FACTORS AFFECTING IN VITRO FERMENTATIONS,

BY RUMEN MICROORGANISMS

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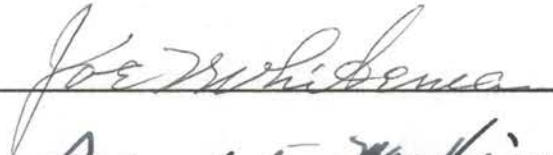DOCTOR OF PHILOSOPHY
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FACTORS AFFECTING <u>IN VITRO</u> FERMENTATIONS
BY RUMEN MICROORGANISMS

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INTRODUCTION

Evaluation of forages _in vitro_ has become a useful tool for the study of digestive processes in the rumen. As compared to conventional digestion trials, time and labor and the amounts of forage and other samples required for analysis are greatly reduced. However, _in vitro_ systems are not without limitations and they have been criticized in that the complexities of the rumen and its microbial population may not be paralleled in the laboratory. There is always the question of how closely results obtained via these procedures approach those obtained by using the intact animal.

Many factors which may influence the digestibility of feedstuffs in the artificial rumen are not encountered _in vivo_. Some of these are: (1) Air; (2) kind and amount of various nutrients which must be supplied; (3) combination of substrate, nutrient solution, and inoculum; and (4) accummulation of the end products of digestion. The composite effects of the variables encountered in _in vitro_ procedures make it necessary to exercise good judgment in the application of the results to practical nutrition.

A study was undertaken to investigate several factors which may influence the digestibility of various substrates _in vitro_. A comparison was made of the ability of _in vitro_

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rumen fermentations to simulate _in vivo_ fermentations. A technique was developed which would facilitate repeatable estimates of cellulose and dry matter digestion.

REVIEW OF LITERATURE

Historical

Tappeiner (as quoted by Marston, 1948) appears to have been the first investigator to assemble a crude type of in vitro fermentation apparatus. He incubated cotton wool and paper pulp with rumen juice under anaerobic conditions and noted the disappearance of cellulose and the evolution of carbon dioxide and methane.

Marston (1948) apparently was one of the first to attempt to duplicate rumen conditions in vitro. He provided temperature and pH control, anaerobic conditions, stirring, buffer salts, and a large inoculum from the rumen of freshly slaughtered sheep. Although his apparatus was quite complex, a series of successful fermentations were carried out.

Louw et al. (1949) modified Marston's (1948) technique by introducing into the system a semipermeable sac which removed much of the volatile fatty acids and other end products from the reaction mixture.

The apparatus of both Marston (1948) and Louw et al. (1949) were very elaborate and not well suited for the routine testing of large numbers of forage samples. Burroughs et al. (1950a) developed a simple, all-glass artificial rumen which would facilitate the fermentation of a number of samples simultaneously.

Other techniques were developed at about this same time. Hungate (1947), Sijpesteijn (1951), and Bryant and Burkey (1953) used pure cultures. Bentley et al. (1954) employed centrifuged unwashed bacterial cells while Cheng et al. (1955) used a washed cell suspension. Gerhardt (1946) was one of the first to describe a completely continuous system. In vivo artificial rumen techniques have been employed whereby the substance to be digested is placed directly into the rumen (Quin, 1943; Hoflund et al., 1948; Balch and Johnson, 1950; Fina et al., 1958) or into semipermeable bags (Quin et al., 1938; McAnally, 1942).

Incubation Techniques

Effect of Temperature

In early experiments Viljoen et al. (1926) reported that the temperature range over which the fermentation of cellulose in vitro could occur was from 43° to 65°C. They found that the microorganisms were able to live at 38°C. but could not ferment. Woodman and Stewart (1932) used incubation periods of 14 days at a temperature of 65°C.

In recent studies the incubation periods have generally ranged from 12 to 48 hours for purified cellulose and 24 to 72 hours for roughages at temperatures of from 38° to 40°C.

Maintenance of Anaerobic Conditions

A considerable amount of variation is found among researchers in the manner in which anaerobiosis is maintained. Most workers have maintained anaerobic conditions using carbon

dioxide, but Marston (1948), Louw et al. (1949), and McNaught (1951) maintained satisfactory anaerobic conditions using nitrogen gas while Walker (1959) used 95 percent carbon dioxide and 5 percent nitrogen gas. Huhtanen et al. (1954) used neither carbon dioxide nor nitrogen gas in the miniature artificial rumen, but the system was closed.

The rate at which the carbon dioxide is supplied to the fermenting mixture varies greatly among researchers. Reports in the literature do not often state the rate of gas flow, but, in general, the rate of bubbling ranges from 60 to 160 bubbles per minute (Burroughs et al., 1950a; Cardon, 1953; Barnett, 1957; Walker, 1959; Donefer et al., 1960; Taylor et al., 1960). Stewart and Schultz (1958) supplied carbon dioxide at the rate of three to six bubbles per minute.

The effect of rapid movement of the reaction mixture has been investigated. Pigden (1955) found that flasks which were not shaken and given an initial carbon dioxide aeration for 2 hours fermented carbohydrate slightly better than flasks which were continuously aerated with shaking or continuously aerated without shaking. The flasks which were continuously aerated but not shaken fermented at least as well as those flasks shaken and continuously aerated.

Walker (1959) reported that the rate of gas bubbling had no effect on the digestibility of the dry matter of the "standard hay," but noted a decrease in digestibility of 7 percent if no gas was bubbled through the fermenting mixture.

Fermentation Vessel

Basically, two main types of fermentation vessels have been employed; those with a semipermeable sac and those without. Wasserman et al. (1952), Huhtanen et al. (1954), Baumgardt (1956), Huhtanen and Elliott (1956), Salsbury et al. (1958), Stewart and Schultz (1958), and Rice et al. (1962) have used the semipermeable sac in their experiments.

Since Burroughs et al. (1950a) developed an all-glass procedure using 500-ml. flasks as fermentation vessels many workers have devised various modifications. Arias et al. (1951), Cardon (1953), Belasco (1954), and Bentley et al. (1954) used 1000-ml. Erlenmeyer flasks. Smaller flasks (125- or 250-ml.) have been employed by Stanley and Kesler (1959), Washburn and Thrall (1961), Dehority (1961), and Baumgardt et al. (1962a). Test tubes or centrifuge tubes of from 50 to 100 ml. capacity have been used by many workers (MacLeod and Brumwell, 1954; Barnett, 1957; Belasco, 1958; Quicke et al., 1959; Donefer et al., 1960; Kruse, 1960; Hubbard, 1960). Stewart and Schultz (1958) used pint jars as the fermentation vessel. Walker (1959) used 500-ml. centrifuge bottles and Bowden and Church (1962a) used centrifuge bottles of 250 ml. capacity. Barnett and Reid (1957a) used a one liter, round-bottom, three-necked flask fitted with two small funnels into the center neck.

Several comparisons of different types of fermentation vessels are found in the literature. Stanley and Kesler (1959) compared 250-ml. Erlenmeyer flasks with 40-ml. test tubes and

found pronounced differences between them, especially as substrate level was increased. As the substrate level was increased from 0.2 g. to 0.8 g. the amount of sugar per 5 ml. of contents increased from 8.2 to 21.1 mg. in the flasks, but the increase was only from 5.3 to 6.7 mg. in the 40-ml. test tube. Dehority (1961) reported nearly identical amounts of cellulose digestion whether 125- or 250-ml. Erlenmeyer flasks were used. Earlier, Pigden (1955) reported only slight differences between 500- and 50-ml. Erlenmeyer flasks.

el-Shazly et al. (1960) studied cellulose digestion, total volatile fatty acid production and ammonia nitrogen production with the three types of apparatus commonly used for fermentation in vitro and found no major differences between them. Walker (1959) and Huhtanen and Elliott (1956) encountered difficulty with rupture of the semipermeable sacs. Baumgardt et al. (1962a) reported no appreciable differences in cellulose digestion or volatile fatty acid patterns in the all-glass system compared to the semipermeable system. Earlier, Gall and Glaws (1951) and Huhtanen et al. (1954) reported similar results but found the microbial population to be more representative of the original rumen inoculum when a dialysing membrane system was used as compared to the all-glass system. Louw et al. (1949) found rate and extent of cellulose breakdown to be slightly better with the semipermeable type. The advantages were very small when particularly active inoculum was taken from cows consuming only hay.

Preparation of Rumen Fluid for Use in In Vitro Systems

Classifications for in vitro rumen procedures have been given by Warner (1956) as follows: (1) Undiluted or slightly diluted rumen liquor incubated with substrate in an impermeable all-glass system; (2) whole rumen liquor diluted to half strength with mineral solution and incubated as in (1); (3) various fractions (washed cell suspensions and centrifuged cells) used in an impermeable system; and (4) rumen liquor, usually whole and undiluted, incubated with substrate in a semipermeable system.

In recent years researchers at the Ohio Station have developed new techniques for the preparation of the inoculum. Bentley et al. (1954) centrifuged the strained rumen juice in a Sharples supercentrifuge and the sediment resuspended in a phosphate buffer at pH 7. Dehority et al. (1957) modified this technique by using only the sediment 1 inch above the celluloid liner. The final modification was described by Johnson et al. (1958). The liquor first expressed from the rumen contents was discarded and the remaining pressed pulp remixed in phosphate buffer and re-expressed. The liquor thus obtained was filtered and then handled as described by Bentley et al. (1954).

Quicke et al. (1959) compared strained rumen juice, phosphate buffer extract, and resuspended ruminal microorganisms and found that strained rumen juice gave the lowest 48-hour cellulose digestion. The method of using resuspended ruminal microorganisms resulted in nearly a 2 percent

increase in cellulose digestion compared to the phosphate buffer extract. These results are somewhat surprising in view of the findings of Johnson et al. (1958) in which 40 percent of the activity of the rumen fluid was lost upon standing at room temperature for 15 minutes. Apparently extremely good temperature control and speed was utilized in transferring and centrifuging in order to maintain the temperature near 39°C.

