for range forages than for the alfalfa standards.
203. **Van Dyne, G. M., and W. C. Weir.** 1964. Microdigestion of grazed annual forage, clipped herbage, and standard samples by cattle and sheep. *J. Range Manage.* 17:327--332. — Six grazed forages, an alfalfa sample, and Solka-floc were not ranked in the same order by three microdigestion techniques. Solka-floc always had the highest digestibility, but forages varied in rank. Cellulose digestibility, but not dry matter digestibility, was higher in cattle-grazed than in sheep-grazed forages. Forages grazed by either cattle or sheep in mid-summer were more digestible by all techniques than forages grazed in early or late summer. Over all techniques, the correlation between microdigestion and macrodigestion estimates was about 0.72. Adjusting microdigestion of range forages to that of a standard sample decreased the range of estimates but did not improve the correlation. The correlations found were not as high as many of those in the literature for farm roughages. Reasons are discussed for possible differences.
204. **Van Dyne, G. M., and W. C. Weir.** 1966. Comparison of microdigestion techniques under range and drylot conditions. *J. Agr. Sci.* 67: 381--387. — Digestion of cellulose and dry matter in nylon bags suspended in the rumen and the digestibility of cellulose in the artificial rumen were compared during three experimental periods on the range and one period on drylot. Nine each of ruminal fistulated wethers and steers provided inocula for artificial rumen studies and carried nylon bags. The same animals were used in all four experimental periods. Solka-floc and a sample of alfalfa were used as standards in all trials. Forage samples were collected from esophageal-fistulated animals which grazed the same range as the rumen-fistulated animals. These forage samples also were digested by *in vitro* and *in vivo* techniques. The *in vivo* digestion results appeared to reflect changes in the quality of the base diet more than did *in vitro* results. Regression equations and correlations between microdigestion estimates by the different techniques were significant if samples within a wide range of digestibilities were included in the

analysis. Within ranges of digestibilities of 10% or less, the equations were not useful for predictive purposes. Range forage samples taken from esophageal-fistulated cattle or sheep were digested better when the base feed was pelleted alfalfa than when it was range forage. Digestion of an alfalfa sample was less affected by the base diet. Solka-floc was digested better *in vitro* when the inocula came from animals grazing on the range than from animals fed pelleted alfalfa.

205. **Van Soest, P. J.** 1965. Symposium on factors influencing the voluntary intake of herbage by ruminants: voluntary intake in relation to chemical composition and digestibility. *J. Anim. Sci.* 24: 834-843. — Although the paper is not primarily concerned with *in vitro* techniques there is a short section concerning them. When *in vitro* fermentation is used, alfalfas are characterized by a high initial gas production followed by a leveling off, while grasses ferment at a slower rate and do not level off to the same extent. One would expect with legumes, because of a large available fraction of highly digestible material, a rapid burst of fermentation initially followed by a plateauing as the soluble cell contents are exhausted. With grasses a slower start is observed, because of the smaller amount of the highly digestible cell contents, and the fermentation continues steadily as the lightly-lignified holocellulose continues to ferment at an appreciable rate. One would expect that a short fermentation time would correlate more highly with intake. One would also expect that the optimum fermentation times for prediction of voluntary intake and digestibility would not be the same for different species of forage. While the cell wall constituents of grasses may be greater than those of legumes, resulting in about equal dry-matter digestibility, the voluntary intake is usually lower, especially in the more mature grasses that are highest in quantity and lignification of cell wall constituents. In the case of legumes, the fibrous mass ingested is not large enough to inhibit intake, and legumes as a class are characterized by optimum intakes.
206. **Van Soest, P. J., and R. H. Wine.** 1965. Development and use of chemical methods for determining the nutritive value of forages. Northeastern-24 Regional Research Project, Annual Report. — In this work the second stage of the Tilley *in vitro* rumen fermentation method was modified by replacing the acid-pepsin digestion with a neutral-detergent treatment of the fermentation mixture. This was done because the feces of herbivora contained (1) endogenous excretions from the animal, (2) bacterial residues, and (3) undigested forage. With the Tilley method there can be no endogenous residues. Therefore one might expect the Tilley method *in vitro* digestibility to be higher than the digestibility *in vivo*. The modified Tilley method was compared with 20 hays including 12 grasses and 8 legumes for which the *in vivo* dry matter digestibility varied from 45 to 80. True digestibility of these forages was estimated by taking apparent digestibility data and correcting them for the fecal non-cell walls, which represent the combined bacterial and endogenous losses from the animal. Results from the *in vitro* digestion, followed by a determination of undigested cell-wall material, yielded digestibilities greater than those obtained by the Tilley procedure. The *in vitro* digestibility by cell walls yielded values nearly equal to those of true digestibility *in vivo*. A closer linear relationship was obtained by this technique than by the Tilley technique. These studies indicated that the difference between the two *in vitro* fermentations are due to bacterial residues. The data also suggest differences between sheep and cattle with regard to endogenous and bacterial losses in the feces. With comparison to the Tilley *in vitro* procedure, the new procedure is shorter by two days and requires fewer manipulations.
207. **Van Soest, P. J., R. H. Wine, and L. A. Moore.** 1966. Estimation of the true digestibility of forages by the *in vitro* digestion of cell walls. *Int. Grassl. Congr., Proc.* 10:438-441. — This is a modification of the Tilley and Terry method. The acid pepsin stage is replaced by a cell-wall determination using the neutral detergent procedures. This system requires less time and predicts digestibility more accurately than the unmodified Tilley and Terry method. Their work shows some indication that there are differences between sheep and cattle in regard to endogenous and bacterial losses in the feces.

