A STUDY OF MICROBIAL ACTIVITIES IN THE OVINE RUMEN

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A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

1952

ABSTRACT

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A manometric method employing a constant volume Warburg apparatus has been used in evaluating the fermentation activity *in vitro* of ingesta from sheep with permanent rumen fistulas at various intervals after eating different rations.

A basal fermentation was determined by measuring the gas produced when mixtures of rumen ingesta and bicarbonate or phosphate buffer were allowed to react under anaerobic conditions at 37 C. Glucose and other fermentations were investigated when an excess of substrate was added.

The glucose fermentation exceeded the basal by 10 to 20 times with samples obtained 12 to 15 hours after eating. Shortly after consuming hay and water, the basal rate increased and glucose fermentation decreased. As the period following eating lengthened, the basal rate dropped and the glucose rate rose.

Sucrose fermentation exceeded slightly the activity on glucose. Glucose fermentation was 2 to several times greater than D-xylose, hemicellulose, soluble starch and corn starch, although this varied with the time after eating.

When barley supplemented the alfalfa diet, an increase in the fermentation of all substrates was noted although there was no appreciable change in the number of bacteria counted in the rumen fluid by direct microscopy employing a diluted carbol fuchsin stain. The pH of all samples was lower with barley in the diet, dropping to about 6.0 shortly after eating.

An oat straw diet for 15 days resulted in little change in the basal rate of samples collected 12 to 15 hours after eating, but a decrease in the

fermentation of glucose, corn starch, ground barley and alfalfa substrates was noted. When corn starch was added to the rumen for 9 days, there was some increase in the fermentation of the substrates but not the basal. The number of organisms declined from about 30 billion bacteria per ml on an alfalfa diet to about 10 billion per ml on the straw-starch ration.

Phosphate and bicarbonate were employed as buffers in the Warburg flasks. In general, greater volumes of gas were recorded with the bicarbonate.

One ml of rumen ingesta treated with acid released about 1600, 800, and 1300 ul CO_2 when obtained 15, 1, and 6 hours respectively after eating. The amount of bicarbonate in the anterior and posterior of the rumen varied with the time in which the saliva had been mixed with the ingesta.

The initial and residual bicarbonates determined in the flasks with early morning samples were about twice as great with the bicarbonate buffer as with the phosphate. These values varied at different times of the day. Without exception, the glucose fermentation and acid CO_2 were higher with the bicarbonate buffer. However, there was no consistent difference between the metabolic CO_2 values.

The fermentation capacity of a 6-year-old sheep with an open fistula was about 50 percent less than that of a 3-year-old ewe with a fistula closed by a lucite plug. The average number of bacteria in the rumen fluid for 12 consecutive days on an alfalfa diet were 18.5 billion for the sheep with the open fistula and 22.6 billion bacteria per ml for the other.

The numbers of bacteria per ml of ingesta from 2 ewes under a variety of conditions over a period of 2 years were between 5 and 50 billion with an average of about 30 billion.. In general the counts showed little correlation with the fermentation capacity of the ingesta.

The manometric method warrants further investigation for possible uses in evaluating rations, in determining the effects of therapeutic agents on the rumen microflora, and in obtaining additional information on ruminant digestion.

Approved
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July 30, 1952

William Goff Walter was born November 1, 1914 in Lake Placid, N. Y., son of the late John F. and Myrtle Goff Walter. He received the Bachelor of Science degree in Bacteriology from Cornell University in 1938. From 1938 to 1942, he was a graduate assistant and an assistant in research at the New York State Agricultural Experiment Station, Geneva, N. Y. During this period, he earned the Master of Science degree in Bacteriology from Cornell University. In 1942 he went to Montana State College, Bozeman, Montana, as an assistant professor in the Department of Botany and Bacteriology and at present has the rank of professor.

ACKNOWLEDGEMENTS

The author expresses his appreciation to Dr. F. W. Fabian for his continued interest and encouragement in this work done *in absentia* and to Dr. R. E. Hungate at the State College of Washington who read parts of the manuscript and made several helpful suggestions.

Thanks are extended to Professor J. L. Van Horn of the Department of Animal Industry for furnishing the sheep and to Drs. H. Marsh, L. Seghetti, J. W. Safford and E. A. Tunnicliff of the Veterinary Research Laboratories at Montana State College for making the rumen fistulas and providing convenient quarters for the experimental animals.

The author is most grateful to Dr. R. H. McBee, a colleague at Montana State College, for his numerous suggestions regarding technics and for the many hours spent discussing various aspects of rumen microbiology.

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INTRODUCTION

The controversial subject of ruminant digestion has attracted the attention of many investigators in recent years. Since ruminants serve as valuable sources of food, clothing and power, it is not surprising that nutritionists, microbiologists, physiologists and biochemists have been curious about the symbiotic relationship of microorganisms and their hosts, the sheep, cow, goat, ox and other herbivora. Many fundamental and applied studies have been undertaken during the last 100 years in an effort to delve into the perplexing problems of making these animals more productive for man's ever-increasing needs.