Barnett (1957) stated that the results most akin to in vivo are obtained in vitro by the use of the chemically untreated feeding stuff and whole, filtered rumen liquor, and Bentley (1959) stated that washing of cells or centrifuging to separate cells from rumen juice seems unnecessary if the cellulose digestion of a forage is under study.

Walker (1959) employed several of the published procedures and concluded that complexity of the procedure was no criterion for obtaining digestibilities in vitro which agreed closely with conventional in vivo determinations. Van Dyne (1962) reported that inoculum prepared by simply straining rumen contents through several layers of cheesecloth gave as high and as uniform cellulose digestibility values as did more elaborate procedures of inoculum preparation.

Amount of Inoculum Used for In Vitro Fermentations

The amount of strained rumen fluid used to inoculate the fermentation flasks has been quite variable among research

workers using this technique. For example, Cardon (1953)
used 600 ml. of rumen fluid while Bowden and Church (1962a)
used 6 ml. of fluid obtained from the lower regions of a
flask after the contents had been allowed to settle for 30
minutes. Barnett (1957) used 5 ml. of strained rumen fluid.

In order to obtain relative comparisons from the litera-
ture, it is necessary to calculate the percentage of rumen
fluid in the total volume of contents. Some values deter-
mined from the information in the literature are 6 (Bowden
and Church, 1962a), 8 (Walker, 1959), 10 (Barnett, 1957), 20
(Meites, 1951), 23 (Kruse, 1960), 40 (Hershberger et al.,
1959), 40 (Clark and Mott), 40 (Burroughs et al., (1950a), 45
(Baumgardt et al., 1962a), 50 (Belasco et al., 1958), 62
(Hubbard, 1960), and 98 percent (Cardon, 1953). Thus, it is
evident that no well defined pattern is followed by the
various investigators.

Gray, Pilgrim, and Weller (1951) compared cellulose
digestion using either 750 or 25 to 50 ml. of rumen fluid.
When small amounts of inoculum were used less than 10 percent
of the cellulose of wheaten hay was digested in 48 to 72
hours while 34 to 44 percent of the cellulose was digested
in 48 hours when the larger amount of rumen fluid was used.
A portion of the discrepancy noted may have been caused by
the dilution of the small volume of inoculum with water
(amounts added not stated) whereas no water was added to
the large inoculum.

Meites (1951) and Cheng et al. (1955) reported enhanced cellulose digestion when larger amounts of rumen fluid were used. Church and Petersen (1960) varied the rumen liquor level from 20 to 120 ml. and obtained nearly linear increases in percent cellulose and dry matter digestion.

Walker (1959) found that within wide limits the volume of rumen juice did not affect percentage dry matter digestibility in a 72-hour fermentation, but Tilley et al. (1960) obtained decreases in dry matter digestibility when the volume of rumen liquor incubated with a given weight of substrate was reduced.

Baumgardt (1956) used 10 or 20 ml. of rumen fluid to prepare 10 ml. of inoculum (washed cell suspensions) and noted that cellulose digestion was not significantly affected if days were considered random. If days were considered fixed, the difference in cellulose digesting capacity was highly significant in favor of the 20 ml. of rumen fluid.

Time of Collection of Rumen Fluid

The time at which the rumen fluid is obtained in relation to feeding has received little attention as most in vitro fermentations have been conducted with the inoculum being obtained at a more or less uniform time after feeding.

Baumgardt (1956) found rumen fluid drawn before feeding (14 hours since previous feeding) digested a significantly greater amount of cellulose than rumen fluid obtained 2 hours after feeding. Digesting ability of rumen fluid drawn 4 and

6 hours after feeding was higher than at 2 hours after feed-
ing and slightly lower than before feeding. Although signifi-
cance was obtained only two samples were used for each
determination. McBee (1953) stated that for consistent results
all samples must be taken from the same part of the rumen and
thus he considers the use of the stomach tube to be unsatis-
factory for sampling. He also found large differences
between whole rumen fluid, the solid fraction, and the liquid
fraction in the ability to ferment. Nottle (1956) observed
significantly different bacterial concentrations at 0, 3, and
6 hours after feeding, but apparently the concentration of
protozoa are unaffected before or 1 hour after feeding at
various locations in the rumen (Purser and Moir, 1959).
Pearson and Smith (1943) found no significant difference in
the ability of rumen liquor to convert urea to ammonia whether
removed 1 hour after feeding or after a 16-hour fast.

Effect of Variable Water Intake on Cellulose Digestion

From the reports of most workers involved with in vitro
fermentations it can be noted that many follow the procedure
of withholding water for a certain period of time before
inoculum is obtained while others disregard water consumption
of their donor animals.

Balch and Johnson (1950) and Balch (1950) found that a
low dry matter content of the rumen ingesta favored the rapid
breakdown of cellulose. The rate of breakdown of the dry
matter of hays placed in the rumen in silk bags was faster

in the ventral sac of the rumen (which is more liquid) than in the dorsal sac. Miles (1951) noted that the apparent digestion of dry matter and cellulose was more complete in the ventral part of the rumen than in the dorsal.

Huhtanen and Elliott (1956) studied the effect of dilution of rumen samples in the laboratory by the addition of water to fermentation flasks and found that dilutions up to one-fifth of the original fluid had no effect on cellulose digestion but further dilutions resulted in a gradual decline in cellulose digestion.

Effect of Aeration of Rumen Fluid Upon Cellulose Digestion

The effect of contamination of the rumen fluid with air on its subsequent ability to digest cellulose in vitro has received considerable study, and the results obtained have been quite variable.

Fina et al. (1958) reported that digestion coefficients were considerably lower if the rumen fluid was not collected under strict anaerobic conditions and if oxygen entered the outlet tubing at any time cellulose decomposition ceased completely. Wasserman et al. (1952) and MacLeod and Brumwell (1954) maintained near anaerobic conditions during collection of the rumen fluid by bubbling carbon dioxide into the collecting flask.

Most workers do not take special precautions to maintain complete anaerobic conditions during sampling but attempt to handle the rumen fluid as quickly as possible until it is again placed under anaerobic conditions.

Several interesting experiments have been conducted to determine the effect of aeration of rumen fluid upon its subsequent ability to digest cellulose. Hoflund et al. (1948) reported that cotton threads emersed in ruminal ingesta were digested to the same extent when the tubes were placed under anaerobic conditions or remained in the open, but cellulose digestion was completely inhibited when air was continuously bubbled through the mixture. Huhtanen and Elliott (1956) dialyzed rumen fluid for 4 hours in a refrigerator against 10 volumes of distilled water with no loss of cellulose digesting activity.

Hungate et al. (1960), using a manometric technique, found that samples of rumen fluid held at room temperature for 45 and 85 minutes were 20 percent slower in activity during the first 30 minutes of the run and slightly higher during the remaining 40 minutes than samples incubated immediately. They concluded that apparently oxygen does not penetrate deeply enough into large samples of rumen fluid held at room temperature to kill the anaerobic microorganisms. Normal activity was resumed when favorable temperatures were restored.

Johnson et al. (1958) found that only about 60 percent of the cellulolytic activity of the inoculum (second extraction inoculum) remained after 15 minutes aeration at room temperature and at the end of 30 minutes cellulose digestion had ceased, but when the inoculum was held at 37°C. the activity was decreased only 23 percent after 2 hours. Aeration beyond 2 hours caused a rapid decrease in activity and the ability to digest cellulose was lost after 6 hours.

Meites (1951) stirred rumen liquor for as long as 30 minutes in air with no subsequent loss of cellulolytic activity. Rumen liquor allowed to stand for 48 hours at 40°C. in the presence of toluene lost nearly all of its capacity to digest cellulose.

Comparison of Digestibility Coefficients as Determined by In Vitro and In Vivo Techniques

Successful in vitro procedures employed in forage evaluation depend upon the accuracy with which they can be used to predict the value of the forage to the animal.

In 1932, Woodman and Stewart attempted to correlate in vitro fermentation data with fiber digestion in conventional trials but were unable to obtain any consistent relationships between the two.

Baumgardt et al. (1958) reported a correlation coefficient of .81 between in vitro and in vivo methods for estimating the dry matter digestibility of 11 forages. The 11 forages included eight grass and three alfalfa hays. The correlation coefficient of in vitro cellulose digestion with in vivo cellulose digestion was .50 for the 11 forages and .90 (P<.01) for the eight grass forages only.

Hershberger et al. (1959) reported a correlation coefficient of .97 in a comparison of in vivo and in vitro cellulose digestibility of 35 forages.

Asplund et al. (1958) and Clark and Mott (1960) determined dry matter digestibility in vitro and in vivo of a number of forages and found highly significant correlations of .71 and .77, respectively.

Walker (1959) and Reid et al. (1959) reported close agreement between dry matter digestibilities in vitro and in vivo, and Barnett (1957) found a similar relationship between the digestibility of cellulose and that of crude fiber in silage as determined conventionally with sheep.

Baumgardt et al. (1962b) reported correlation coefficients of .84 and .81 for cellulose digestibility in vitro and digestibility of organic matter and dry matter in vivo, respectively.

Bowden and Church (1962b) reported a number of correlations of in vitro digestibility of dry matter and cellulose with certain chemical components and in vivo digestion coefficients of tall fescue. The correlation coefficient (pooled data from four years) between in vitro dry matter and in vivo dry matter digestibility was .73 (P<.01). The correlation coefficient between in vitro cellulose and in vivo cellulose digestibility was .89 (P<.01) for only one year's data.

PART I.   DEVELOPMENT OF A SUITABLE TECHNIQUE FOR
IN VITRO FERMENTATIONS

Experimental Procedure

An attempt was made to develop a simple technique for determining in vitro dry matter and cellulose digestibility and to determine the influence of several variables upon cellulose digestion. The general procedure described here will apply to all experiments, and specific details will be presented where required.