208. **Virtanen, A. I.** 1946. Fermentation of wood-dust by cellulose bacteria. *Nature* 158:795. — Birch, aspen, and pine dusts were digested by bacteria. The finer the wood was ground, the more cellulose was fermented.
209. **Waldern, D. E., W. K. Roberts, T. H. Blosser, and I. A. Dyer.** 1961. Effect of levulinic acid on digestibility, fatty acid production and microbial activity in dairy heifers and wether. *J. Anim. Sci.* 20: 429–432. — *In vitro* digestion was evaluated manometrically with a 55-minute incubation period. Gas production was significantly increased by adding alfalfa to the diet of animals on wheat straw. Adding levulinic acid to the diets of either group of animals significantly decreased microbial activity. This suggests that levulinic acid might function as an antimetabolite for rumen microorganisms.
210. **Walker, D. J.** 1961. Isolation and characterization of a hemicellulose-fermenting bacterium from the sheep rumen. *Australian J. Agr. Res.* 12: 171–175.
211. **Walker, D. J., and W. W. Forest.** 1964. The application of calorimetry to the study of ruminal fermentation *in vitro*. *Australian J. Agr. Res.* 15:299–315. — A calorimeter was designed in which rumen fermentations could be undertaken and heat production measured continuously. These studies were undertaken because there was no conclusive evidence that *in vitro* techniques support the same fermentation as occurs *in vivo*. Also, as done in many kinetic studies, it was not possible to dissect the complex biological system and study each component individually. Such a method gives no real indication of how the system behaves as a whole, especially in the case of a rumen fermentation where the extent of synergistic and antagonistic effects are unknown. Heat production is a quantitative measure of the overall rate of metabolism and may be obtained no matter how complex the system under consideration. The authors recognized the difference of activity of microbes at different points in the rumen and obtained samples from ten geometrically spaced points rather than a single sample. Gas production was measured in the calorimeter by collecting fermentation gases in a closed buret inverted over, and filled with, acidified water. A comparison was made of the solid portion of the rumen contents with the liquid portion in its ability to ferment cellobiose. The solid portion had a much greater ability to ferment cellobiose, and lactate accumulated as a transitory intermediate during cellobiose fermentation. With whole contents, lactate breakdown is the rate-limiting step in cellobiose fermentation, whereas with the rumen fluid only cellobiose breakdown itself is the limiting factor. Cellobiose is probably an intermediate in cellulose breakdown. The rate of heat and gas production varied directly with the solid content of the sample. At 24 hr post-feeding the heat production by ruminal fermentation in the sheep used was calculated to be between 480 and 1360 cal/hr. There was an additional large amount of heat produced in the first few hours after feeding.
212. **Walker, D. M.** 1959. The *in vitro* digestion of roughage dry matter. XV. *Int. Dairy Congr., Proc.* 1:190–195. — In this *in vitro* study a complex mineral and buffer solution was utilized with 20 ml of strained rumen juice, 1 g of ground roughage, and a 72-hr digestion period. This system was continuously gassed with CO<sub>2</sub> and N<sub>2</sub> with the gas line passing from one tube to the next in an inverted U to act as a condenser to prevent liquid being carried from one bottle to another. Protozoal activity ceased after 36 hr but bacterial activity continued up to 72 hr, after which no further digestion occurred. Comparisons were made with dry matter digestibility *in vitro* and *in vivo*. The *in vitro* data were slightly lower than the *in vivo* data but there was relatively good agreement throughout. A standard hay was tested in a large number of trials and showed a variation of 52–57%, due to variations in the rumen juice obtained from the sheep. The rumen juice varied in its content of dry matter from one experiment to another. The sheep was fed only hay at the beginning, but later the diet varied because it was found digestibility *in vitro* was unaffected by variations in the diet of the sheep which provided the