Three rumen-fistulated steers were used as donors of rumen fluid. One set of twin Angus steers (steers 1 and 2) were penned together and allowed to eat pelleted, mature prairie hay ad libitum plus 2 pounds of cottonseed meal per head daily. Steer 3 (Hereford) was allowed good quality prairie hay, ad libitum, and 3 pounds daily of a pelleted complete ration containing cottonseed meal, dehydrated alfalfa meal, and milo.

The Angus steers were fed the protein supplement in the morning. Inoculum was obtained 6 hours after feeding, squeezed through eight layers of cheesecloth into a warmed (39°C.) thermos jug, and brought to the laboratory. In the laboratory, the rumen fluid was strained through eight layers of cheesecloth and added to fermentation vessels which contained nutrient solution. The composition of the nutrient

solution (table I) was similar to that described by Cheng
et al. (1955).

The fermentation vessels were placed into the water bath
(39°C.), attached separately to carbon dioxide gas outlets
until the contents of all vessels were under anaerobic condi-
tions, after which, several (usually four) vessels were
connected in series to a single carbon dioxide gas outlet.
In this manner the gas flow could be regulated more uniformly
between groups of vessels and also within the vessels in the
series. Gas flow was approximately 175 bubbles per minute.

Residual cellulose was determined by the method of
Crampton and Maynard (1938) with slight modifications. These
modifications included use of glacial acetic acid instead of
80 per cent acetic acid and boiling of the cellulose-containing
substance in a water bath instead of refluxing. Amount of
acetic and nitric acid used exceeded the amount proposed by
Crampton and Maynard (1938) because larger samples were
analyzed. Proportions of the acids used were not changed.

Forage cellulose determinations were made upon the
samples which had previously been dried for dry matter
digestibility determinations. The acids were added to the
tubes containing the undigested forage, heated with occasional
stirring in a boiling water bath for 30 minutes, allowed to
cool, centrifuged, and the supernatant discarded. The resi-
dues were transferred into Gooch crucibles lined with asbestos,
vacuum filtered, washed with hot ethanol and hot benzene, and
finally, with ether. After the ether vapor had escaped, the

crucibles were oven-dried at 100°C., cooled in a desiccator,
weighed, ashed for 4 hours in a muffle furnace (600°C.),
cooled in a desiccator, and reweighed. Loss in weight was
considered the amount of cellulose remaining after the
incubation.

TABLE 1

COMPOSITION OF NUTRIENT SOLUTION

Ingredient	Grams per liter
Na_2HPO_4	0.631
KH_2PO_4	0.303
$NaHCO_3$	5.250
KCl	0.750
NaCl	0.750
$MgSO_4$	0.225
$CaCl_2$	0.075
$FeSO_4 \cdot 7H_2O$	0.015
$MnSO_4$	0.008
$ZnSO_4 \cdot 7H_2O$	0.008
$CuSO_4 \cdot 5H_2O$	0.004
$CoCl_2 \cdot 6H_2O$	0.002
Urea	2.000

In all experiments, either the dry matter or cellulose
(or both) content of the rumen fluid was determined. Two or
three samples of rumen fluid were centrifuged at 450 x g. for
5 minutes. The amount of cellulose in 120 ml. of strained
and centrifuged rumen fluid varied from about 50 to 150 mg.

This is within the range reported by Baumgardt et al. (1962b).
Dry matter content of 120 ml. of strained and centrifuged
rumen fluid ranged from 0.6 to 1.5 g. This is slightly less
than reported by Baumgardt (1956). Average cellulose and dry
matter content of each day's rumen fluid were used in the for-
mulae for calculating the percent digestion. The amount of
dry matter in the nutrient solution was determined by evap-
orating 40 ml. of the solution to dryness in an oven heated
to 100°C.

Forages were oven-dried at 100°C., allowed to cool in a
desiccator, 2-gram samples weighed on a Mettler electronic
balance and placed into the fermentation vessels. Early in
these studies, pH was determined. The average pH of the rumen
fluid was 6.4. Several researchers (Meites et al., 1951;
Cheng et al., 1955; Kamstra et al., 1958; Hershberger et al.,
1959) have indicated that the optimum pH is 6.8 to 7.0 and
have maintained the pH within this range during the fermenta-
tion. In these studies it seemed desirable not to elevate the
pH and immediately subject the microorganisms to an environ-
ment in which the pH was different from that found in the
rumen. Buffering capacity was apparently adequate as final
pH values were only slightly lower than initially. Kitts and
Underkofler (1954) and Stanley and Kessler (1959) indicated
that the optimum pH of cellulase preparations was near 6.0.
Similar cellulose digestibilities were obtained by Meites
et al. (1951) in the pH range of 4.5 to 7.3.

The length of the fermentations was 24 hours for
Solka-floc digestion and 24 or 48 hours for forage dry matter

and cellulose digestion. At the end of the fermentation the
sides of the fermentation vessel were washed with a small
stream of water, the vessels centrifuged at 450 x g. for 5
minutes, and the contents transferred to a tared 50-ml.
centrifuge tube. These tubes were centrifuged, the super-
natant discarded, and the residue oven-dried at 100°C.,
cooled in a desiccator, and weighed. The formula for obtaining
percent dry matter digestion is as follows:

Percent dry matter digestion $= \dfrac{a + b + c - d}{a + b + c} \times 100$

a = initial weight of the forage
b = dry matter content of the rumen fluid
c = dry matter content of the nutrient solution
d = ending weight of the residue

The formula for calculating cellulose digestion when
Solka-floc was the substrate is as follows:

Percent Solka-floc digestion $= \dfrac{a + b - d}{a + b} \times 100$

a = initial weight of Solka-floc
b = cellulose content of the rumen fluid
d = amount of cellulose left at the end of the incubation

Forage cellulose digestion was calculated by the following
formula:

Percent cellulose digestion $= \dfrac{a + b - d}{a + b} \times 100$

a = amount of cellulose in the forage
b = amount of cellulose added by the rumen fluid
d = amount of cellulose remaining at the end of the
    incubation

The cellulose content of the oven-dried forage was
determined on four, 2-gram samples by the method previously
described.

Results and Discussion

Several preliminary experiments were conducted in order to find the type of fermentation vessel best suited for the in vitro fermentations. The method in use at this station (Hubbard, 1960; Harbers, 1961) was first employed in a uniformity trial. Solka-floc levels of 100 and 120 mg. were used with either 10, 15, or 20 ml. of rumen fluid. The fermentation vessels were 50-ml. centrifuge tubes and each contained 5 ml. of double strength nutrient solution.

Data presented in table 2 show that significantly ($P<.05$) more Solka-floc was digested when 20 ml. of rumen fluid was used in the fermentation than when 10 ml. was used. There was no significant difference in cellulose digestion due to level of substrate. Four observations were included in each mean and the range of cellulose digestion was very large, as indicated by the standard errors. It was observed that considerable material was carried from the first tube to the others in the series. This was probably the result of a large volume in the tube and the rate of gas flow which could not be reduced to a rate slow enough to prevent any carry-over.

It was apparent that larger fermentation vessels must be employed if small treatment differences were to be detected. Gray et al. (1951) indicated that large amounts of rumen fluid must be used in order to reproduce rumen fermentation in vitro. Most workers have used larger amounts of rumen fluid than 10 to 20 ml. The proportions of the components remained nearly the same and larger amounts of rumen fluid

and larger fermentation vessels were used in subsequent studies. Since urea utilization studies involving twin steers were to be initiated, it was also decided to determine if differences existed between the fermenting ability of rumen fluids obtained from identical twins.

TABLE 2

CELLULOSE DIGESTIBILITIES WHEN 50-ML. CENTRIFUGE TUBES
WERE USED AS THE FERMENTATION VESSELS

Substrate[1] level (mg.)	Rumen fluid (ml.)		
	10	15	20[2]
	%	%	%
100	20.9±2.0[3]	41.4±9.4	49.6±13.3
120	30.7±9.7	37.6±3.9	55.8±6.1
x̄	25.8±5.8	39.5±6.6	52.7±9.7

1. Solka-floc.
2. Digestion with 20 ml. of rumen fluid significantly greater ($P < .05$) than with 10 ml.
3. Standard error of the mean.

In the second preliminary trial, identical twin Angus steers receiving the same feeds supplied the inoculum samples, and 250-ml. Erlenmeyer flasks were used as the fermentation vessels. The amount of Solka-floc was increased to 1 gram and incubated with 150 ml. of rumen fluid and 50 ml. of double strength nutrient solution.

The results of this experiment are summarized in table 3. The repeatability of results obtained within a run (four flasks per run) was considerably better than that obtained when the

fermentations were carried out in 50-ml. centrifuge tubes and was considered satisfactory. Differences between the twin steers in ability of their inoculum to ferment cellulose were highly significant (P<.01). Church and Petersen (1960) reported significant differences in digesting capacity between the rumen fluids of twin steers in some of their experiments. Steer differences may have been influenced to an unknown degree because the fistulae were of different types, thus, sampling was slightly different. It is believed, however, that sampling errors were slight, if present, since the same area of the rumen of each steer was sampled. More important is the fact that these steers were at least 7 years old and, therefore, cannot be considered any more alike in their rumen population than any other two steers fed alike.

Since the base of the Erlenmeyer flasks was quite large, gas flow was increased in order to prevent settling of materials in certain areas. Round-bottom centrifuge bottles of 250 ml. capacity were used as the vessels in another fermentation. The results of this experiment are shown in table 4. Gas flow was reduced below that used in the previous experiment. Steers 1 and 2 (twins) retained their relative position in their ability to digest cellulose in vitro. Steer 3, fed a different ration, was intermediate. The within-run variation was reduced slightly by using the centrifuge bottle. In this trial, the volume of rumen fluid was reduced to 120 ml. and the Solka-floc reduced to 800 mg.

No changes were made in the volume of nutrient solution. This procedure was then adopted for all subsequent experiments.

TABLE 3

CELLULOSE DIGESTIBILITIES WHEN 250-ML. ERLENMEYER FLASKS
WERE USED AS THE FERMENTATION VESSELS

Steer	Run	Cellulose digestion (%)[1]
1	1	54.3 ± 1.42[2]
	2	56.9 ± 2.39
	3	48.9 ± 0.97
	$\bar{x}$	53.4 ± 1.59
2[3]	1	67.8 ± 1.46
	2	66.6 ± 1.18
	3	71.4 ± 1.15
	$\bar{x}$	68.3 ± 1.26

1. Four flasks in each run.
2. Standard error of the mean.
3. Significant (P<.01) difference in cellulose digestion between steers.

TABLE 4

CELLULOSE DIGESTIBILITIES WHEN 250-ML. ROUND-BOTTOM CENTRIFUGE
BOTTLES WERE USED AS THE FERMENTATION VESSELS

Steer	Cellulose digestion (%)[1]
1	67.1 ± 1.35[2]
2	84.4 ± 0.65
3	74.0 ± 0.44

1. Average of four observations.
2. Standard error of the mean.

PART II.   EFFECT OF SEVERAL VARIABLES UPON CELLULOSE AND
DRY MATTER DIGESTION IN VITRO

Trial I.   Effect of Dilution of Rumen Fluid Upon
Cellulose Digestion

Experimental Procedure

        Rumen fluid was obtained and handled as described in
Part I.   Rumen fluid was added to the bottles which contained
40 ml. of nutrient solution and .8 g. of Solka-floc.   To the
control bottles 120 ml. of rumen fluid was added.   Dilutions
were made by replacing 20, 40, 60, 80, and 100 ml. of the
rumen fluid with an equal volume of water.   In another trial,
the donor animal was fed as usual but not allowed to drink
until a sample of rumen fluid had been obtained 6 hours later.
After the steer had consumed water, rumen fluid was again
obtained.

Results and Discussion

        The results obtained with different dilutions of rumen
fluid are presented in table 5.   Since 40 ml. of nutrient
solution was added to all bottles, the original rumen fluid
was initially diluted by 25 percent.   Cellulose digestion
with this rumen fluid was 62.0 percent.   Cellulose digestion
with dilutions of one-half water and one-half original rumen
fluid was slightly higher than with the original inoculum.
When two-thirds of the original inoculum had been replaced

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with water cellulose digestion decreased (P<.05), and
further dilution caused further decrease. Although the
percent digestion was considerably higher, the trend of
these results agree very closely with those of Huhtanen and
Elliott (1956).

TABLE 5

EFFECT OF DILUTION OF RUMEN FLUID ON CELLULOSE DIGESTION

Treatment[1]	Rumen fluid:water (ml.)	Cellulose digestion (%)
1	120:0	62.0 ± 0.76^{2}
2	100:20	64.6 ± 0.69
3	80:40	62.9 ± 0.82
4	60:60	63.4 ± 1.39
5	40:80	49.3 ± 3.75
6	20:100	32.0 ± 1.19

1. Treatments 1, 2, 3, and 4 significantly different
    from 5 and 6 (P<.05). Treatment 5 significantly
    different from 6 (P<.05).

2. Standard error of the mean computed on a within
    replication basis (two replicates).

The effect of dilution of rumen contents with water
upon cellulose digestion are shown in table 6. Although
differences were not large, cellulose digestion was signifi-
cantly greater (P<.01) with rumen fluid obtained from the
steer after drinking. The reasons for this slight increase
are not known. Other workers (Balch and Johnson, 1950;
Balch, 1950; Miles, 1951) noted higher cellulose digestion
in that region of the rumen which is the more liquid. The

increase in cellulose digestion cannot be explained by a
later withdrawal of the inoculum, and thus less time lapse
before anaerobic conditions were restored, as the time of
the delay from one sampling to another was taken into account
(by a similar delay) before the inoculum was added to the
substrate. The inoculum remained in the thermos during this
time.

TABLE 6

EFFECT OF DILUTION OF RUMEN CONTENTS WITH WATER UPON
CELLULOSE DIGESTION

Day	Cellulose digestion (%)[1]	
	Before drinking	After drinking[2]
1	70.9 ± 0.81[3]	73.2 ± 0.28
2	64.0 ± 1.13	72.1 ± 0.71
$\bar{x}$	67.4 ± 0.97	72.6 ± 0.50

1. Average of four observations.
2. Significantly greater (P<.01) amount of cellulose
   digested after the animal had consumed water.
3. Standard error of the mean.

The results of these dilution studies suggest that cellu-
lose digestibility in vitro may be slightly influenced by
concentration of the microorganisms in the rumen fluid.
Since a relatively large amount of water was added, both in
the laboratory and by the animal, the differences obtained
might be considered to be extreme, and thus, normal consump-
tion of water by the animal causing slight fluctuations in
the number of microorganisms per volume of rumen fluid from

day to day would not seriously affect the results obtained.
Apparently, as long as the number of microorganisms are not
drastically reduced, in vitro digestion is not hindered.
Results presented in table 6 also indicate that for most
consistent day-to-day results some dilution of the rumen
fluid is beneficial. Cellulose digestion by the inoculum
obtained after drinking was nearly the same for both days
but the digestion of cellulose by inoculum obtained before
the steer consumed water was significantly ($P<.01$) different
between days.

These results indicate that the practice of withholding
water from the donor animal for a period of time before
sampling increases, rather than decreases, the day-to-day
variation of cellulose digestion in vitro.

Trial II.   Effect of Rate of Carbon Dioxide Flow Upon Cellulose Digestion

Experimental Procedure

Three rates of carbon dioxide flow were employed. The
contents of one set of bottles were agitated by bubbling
carbon dioxide through the mixture at a rate of about 150 to
175 bubbles per minute and through another set at a rate
which caused considerable agitation of the contents. The
latter flow rate was estimated to be approximately 300
bubbles per minute. The third set of bottles was initially
flushed for 30 minutes with carbon dioxide, after which no
more gas was passed through the mixture.

Results and Discussion

Results are shown in table 7. Cellulose digestion was 4.1 percent higher when the contents were continuously agitated at the slower rate as compared to the rapid rate of carbon dioxide flow. This difference was not significant. Stopping the carbon dioxide flow after 30 minutes produced significantly less (P<.01) cellulose digestion. These results indicate that small variation in rate of gas flow would not seriously affect the degree of cellulose digestion. Settling of the substrate occurred in those flasks which were not continuously flushed with carbon dioxide, thus exposing less of the cellulose to the microorganisms and the nutrients supplied by the nutrient solution and the rumen fluid. Walker (1959) noted only a slight decrease in dry matter digestion when no gas was bubbled through the fermenting mixture. Pigden (1955) found that non-shaken, continuously aerated flasks fermented as well as continuously aerated, shaken flasks. Apparently, sufficient agitation to keep the solid contents of the bottle suspended is all that is necessary.

TABLE 7

EFFECT OF RATE OF FLOW OF CARBON DIOXIDE UPON
CELLULOSE DIGESTION

Treatment	Cellulose Digestion (%)
Continuous aeration (rapid bubbling)	75.7 ± 1.15[1]
Continuous aeration (normal bubbling)	79.8 ± 0.88
Normal aeration (stopped after 30 min.)	58.5 ± 1.35**

1. Standard error of the mean.
** P<.01

Trials III and IV. Effect of Animal Diet, Exposure to Air, and Volume of Nutrient Solution Upon Cellulose Digestion

Experimental Procedure

The effect of volume of nutrient solution was studied in combination with other variables in both trials. In trial III the effect of two levels of nutrient solution and three exposure times on cellulose digestion in vitro was studied, and in trial IV, the effect of animal diet upon in vitro digestion of the dry matter and cellulose of two different quality hays was studied in combination with two levels of nutrient solution.

The same steer supplied the inoculum for all fermentations. Solka-floc was the substrate in trial III and the animal was fed as described in the experimental procedure section of Part I. In trial IV the dry matter and cellulose of a high-quality alfalfa hay and of a low-quality, mature and weathered prairie hay were fermented in vitro by inoculum obtained from the steer fed the alfalfa hay and, after a two-week interval, the prairie hay. The steer had access to salt when fed the alfalfa hay and to a salt and bonemeal mixture (2:1) when fed the prairie hay. Two pounds of soybean meal was fed daily to supplement the prairie hay. The hays used as substrates were ground through a 40-mesh screen in a Wiley mill. Two fermentations were conducted with triplicate bottles in each fermentation.

All fermentations were of 24 hours duration. The usual procedure was followed with the exception that 100 ml.

of rumen fluid was used. The donor animal was fed the hays free choice and rumen fluid was collected at a constant time each afternoon. Since differences between runs were small the results of both runs were combined in the analysis.

Exposure to air was of 0, 20, and 40 minutes duration. The time at which the first set of bottles was placed under anaerobic conditions was considered to be zero time. The other sets of bottles were put into the water bath 20 and 40 minutes later. The rumen fluid was kept in a 2000-ml. Erlenmeyer flask and swirled every few minutes in order to incorporate air. The temperature of the rumen fluid was noted at each time a set of bottles was placed into the water bath.

Results and Discussion

The results of exposure of rumen fluid to air are shown in table 8. No significant differences were found between zero and 20 minutes exposure to air but significantly less (P<.05) cellulose was digested after the inoculum had remained open to the air for 40 minutes. The temperature of the rumen fluid dropped from 38° to 34°C. during the first 20 minutes and to 32°C. the last 20 minutes. Smaller and nonsignificant differences were noted in a second trial in which the only variable was time of exposure to air. These findings are in agreement with those of Hoflund et al. (1948), Huhtanen and Elliott (1956), and Hungate et al. (1960). Johnson et al. (1958) noted greater losses in

cellulolytic activity after comparable times of exposure to
air and that these losses were considerably reduced if the
inoculum was kept warm. Apparently little deterioration of
rumen fluid occurs after short exposure to air. The results
of these experiments substantiate the supposition of Hungate
et al. (1960) that in short times of exposure, air does not
penetrate deeply enough into large amounts of rumen fluid to
kill the microorganisms. Normal activity is resumed when
favorable conditions are restored.