rumen inoculum. Yet their data show 5% variation in digestion of the standard sample from period to period, perhaps related to dietary variations.

213. Ward, J. K., D. Richardson, and W. S. Tsien. 1961. Volatile fatty acid concentrations and productions in the gastrointestinal tract of fullfed beef heifers. *J. Anim. Sci.* 20:830–832.
214. Warner, A. C. I. 1956. Criteria for establishing the validity of *in vitro* studies with rumen micro-organisms in so-called artificial rumen systems. *J. Gen. Microbiol.* 14:733–748. — Warner's system was composed of dialyzing against a standard mineral solution a fermentation enclosed in a cellophane sack. Both the dialyzing solution and the fermentation solution had N<sub>2</sub> containing 5% CO<sub>2</sub> slowly bubbled through them. pH was adjusted periodically in both compartments using phosphate and carbonate buffers. When the substrate approximated the composition of a normal diet, little adjustment of pH was required, but when single substrates such as starch were used, considerable alterations of pH occurred. The whole apparatus was held at 39°C. A complex dialyzing solution was utilized and approximated the composition of the ruminal liquor. Warner's criteria for a successful system include (1) the maintenance of numbers and normal appearance of bacteria and protozoa, (2) the maintenance of normal rates of digestion of various food components, and (3) the ability to predict quantitative results *in vivo*. His system met the criteria suggested with reasonable success for periods of about 8 hr. For microbial population to remain normal in numbers and activities it was necessary to use as test substrate *in vitro* only substances similar to the diet fed to the animal from which the rumen liquor inoculum was taken. Although cellulose digestion is the most commonly used criterion of functioning of artificial rumen systems, there are some disadvantages to its use. The rate-limiting step occurred at a very early stage where the products would still be measured as cellulose by most techniques in common use. In the rumen, samples may remain 24 hours or more. Secondly, it is practically essential to strain the rumen liquor for use *in vitro*, and some diminution of the rate of digestion may be expected because many of the cellulolytic microorganisms remain attached to the large plant particles removed in straining. In one experiment a low nitrogen content of the diet affected *in vitro* results because it is presumed to have supported a less numerous, and hence less active, microbial population than other diets. Thus, there is danger in relying on *in vitro* tests to predict behavior *in vivo* where the substrate tested is very different from the diet of the animal used to supply the rumen liquor.
215. Warner, A. C. I. 1956. Proteolysis by rumen micro-organisms. *J. Gen. Microbiol.* 14:749–762.
216. Warner, A. C. I. 1962a. Enumeration of rumen micro-organisms. *J. Gen. Microbiol.* 28:119–128. — He found no major difference in microbial concentrations in different positions in the rumen of sheep. This is in contrast to other studies with cattle in which there is a positional variation. In microscopic examinations he found variations, however, in the location of microbes in the sample. Sometimes there were dense concentrations around feed particles. He furthermore postulated that when conditions in the rumen are such that different kinds of feed particles begin to separate, it can be expected that both the microbial and the solute concentrations will differ from place to place. The feed and rations used in his experiments did not cause layering in the rumen. It is not yet possible to apportion a given reaction between different organisms present that are capable of carrying out the reaction. In a microbial population where members differ greatly in size and metabolic activity, a figure for total numbers alone is relatively meaningless. Only a moderate variable error is incurred in making the assumption that the number of microorganisms is indicative of the true total counts of the entire rumen contents.