TABLE 8

EFFECT OF AERATION AND VOLUME OF NUTRIENT SOLUTION
UPON CELLULOSE DIGESTION

Nutrient solution (ml.)	Cellulose digestion (%) Aeration time (min.)		
	0	20	40[1]
40	71.1 ± 0.67[2]	67.8 ± 0.55	66.9 ± 1.40
50[3]	80.6 ± 0.20	78.1 ± 0.39	75.2 ± 0.22
$\bar{x}$	75.8 ± 0.44	73.0 ± 0.47	71.0 ± 0.81

1. Significant difference (P<.05) in cellulose diges-
tion between zero and 40 minutes aeration.
2. Standard error of the mean.
3. Significantly greater (P<.01) amount of cellulose
digested with 50 ml. of nutrient solution.

Data presented in tables 8 and 9 show the effect of
amount of nutrient solution upon the digestion of Solka-floc,
forage dry matter, and forage cellulose. Fifty ml. of
nutrient solution promoted significantly greater (P<.01)
Solka-floc digestion than 40 ml. (table 8). The statistical

TABLE 9

EFFECT OF ANIMAL DIET AND VOLUME OF NUTRIENT SOLUTION UPON THE DRY MATTER DIGESTIBILITY OF GOOD AND POOR QUALITY FORAGES

Forage fed to steer	Sub-strate	Nutr. soln. (ml.)	Digestion coefficients (%)					
			Dry matter			Cellulose		
			Run 1[1]	Run 2	$\bar{x}$	Run 1	Run 2	$\bar{x}$
Alfalfa	Alfalfa	40	45.5±0.48[2]	47.6±0.02	46.6±0.25	53.2±0.59	51.4±0.45	52.3±0.52
		60	48.3±0.29	50.4±0.14	49.4±0.22	60.7±1.94	52.4±0.52	56.6±1.23
	Weathered prairie	40	33.3±0.18	30.5±0.16	31.9±0.17	37.5±0.94	23.7±0.78	30.6±0.86
		60	36.4±0.05	33.8±0.21	35.1±0.13	37.6±2.07	24.7±0.34	31.2±1.20
Weathered prairie	Alfalfa	40	41.0±0.64	39.7±0.79	40.4±0.72	42.5±3.00	51.3±1.10	46.9±2.05
		60	44.9±0.64	43.0±0.32	44.0±0.48	52.2±1.01	56.7±0.36	54.4±0.68
	Weathered prairie	40	31.0±0.95	31.3±0.66	31.2±0.80	31.3±3.21	42.2±0.73	36.8±1.97
		60	35.0±0.91	35.4±0.03	35.2±0.48	34.3±0.87	43.4±0.39	38.8±0.63

1. Triplicate bottles in each run.
2. Standard error of the mean.

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analysis of trial V is shown in table 10. Significantly more forage dry matter (P<.01) and cellulose (P<.05) were digested when the larger volume of nutrient solution was used in the fermentations. These differences, although significant, were not large. The average increase in dry matter and cellulose digestibility was 3.4 and 3.6 percentage units, respectively when 60 ml. of nutrient solution was used instead of 40 ml. (table 9). Thus, either volume would be satisfactory for use in in vitro fermentations, but for best results the volume used must remain constant within an experiment.

TABLE 10

SUMMARY OF THE ANALYSIS OF VARIANCE FOR EXPERIMENT IN
WHICH EFFECT OF ANIMAL DIET AND VOLUME OF
NUTRIENT SOLUTION WAS STUDIED

Source of variation	Degrees of freedom	Mean squares	
		Dry matter digestion	Cellulose digestion
Diet (D)	1	114.0**	29.5
Substrates (S)	1	1640.3**	3982.2**
Nutrient solution	1	138.0**	156.3*
D x S	1	90.3**	341.3**
D x M	1	2.3	17.4
S x M	1	0.7	63.9
D x S x M	1	0.0	2.2
Error	40	1.24	32.4

** P<.01
 * P<.05

The effect of animal diet upon forage dry matter and
cellulose digestibility has received little attention by
most workers in this field. Warner (1956), Baumgardt (1956),
Asplund et al. (1958), and Taylor et al. (1960) indicated
that the inoculum should be supplied by animals receiving
the same forage as the one under study, whereas Barnett (1957),
Quicke et al. (1959), and Walker (1959) found that in vitro
digestion of forages was not affected by animal diet.
Van Dyne (1962) noted that animal diet significantly influ-
enced Solka-floc and forage digestion.

Dry matter digestion was significantly greater (P<.01)
when the inoculum was obtained from the steer fed alfalfa
hay compared to when the same steer was fed prairie hay, but
there was no significant difference in cellulose digestion.
Highly significant differences (P<.01) in dry matter and
cellulose digestion (percent and total) were found between
forages. Considering the differences in quality of the for-
ages involved, these results were to be expected. A graph-
ical representation of the data in table 9 is shown in figure 1.
Each value is the average of 12 observations.

A significant interaction was found between roughage fed
to the steer and roughage fermented for both dry matter and
cellulose digestion. The interaction (in the case of dry
matter digestion) was caused by the increase in dry matter
digestibility of the alfalfa hay over the prairie hay when
the inoculum was obtained from the steer fed alfalfa hay,
whereas the digestibility of the dry matter of the prairie
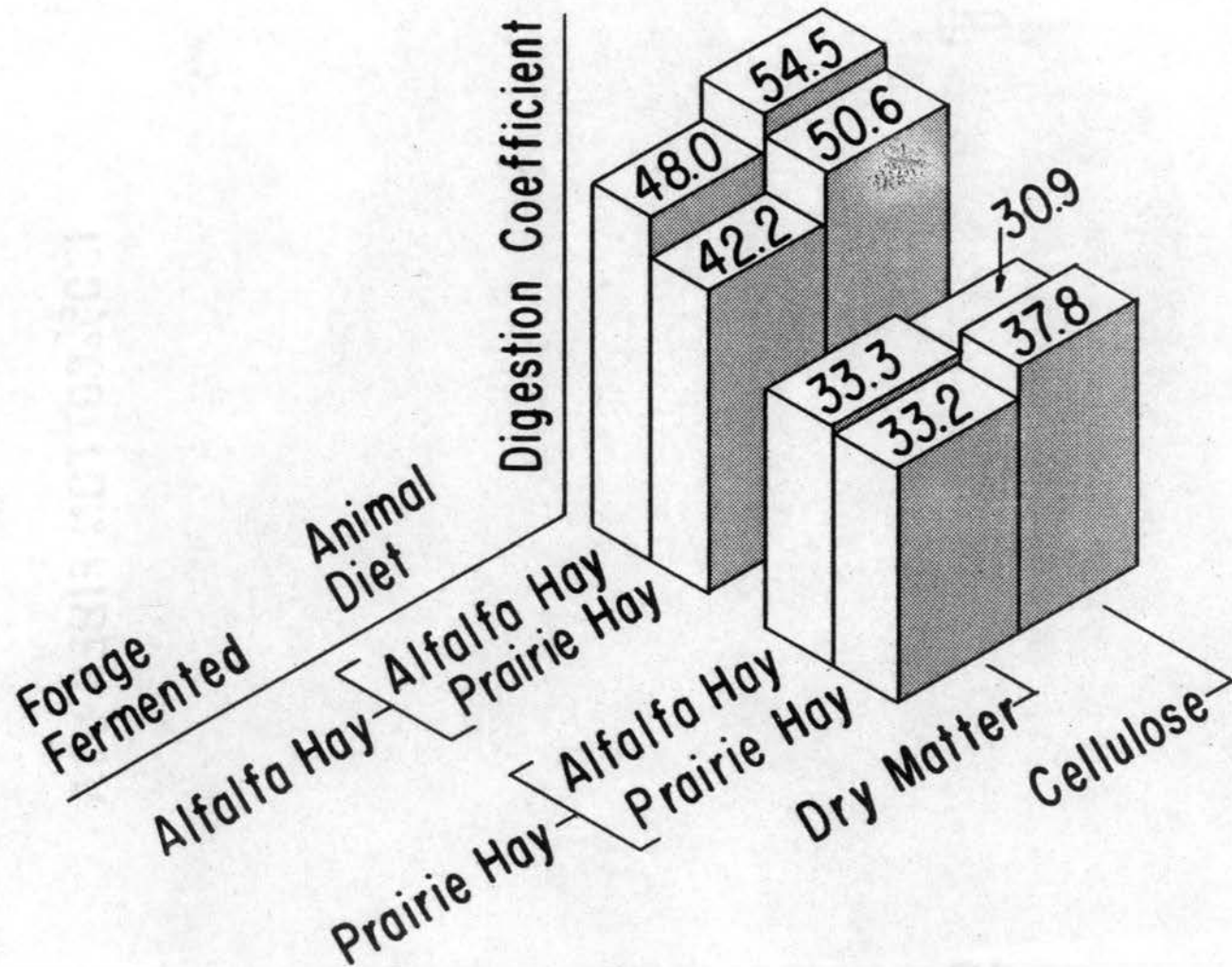
Figure 1. Comparison of Percent Dry Matter and Cellulose Digestion of Alfalfa Hay and Prairie Hay _In Vitro_ by Inoculum from Steer Fed Alfalfa Hay or Prairie Hay

hay was about the same regardless of the source of rumen fluid. In other words, the _in vitro_ digestibility of the dry matter of the prairie hay was much less affected by animal diet than was the dry matter digestibility of the alfalfa hay.

Cellulose in the alfalfa hay was digested to a greater degree when the inoculum was supplied by the steer consuming the alfalfa hay; cellulose digestibility of the prairie hay was greatest when the rumen fluid was obtained from the same steer fed prairie hay. A large part of this interaction was apparently due to the low cellulose digestibility of the prairie hay in run 2 when the inoculum was obtained from the steer fed alfalfa hay.

It is interesting to note that forage cellulose digestion coefficients from day to day were less repeatable compared to a relatively high repeatability of day-to-day dry matter digestion coefficients (table 9). Similar results have been noted by Bowden and Church (1962a) for various tall fescue samples. They have suggested an interaction between the substrate and the inoculum which results in a greater or lesser digestibility of some fraction of the dry matter at the expense of cellulose.

These data indicate that for the most meaningful results the forage which is fermented _in vitro_ should be incubated with inoculum obtained from the donor animal receiving the same forage. If this is not possible, a forage similar to the one which is digested _in vitro_ should be fed. It is

evident from these data that the inoculum used exerts a
large influence upon the dry matter and cellulose digesti-
bility of the forage in the _in vitro_ system.

PART III. EVALUATION OF _IN VITRO_ FERMENTATIONS

Trial V. Comparison of _in vitro_ dry matter and cellulose
digestion with _in vitro_ volatile fatty acid production

Experimental Procedure

Upon completion of trial IV, rumen fluid was obtained to
inoculate several bottles, each containing 2 grams of finely
ground, dried alfalfa hay. One-hundred ml. of strained rumen
fluid and 40 ml. of nutrient solution were used. Two bottles
were randomly selected and removed at various times during a
48-hour fermentation and handled as previously described.
After the initial centrifugation, the supernatants from the
two bottles were combined and diluted to 500 ml. with water,
a small aliquot removed, placed in a stoppered 50-ml.
Erlenmeyer flask, and frozen. Dry matter and cellulose
digestibilities were determined on the residues at each
stage of the fermentation. This same procedure was used when
the weathered prairie hay was fed to the steer and the inoc-
ulum used to ferment 2 grams of the prairie hay _in vitro_
during a 48-hour fermentation. Samples of the inoculum were
saved for dry matter, cellulose, and volatile fatty acid
analyses.

The samples for volatile fatty acid determination were
stored in a frozen state until prepared for analysis. Five
ml. of the solution was mixed with 1 ml. of a 25 percent

solution of metaphosphoric acid and allowed to stand in a stoppered tube for 30 minutes (Erwin et al., 1961). The tubes were then centrifuged in an ultracentrifuge at 8590 RCF for 10 minutes. The supernatant was poured off and refrigerated in capped vials until the analyses were made. The component volatile fatty acids were separated using an Aerograph model A-600-B "Hy-Fi" gas chromatograph with a hydrogen flame ionization detector. Nitrogen gas was used as the carrier.

Results and Discussion

Dry matter and cellulose digestibilities of each of the forages at each stage of the fermentation are shown in table 11 and reproduced graphically in figure 2. Dry matter digestion proceeded rapidly during the early part of the fermentation. Dry matter digestion of the alfalfa hay was 41.1 percent in the first 12 hours. Small increases were noted up to 30 hours but essentially no dry matter was digested thereafter. Dry matter digestion of the prairie hay proceeded at a slower rate than the alfalfa hay during the entire fermentation. Nearly half of the dry matter digestion occurred after 12 hours and thereafter the increases in digestion were gradual.

Cellulose digestion of the alfalfa hay was 16.2 percent at the end of 3 hours with another large increase in the following 3 hours; it virtually ceased at the end of 24 hours. Donefer et al. (1960) obtained essentially no increase in cellulose digestion of legume forages between 24

TABLE 11

DRY MATTER AND CELLULOSE DIGESTION COEFFICIENTS OBTAINED
AT VARIOUS INTERVALS DURING 48-HOUR FERMENTATIONS OF
ALFALFA HAY AND PRAIRIE HAY

Incubation time (hr.)[1]	Dry matter digestion (%)		Cellulose digestion (%)	
	Alfalfa hay	Prairie hay	Alfalfa hay	Prairie hay
2		12.2±0.16		0.3±0.15
3	28.8±0.32[2]		16.2±0.36	
4		13.8±0.20		4.8±0.55
6	34.2±0.16	15.6±0.26	29.8±0.87	10.6±0.46
12	41.1±0.20	21.6±0.07	43.3±1.10	26.0±2.98
18	45.6±0.28	26.1±0.26	47.5±0.59	33.9±1.10
24	48.4±0.26	29.7±0.07	57.1±0.32	42.1±0.40
30	51.2±0.14	31.9±0.12	57.4±0.42	42.6±2.95
39	52.4±0.50	34.4±0.07	59.8±0.71	47.0±2.45
48	51.4±2.10	37.2±0.14	59.6±1.25	49.2±0.71

1. Two bottles removed at each time interval.
2. Standard error of the mean.

PERCENT
DIGESTION
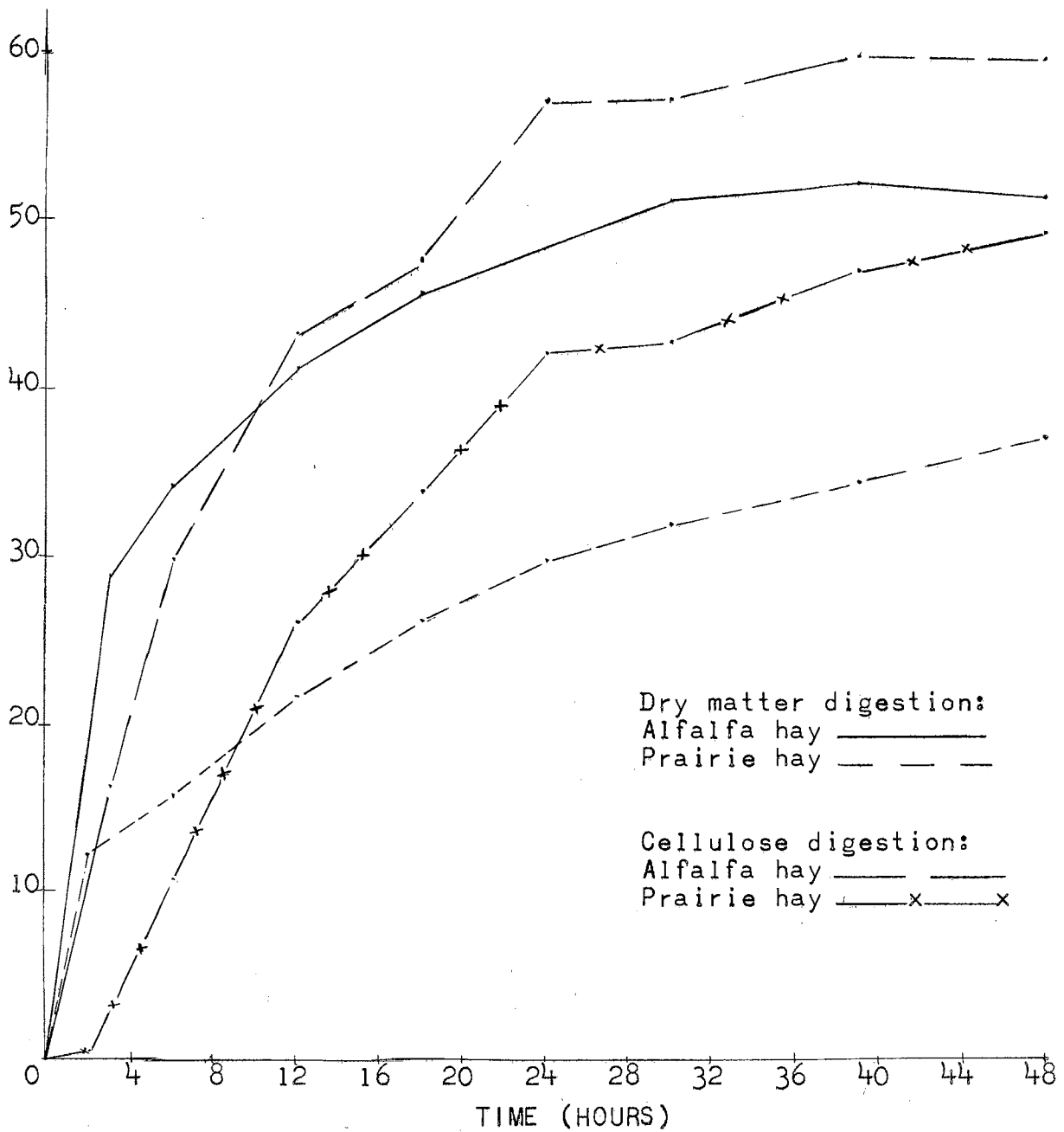
Figure 2. Dry Matter and Cellulose Digestion of Alfalfa Hay
and Prairie Hay at Various Times During a 48-Hour
In Vitro Fermentation

and 48 hours. In contrast to alfalfa cellulose digestion,
prairie hay cellulose digestion proceeded slowly during the
first 4 hours of the fermentation. The greatest amount of
cellulose was digested between the sixth and 24th hours.
Small amounts were digested at each stage during the last 24
hours of the fermentation. These data suggest that 48-hour
fermentations are not superior to 24-hour fermentations,
especially when high quality forage such as alfalfa hay is
fermented. Donefer et al. (1960) concluded that in vitro
cellulose digestion beyond 24 hours essentially measured the
ultimate in vivo energy availability.

Hershberger et al. (1959), Donefer et al. (1960), and
Rice et al. (1962) noted that lag periods in the early part
of in vitro fermentations of cellulose were related to for-
age species. In this study the cellulose of the highly
lignified prairie hay appeared to be more resistant to early
microbial attack than that of the alfalfa hay. The curves
for cellulose digestion of both hays are nearly parallel
after the first 4 hours (figure 2). Similar results have
been noted by Barnett and Reid (1957b) and Rice et al. (1962)
with forages of similar quality. In general, the shape of
the curves for the digestion of alfalfa hay cellulose and
dry matter agree with those of Hershberger et al. (1959),
Walker (1959), and Donefer et al. (1960).