217. Warner, A. C. I. 1962b. Some factors influencing the rumen microbial population. *J. Gen. Microbiol.* 28:129-146. — In sheep fed once daily the concentrations of microorganisms in the rumen changed with the time after feeding, and there were variations among organisms. Peak concentrations were reached at different times for different organisms. One animal at different times, or different animals, on the same ration had very different ruminal microbial populations; these differences were variable among different microorganisms. Feeding different quantities of the same ration had little effect on the concentration of ruminal microbes, provided the ration was above a minimal level. Starvation for a few days or prolonged under-nutrition had a marked effect causing some organisms to be drastically reduced in numbers or to die out completely. When the qualitative nature of the diet was changed, about 10 days were needed to complete the major adjustments in the rumen microbial population. There were some indications of specific animal effects. When rumen contents were interchanged between two sheep, there appeared to be a tendency for some organisms to reestablish in concentrations characteristic of the host animal. No specific factor could be found in the saliva or the rumen liquor which would cause these variations in species composition of the microflora. The microbial protoplasm was estimated to occupy no more than 10% of the volume of strained rumen liquor. Relatively large quantitative and qualitative changes occurred in microbial populations as compared to changes in chemical activity. The major end products of fermentation are substantially the same with different microbial populations. This is also true when comparing faunated and defaunated ruminants. The causes of differences in microbial populations remain obscure. The most likely general explanation would seem to be that there are a large number of possible, more or less stable, microbial populations for any one combination of ration, eating and drinking routine, and perhaps animal. Relatively small causes can start a process of change from one such population to another.
218. Warner, A. C. I. 1965. Factors influencing numbers and kinds of microorganisms in the rumen. p. 346-359. *In: Dougherty et al. Physiology of digestion in the ruminant.* Butterworths, Washington. 480 pp. — There are wide diurnal changes in microorganism concentrations. The patterns vary with the specific organism and the diet. It is doubtful whether use of an average value for the pattern has any advantages over the use of concentration under standard conditions, such as just before feeding. Probably the metabolic activity of the microorganisms is greatest at the time of peak concentration. It would be valuable to express numbers of bacteria as volume of microbial protoplasm per ml of fluid. Microbial protoplasm is usually about 10% of the volume of rumen fluid. Different animals given the same dietary and environmental treatment may have very different rumen microbial populations both quantitatively and qualitatively. Even for the same animal kept under constant conditions, there may be from time to time very different rumen microbial populations. The amount of diet given to an animal has, within wide limits, little effect on the concentrations of the microorganisms in its rumen. However, the volume of rumen contents, the rate of flow of saliva, and probably the rate of flow of digestion from the rumen increase when the amount of feed is increased. Thus, there would be an increase in output of both microorganisms and their fermentation products despite relatively constant concentration. This suggests microorganisms multiply more rapidly on higher feed levels. The feeding frequency has an influence; to make comparisons between grazing animals and penned animals it is necessary to take into consideration the behavior patterns. Under most grazing conditions there is a 6-8 hr period before dawn when little or no grazing takes place. A sample of rumen contents taken then would most closely correspond to a before-feeding sample from pen-fed animals. In grazing studies penning the animals overnight would insure the proper concentration in early morning. High concentrations of holotrich protozoa are found in animals fed hay or forages rich in soluble sugars. Animals fed diets rich in starch often have high concentrations of Entodinia in their rumens. Bacterial numbers vary widely, but in general diets richer in readily fermented nutrients support greater total numbers of bacteria than do other diets.