Lag periods similar to the one obtained with cellulose
digestion of the prairie hay were noted by Barnett (1957)
with oat silage as the substrate, by Hershberger et al. (1959)

with timothy hay, and by Rice et al. (1962) using oat straw.
Shorter lag periods are most desirable since this is an indi-
cation of early microbial activity and also evidence that the
microbial population is not being altered (Hungate, 1950).

Total volatile fatty acid production, acetic to propi-
onic ratios, and molar percentages of the volatile fatty
acids produced at the various stages during the 48-hour
fermentations are presented in table 12. Total weight of the
volatile fatty acids (acetic, propionic, butyric, and valeric)
produced was similar with both forages. Remarkably close
agreement in weight of cellulose digested was noted between
forages (714 vs. 721 mg. for prairie and alfalfa,
respectively).

The correlations of total weight of volatile fatty acids
produced at the various stages during the fermentation with
percent dry matter and percent cellulose digestion at the
same stages were highly significant (P<.01). These correla-
tions are shown in table 13. If total moles of volatile
fatty acids produced were used instead of total weight,
correlations were slightly higher than shown in table 13 for
the prairie hay but were not altered for the alfalfa hay.

The variables would be expected to be perfectly corre-
lated if errors were not made in the measurement of the
variables involved. The high correlations indicate that
repeatable estimates of dry matter and cellulose digestion
and volatile fatty acid production were made, which in turn
is a check upon technique.

TABLE 12

TOTAL VOLATILE FATTY ACIDS PRODUCED, ACETIC TO PROPIONIC RATIO, AND MOLAR
PERCENTAGES OF VOLATILE FATTY ACIDS PRODUCED AT VARIOUS TIMES DURING A
48-HOUR FERMENTATION OF ALFALFA AND PRAIRIE HAY

Hours fermented	Total VFA produced (mg.)[1]		Acetic to propionic ratio		Molar percent of each acid produced							
					Alfalfa				Prairie			
	Alfalfa	Prairie	Alfalfa	Prairie	C_2	C_3	C_4	C_5	C_2	C_3	C_4	C_5
Original rumen fluid			4.37	5.08	74.8	17.1	7.0	1.1	77.8	15.3	6.5	0.4
2		158		2.60					66.3	25.5	8.0	0.2
3	318		3.70		73.0	19.7	4.6	2.7				
4		259		1.23					49.3	40.1	10.4	0.2
6	403	308	3.49	1.48	72.8	20.9	5.0	1.3	54.8	37.0	7.9	0.3
12	604	367	3.40	2.60	72.4	21.3	5.9	0.4	64.3	24.7	10.8	0.2
18	1007	442	3.60	2.66	72.3	20.1	6.5	1.1	63.9	24.0	11.7	0.4
24	1032	517	3.21	2.88	70.2	21.9	7.0	0.9	65.9	22.9	10.7	0.5
30	1087	982	2.99	3.30	67.2	22.5	9.1	1.2	67.6	20.5	11.0	0.9
39	1257	1190	2.69	5.72	65.7	24.4	8.7	1.2	78.9	13.8	6.8	0.5
48	1353	1200	3.14	4.32	69.0	22.0	7.9	1.1	74.3	17.2	7.8	0.5

1. Acetic, propionic, butyric, and valeric acids produced from the fermentation of 4 grams of forage.

TABLE 13

SIMPLE CORRELATION COEFFICIENTS BETWEEN TOTAL WEIGHT
OF VOLATILE FATTY ACIDS PRODUCED AND PERCENT DRY
MATTER AND CELLULOSE DIGESTION OF EACH FORAGE

Variable	Volatile fatty acid production[1]	
	Alfalfa hay	Prairie hay
Percent dry matter digested	.96	.92
Percent cellulose digested	.94	.86

1. All correlations significant at the .01 level.

It is possible that volatile fatty acids are lost to the atmosphere in the escaping carbon dioxide during fermentation. In order to determine if substantial amounts were so lost, a series of three bottles containing a known amount of water was connected in the same manner as the bottles in the fermentation. A piece of rubber tubing connected these to the last bottle in the fermentation sequence allowing carbon dioxide to escape from the third bottle of water. This system was employed for an hour at three equally spaced times throughout the fermentation. Chromatographic analysis indicated that only traces of volatile fatty acids were carried out of the _in vitro_ system. Thus, losses of volatile fatty acids from the fermentations may be considered nil and values reported may be considered to be good estimates of the volatile fatty acid production _in vitro._

The total production of volatile fatty acids appears rather large but when converted to mg. of acid produced per

gram of substrate dry matter the values obtained at the end of the incubation averaged 319. This value is similar to those reported for tall fescue (Bowden and Church, 1962a) and those of Gray et al. (1951) for wheaten and lucerne hay and Barnett and Reid (1957a,b,c) for ley grass hay. The accumulation of volatile fatty acids during the fermentation may have slowed down the rate of fermentation considerably at the later stages. The increase in volatile fatty acid production during the fermentation was about double that added by the inoculum. The inoculum contained 565 and 693 mg. of volatile fatty acids for the alfalfa and prairie hay diets, respectively. The volatile fatty acid levels reported by Johnson et al. (1958) to be inhibitory to cellulose digestion were not reached. It would, therefore, appear that volatile fatty acid accumulation in these experiments was not large enough to cause inhibition of cellulose digestion.

The possibility also exists that further breakdown was prohibited because of lignification. This possibility appears reasonable in view of the findings that purified cellulose will be nearly completely digested in 30 to 48 hours. (Hershberger et al., 1959; Donefer et al., 1960; Van Dyne, 1962). Additional evidence has been presented by Dehority and Johnson (1961) who found that previously-fermented, finely ground forages subjected to additional ball-milling were digested to a considerable extent in a second fermentation. These workers suggested that lignin in the forage acts as a physical barrier between the cellulose and the rumen bacteria.

Acetic to propionic ratio of the original inoculum from the alfalfa and prairie hay diets was 4.37 and 5.08, respectively. During the fermentation of the alfalfa hay the acetic to propionic ratio gradually declined. In the early part of the fermentation of the prairie hay, large quantities of propionic acid were produced and the ratio declined to 1.23 after 4 hours of incubation. The ratio was only 1.48 after 6 hours but increased rather consistently during the remainder of the fermentation. The decline in acetic to propionic ratio during the fermentation of the alfalfa hay was quite similar to the results obtained by Rice et al. (1962) with alfalfa hay.

Molar percentages of each of the acids produced at the various times are also presented in table 12. A slightly higher percentage of acetic acid was produced in vivo by the prairie hay diet. The percent of acetic acid present in the rumen fluid obtained from the steer fed these diets was slightly higher than values commonly reported for similar type diets (Gray and Pilgrim, 1951; Balch and Rowland, 1957; Archibald et al., 1961) and considerably higher than the values reported by Barnett and Reid (1957a).

In general, the molar percentages of butyric and valeric acids which were produced during the fermentation of both hays remained relatively constant. The productions of acetic and propionic acids from the two hays were greatly different, especially during the early part of the fermentation. The molar percentages of the acids produced after a 48-hour

fermentation are similar to most of the values reported by Bowden and Church (1962a) for a 48-hour fermentation of tall fescue. However, several of the ending molar percentages in the work of Bowden and Church (1962a) are similar to values obtained at the early stages of the prairie hay digestion.

Balch (1958) compared molar percentages of the volatile fatty acids in the inoculum and in the in vitro liquor after a 12-hour fermentation. He found that the molar percentage of acetic decreased about 7.5 units, propionic decreased slightly, while butyric and the higher acids increased sharply. No major differences were noted between morphological types of bacteria in the inoculum and in the liquor after the fermentation. Similar observations were made by el-Shazly et al. (1959) when hay or a complete synthetic diet was used as the substrate in a 24- to 30-hour fermentation. In the study reported herein, the molar percentages of the volatile fatty acids in the liquor at the end of the 48-hour fermentation of the alfalfa hay deviated less from the inoculum than those in the 12-hour study conducted by Balch (1958). This was not true for the fermentation of the weathered prairie hay.

If the hypothesis is advanced that the types of microorganisms present were relatively unchanged throughout the fermentation, data from the fermentation of the two hays are contradictory. The absence of pronounced lag periods at the beginning of the fermentation of the legume hay lends support to this hypothesis. Lag periods in cellulose digestion have been assumed by Hungate (1950) to indicate an alteration of normal rumen activity.

The results of this study are in partial agreement with
the work of Warner (1956) in which it was proposed that fer-
mentations conducted under the most ideal conditions were no
longer representative of conditions in vivo after 8 hours.
Pearson and Smith (1943) found that drastic alterations
occurred in the microbial population on prolonged incubation
but over shorter times (up to 4 hours) little change occurred.
Burroughs et al. (1950a,b) and Brooks et al. (1954) reported
little alteration in number and types of bacteria present
after long fermentations in glass containers.

Barnett and Reid (1957a) noted that cellulose always
yielded propionic acid in the greatest proportion. Gray and
Pilgrim (1952) noted that nearly equal amounts of acetic and
propionic acids were produced from the fermentation of cellu-
lose. Hemicellulose fermented by washed suspensions of rumen
microorganisms also produced nearly equal proportions of
acetic and propionic acids (Gray and Weller, 1958). The
results obtained during the early part of the prairie hay
fermentations are in agreement with these findings, but
since acetic acid was predominately produced throughout the
fermentation of alfalfa hay the results obtained with this
hay are in disagreement. Whether this is a result of the
type of hay fermented or of the technique employed is not
known.

Gray et al. (1962) proposed a very stringent test for
the artificial rumen. They advocated that the formation of
fatty acids from a given substrate should follow the same

course in the artificial rumen and the rumen of the experimental animal at the same time, and also, that over-all rate of fermentation should be similar in each. In the present experiments changes in the rumen were not followed, but since the steer ate hay _ad libitum_ it appears likely that changes in volatile fatty acid composition would not be extremely variable throughout the day. Thus, the system developed could be considered as meeting these requirements, except during the first 6 hours of the prairie hay digestion.

Alfalfa hay dry matter digestion in conventional trials (reported in the following section) with steers and wethers was about 12 percent higher than the dry matter digestion of this same hay _in vitro_. Apparently, dry matter digestion was affected only after an incubation of 24 to 30 hours since it was at this time that further increases in dry matter digestion did not occur.

<div align="center">

Trial VI. Comparison of _In Vitro_ and _In Vivo_ Dry
Matter and Cellulose Digestibilities of
Alfalfa Hay Using Cattle and Sheep

</div>

Experimental Procedure

Six crossbred wether lambs, averaging 105 pounds in weight and about 8 months of age, and six yearling Hereford steers averaging 625 pounds in weight were placed in metabolism stalls (Nelson _et al._, 1954) and fed a high quality, chopped alfalfa hay. Each animal was fed twice daily. A 12-day adjustment period preceded a 6-day period of _ad libitum_ feed consumption and the 7-day collection period. During the

collection period each animal was fed 90 percent of his daily
ad libitum consumption. Feces and urine collections were
begun 2 days after the restricted feeding phase was started.
Hay samples were removed at each feeding for proximate analy-
ses and for use as the substrate for the in vitro fermentations.

Total feces were collected from both wethers and steers.
The total daily collections of sheep feces were dried in a
forced air oven controlled at 70°C. and stored in loosely-
covered metal containers. Samples for proximate analyses
were obtained after thorough mixing of all feces voided.

Steer feces were collected in metal boxes and trans-
ferred several times daily to covered metal containers. The
daily fecal output was weighed and mixed thoroughly with an
electric mixer. A 3 percent sample was removed and stored
in tightly-covered glass jars in a frozen state. At the end
of the trial the composited feces samples were thawed without
loss of moisture, mixed thoroughly, and samples removed for
drying. Nitrogen determinations were made on the wet steer
feces by the Kjeldahl method. Proximate analyses were made
as described by A. O. A. C. (1960).

The fistulated steer which supplied the inoculum for the
in vitro fermentations consumed the chopped alfalfa hay
ad libitum. The fistulated sheep consumed a high quality,
baled alfalfa hay (ad libitum) since they could not be induced
to eat the chopped hay. Rumen samples were obtained in
mid-afternoon. The hay obtained from the samples collected
during the digestion trials was used in 24-hour in vitro
fermentations conducted as previously described.

Results and Discussion

The average apparent digestion coefficients of the alfalfa hay as determined in conventional metabolism trials with sheep and steers are shown in table 14. The steers digested a significantly larger amount of dry matter (P<.05), crude fiber and cellulose (P<.01) than the wethers; protein digestion significantly (P<.01) favored the wethers. The differences in nitrogen-free extract and ether extract digestibilities were not significant. These results are in agreement with those of Swift and Bratzler (1959) and Alexander et al. (1962) who found higher, but non-significant, dry matter digestion coefficients with cattle than with sheep. Jordan and Staples (1951) compared digestion data of steers and sheep fed several prairie hays and noted higher digestion coefficients with steers for all nutrients except protein. Cipolloni et al. (1951) conducted a statistical study of the published data and found that cattle digested all nutrients in dry roughages better than sheep. Protein digestibilities of several forages have been reported to be higher for cattle than for sheep (Watson et al., 1948).

Barnes (1963) using hay cut from the same field as the hay used in the trials at this station found that crude fiber and cellulose digestibilities were slightly higher with steers than with sheep, and that sheep showed an advantage in dry matter and protein digestibility. The apparent digestion coefficients obtained in both laboratories were very similar with the exception of crude fiber and ether extract, which

TABLE 14

AVERAGE APPARENT DIGESTION COEFFICIENTS OF ALFALFA HAY OBTAINED IN DIGESTION TRIALS WITH WETHERS AND STEERS

Species[1]	Hay intake, gm, per day[2]	Average digestion coefficients (%)					
		Dry matter	Crude fiber	Cellulose	N-free extract	Ether extract	Crude protein
Wethers	864.2± 76[3]	62.5±0.74 (2.9)[4]	51.6±1.49 (7.1)	52.6±1.98 (5.4)	73.5±0.57 (1.9)	45.1±1.65 (9.0)	70.9±0.57** (2.0)
Steers	6700.4±295	65.1±0.52* (2.0)	57.8±1.18** (5.0)	58.0±0.74** (3.1)	73.0±0.58 (2.0)	50.0±1.83 (8.9)	65.2±0.53 (2.1)

1. Six animals per trial.
2. Dry matter basis.
3. Standard error of the mean.
4. Coefficient of variation (%).
   * P<.05.
   ** P<.01.

were about 7 and 12 percentage units higher, respectively, in trials at this laboratory.

In general, the standard errors of the digestibility coefficient means and the coefficients of variation were slightly lower for the steers than for the wethers (table 14) and are within the range usually found in digestion trials. Bartlett (1904), Jordan and Staples (1951), and Alexander et al. (1962) noted slightly greater variation in the digestibility of the various nutrients by sheep than by cattle. In the present study there were no significant differences between the variances of wethers and steers with regard to the digestion of a particular nutrient.

Dry matter and cellulose digestion was 47.2 and 53.9 percent, respectively, when the alfalfa hay was fermented in vitro with inoculum obtained from the steer (table 15). These values are nearly identical with the average of the values obtained with sheep. LeFevre and Kamstra (1960) and Van Dyne (1962) noted that in vitro cellulose digestion was nearly the same with rumen fluid obtained from sheep and cattle. In the present experiment cellulose digestion coefficients obtained in vitro were similar to digestion coefficients in vivo, whereas the in vitro digestion of dry matter was consistently lower than that in vivo.

Since only one hay was digested in vitro and in vivo no correlations may be made. Correlations of in vitro dry matter digestibility with in vivo dry matter digestibility reported in the literature have usually been highly

significant (Asplund et al., 1958; Baumgardt et al., 1958; Walker, 1959; Reid et al., 1959; Clark and Mott, 1960; Bowden and Church, 1962b). Likewise, in vitro and in vivo cellulose digestibilities have been shown to be very highly correlated (Barnett, 1957; Baumgardt et al., 1958; Hershberger et al., 1959; Lefevre and Kamstra, 1960; Bowden and Church, 1962b).

TABLE 15

IN VITRO DRY MATTER AND CELLULOSE DIGESTION COEFFICIENTS
OF ALFALFA HAY USING INOCULUM FROM SHEEP AND CATTLE

Item	Run[1]	Sheep		Steer
		1	2	1
Dry matter	1	50.4 ± 0.47[2]		45.8 ± 0.43
	2	49.7 ± 0.85	43.5 ± 0.68	47.7 ± 0.11
	3	49.1 ± 0.20	48.0 ± 0.15	48.1 ± 0.39
	$\bar{x}$	49.7 ± 0.51	45.8 ± 0.42	47.2 ± 0.31
Cellulose	1	57.9 ± 0.78		53.5 ± 0.48
	2	47.0 ± 0.27	51.0 ± 1.71	51.2 ± 0.34
	3	49.6 ± 1.15	59.4 ± 0.41	57.1 ± 0.41
	$\bar{x}$	51.5 ± 0.73	55.2 ± 1.08	53.9 ± 0.41

1. Quadruplicate fermentations per run.
2. Standard error of the mean.

SUMMARY

The effect of several variables upon the digestion of purified cellulose (Solka-floc) and forage dry matter and forage cellulose was studied in a series of experiments conducted _in vitro_. Volatile fatty acid production _in vitro_ was studied in relation to _in vitro_ digestion of dry matter and cellulose of alfalfa and mature, weathered prairie hays. A comparison was made of alfalfa hay dry matter and cellulose digestion _in vitro_ and _in vivo_ using wethers and steers.

The most repeatable results were obtained when 250-ml. round-bottom centrifuge bottles were used as the fermentation vessels. Inoculum obtained after consumption of water by the donor animal, which had not had access to water for several hours, caused small but significant increases in Solka-floc digestibility _in vitro_. Cellulose digestion was not significantly decreased by diluting rumen fluid in the laboratory until 50 percent of the rumen fluid had been replaced by water. Increased rate of carbon dioxide flow into the fermenting mixture reduced cellulose digestion slightly, but significantly less cellulose was digested when no gas was bubbled into the mixture.

The effect of animal diet upon dry matter and cellulose digestion was studied by fermenting alfalfa and prairie hay _in vitro_ with inoculum obtained from a steer fed the

corresponding hays. Dry matter digestion was significantly affected by animal diet, whereas cellulose digestion was not. Diet had a greater effect upon digestion of dry matter of alfalfa hay than on that of prairie hay. Significant diet by substrate interactions were noted for both dry matter and cellulose digestion.

Dry matter digestion of both hays was rapid early in the fermentation period. Digestion of cellulose was slower in the prairie hay than in the alfalfa hay. Total weight of volatile fatty acids produced at the various stages of the 48-hour fermentation were significantly correlated with percent digestion of dry matter and cellulose at these stages. Molar percentages of the volatile fatty acids pro-duced during the fermentation of alfalfa hay were similar to the percentages in the inoculum, but drastic changes were observed early in the fermentation of the prairie hay.

Forage dry matter digestion _in vitro_ was more repeat-able within a given fermentation and from day to day than forage cellulose digestion.

Digestion coefficients of alfalfa hay cellulose deter-mined _in vitro_ with rumen fluids obtained from sheep and cattle were similar. They were about equal to the average cellulose digestion coefficient obtained _in vivo_; however, dry matter digestion coefficients were higher when determined _in vivo_.

Digestibility of dry matter, crude fiber, and cellulose was significantly higher when determined with steers by con-ventional methods than when determined with wethers; digesti-bility of protein was significantly less.

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