219. Warner, A. C. I. 1966. Diurnal changes in the concentrations of microorganisms in the rumens of sheep fed limited diets once daily. *J. Gen. Microbiol.* 45:213-235. — Changes in concentration of different microbes were found to be characteristic of the group, and they were not much a function of time of day, diet, or host animal. The diurnal changes of different types of micro-organisms differed considerably. Some of their evidence suggests that animals which are more rapid eaters tend to have large rumen volumes. An appendix to the article discusses the kinetics of rumen microbes and ingesta flow.
220. Warner, A. C. I. 1966. Diurnal changes in the concentrations of microorganisms in the rumens of sheep fed to appetite in pens or at pasture. *J. Gen. Microbiol.* 45:243-251. — The pattern of changes of concentration of various groups of microorganisms in sheep fed a roughage diet were similar to those for sheep grazing on mixed pasture. The author postulates that these patterns will be present wherever the daily eating behavior of the animal includes a major fairly continuous feeding period.
221. Warner, A. C. I. 1966. Periodic changes in the concentrations of micro-organisms in the rumen of a sheep fed a limited ration every three hours. *J. Gen. Microbiol.* 45:237-241. — A 3-hr rhythm of concentration change was seen for most microorganisms. This concentration change surpassed any diurnal rhythm that might have been present.
222. Wegner, M. I., A. N. Booth, G. Bohstedt, and E. B. Hart. 1940. The "in vitro" conversion of inorganic nitrogen to protein by microorganisms from the cow's rumen. *J. Dairy Sci.* 23:1123-1128.
223. Wilkins, R. J. 1966. The application of the *in vitro* digestion technique to some arid-zone fodders. *J. Brit. Grassl. Soc.* 21:65-69. — Samples evaluated were ground through a 1-mm screen. A fistulated sheep fed lucerne chaff served as the host animal. This animal was fasted overnight prior to collection of the rumen ingesta, which was strained through muslin and used in an *in vitro* system following the procedures of Tilley and Terry. The system contained 0.4 g substrate and 40 ml strained rumen juice and artificial saliva in a 1:4 mixture. It was maintained at 39°C and fermented for 48 hr before it was centrifuged prior to pepsin digestion. Compared to this system was the addition of a fortified medium containing 10 mg glucose, 9 mg urea, and 20 mg Difco yeast extract per fermentation flask. Samples investigated were grasses and shrubby plants. The correlation of percent cellulose digestion to organic matter digestibility was nonsignificant for many grasses. Organic matter digestibility was 30-70%, but cellulose digestibility was less than 30%. Neither *in vivo* nor *in vitro* percent cellulose digestion is a good measure of the nutritive value of these grass-shrub-forb complexes. Digestibility results obtained with a fortified medium were up to 10% higher than for non-fortified media.
224. Williams, V. J., and K. R. Christian. 1956. Rumen studies in sheep. I. Variation in rumen microbial end-products in free-grazing sheep. *New Zealand J. Sci. Techn.* 28:194-200. — Variations were found in the numbers of ruminal microorganisms and in the products of microbial fermentation due to differences between sheep, between days, between times during the day, and between two groups on different pastures. The most practical measure of the mean levels of rumen constituents in a large group of sheep may be obtained by taking single samples from the largest possible number of animals. Where the number of animals in a group is small, sampling once a day on several days is preferable to sampling more than once a day on fewer days.
225. Wright, P. L., A. L. Pope, and P. H. Phillips. 1963. Effect of physical form of ration upon digestion and volatile fatty acid production *in vivo* and *in vitro*. *J. Anim. Sci.* 22:586-591. — Inocula were obtained from lambs, which had been fed pellets, crushed pellets, finely ground, coarsely ground, or baled hay, and from a cow maintained on baled hay. These six different inocula were

used to evaluate digestion *in vitro* of cellulose from both pelleted and non-pelleted hay, each of which was then ground twice through a 20-mesh screen. The *in vitro* technique was that of Baumgardt *et al.* and involved a 24-hour digestion at 38°C. Inocula were obtained from the lambs via a flexible tube placed into the rumen and attached to a vacuum pump. The inoculum was obtained from the cow through a ruminal fistula. Strained rumen fluid was used within 30–45 minutes after collection. Mean cellulose digestion *in vitro* averaged over the three substrates (pelleted hay, ground hay, and alfalfa meal) varied only from 42–45%. Thus, the inocula had no major effect on the cellulose digestion, but here all the diets were of similar chemical composition. Digestion coefficients *in vivo* for fiber for many of these constituents varied from 39–42%. These data show that various means of processing the inocula had little effect on the cellulose digestion. The number or relative activity of the microorganisms present in the inocula appeared to assert a greater effect in controlling the total amount and relative concentration of the volatile fatty acids produced *in vitro* than the processing to which the substrate had been subjected. There was more variation in volatile fatty acid production, total and relative, than in cellulose digestion.

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