Much has been learned but there are still many aspects of ruminant digestion which require further investigation. One of these phases is considered in the present work. A manometric method has been adopted to evaluate the rate of fermentation of ovine rumen ingesta at different hours of the day and under various feeding conditions, as well as with the addition of several carbohydrate substrates. Since many of the reports in the literature regarding the number of organisms and the pH of the rumen contents are conflicting, additional information on these points has also been obtained.

LITERATURE REVIEW

A number of excellent reports and reviews of various phases of rumen microbiology have been published by Marston (1939), Goss (1943), McAnally and Phillipson (1944), Hastings (1944), Cole et al. (1945), Hungate (1946b, 1950a, 1950b), Baker et al. (1947-48), Owen (1947), and Ellsden and Phillipson (1948).

The following review of literature appears pertinent to the present study.

Multiple Stomach of Ruminants.

The ruminant is a cud-chewing animal in which the symbiotic relationship between mammals and microorganisms is carried on at a very high level (Hastings, 1944). These animals have a compound type of stomach with four distinct cavities or compartments known as the rumen, reticulum, omasum, and abomasum. The first three are dilations of the esophagus and secrete no digestive juices. The abomasum or true stomach liberates digestive secretions but even these do not include cellulases. Food rapidly ingested with very little chewing passes from the esophagus into the rumen and reticulum which act as one functional unit. Here it is held while cuds or boli may be regurgitated into the mouth for mastication. The cud is usually of food that has been in the rumen for a number of hours and has been exposed to the action of protozoa and bacteria. The mastication period is about one minute and the bolus, well mixed with saliva, then returns to the rumen and reticulum before passing to the omasum which, according to Garton (1951), presses

and squeezes the food as well as effecting comminution and absorption of water. The material then passes into the abomasum where it is attacked by proteolytic enzymes. Schalk and Amadon (1928) studied the physiology of the bovine stomach and found that the rumen occupies about 80 per cent of this organ which in an adult animal may have a capacity of 20 to 40 gallons. Likewise, they investigated the interesting and unique process of rumination. More recently Schalk and coworkers at Ohio State University have depicted the constant rhythmic contractions and expansions of the bovine rumen in an excellent colored film.

The Nature and Function of Saliva.

The periodic motion of the rumen insures a thorough mixing of the ingesta with water and saliva. Elsden and Phillipson (1948) point out that the saliva of the ruminant provides the basal medium for the rumen microorganisms and that it is well adapted both in composition and volume for this function.

McDougall (1948) studied the composition and output of sheeps' mixed saliva, consisting mainly of parotid saliva, which forms a fluid medium for the transport of ingesta both back to the mouth for remastication and onward through the stomach to the small intestine. He found that the parotid gland secreted at a rapid rate during eating and rumination and at a slower rate during rest with the volume varying between 2 to 4 liters per day. The saliva from this organ owed its alkalinity and buffering capacity to sodium bicarbonate as well as

inorganic phosphorus. The pH of pure parotid saliva, measured at room temperature, was about 8.2. When the composition of parotid saliva and serum from sheep was compared, it was noted that the parotid gland concentrated inorganic phosphate about 10 to 20 times. The principal cations, sodium and potassium, were present in about the same concentration as in the serum, while the other anions of phosphate and of bicarbonate were approximately 15 times and 4 times respectively as concentrated in the saliva.

McDonald (1948) has shown that the nitrogen of sheep saliva is largely in the form of urea, and that 10 mg urea N may be secreted per 100 ml of saliva. Hence urea is a source of nitrogen continually entering the rumen and subsequently providing ammonia for bacterial growth.

It is interesting to compare the information on ovine saliva with that obtained by Reid and Huffman (1949) who studied some of the physical and chemical properties of 77 samples of bovine saliva. They found that the average water content of 36 representative samples was 99.12 per cent. The average pH of 54 samples was 8.53 when protected from CO_2 loss by neutral paraffin oil, whereas unprotected samples gave an average pH of 8.71. They observed no appreciable variation in pH when samples were collected at 6-hour intervals over a 24-hour period.

A cow secreting daily about 56 liters of saliva thus makes available approximately 300 to 350 g of sodium carbonate. Undoubtedly this alkali plays an important buffering role at the time of eating and

subsequently when fermentation is at a peak.

The pH of Rumen Ingesta.

The pH of the ingesta of steers having rumen fistulas varied from 5.5 to 7.7 depending on the ration according to Kick and associates (1938). They found the most alkaline conditions when alfalfa hay alone was fed. Also, they encountered variations in reactions during the day. Monroe and Perkins (1939) studied the pH values of the ingesta of the bovine rumen by collecting 6 samples from different locations and averaging the pH readings. The pH values were determined on the uncentrifuged liquid expressed from the ingesta within one hour or less after samples were taken. These workers report pH values between 6.83 and 7.01 when their animals were fed such roughages as corn or A.I.V. silage and alfalfa hay in fair amounts. On pasture, both blue grass and alfalfa, the ingesta averaged pH 6.47 to 6.66. Samples taken at 2-hour intervals from 5 a.m. to 5 p.m. showed the ingesta most alkaline (average 7.19) just prior to feeding in the morning. Samples of rumen contents of 16 animals slaughtered 12 to 18 hours after the last feeding had an average pH value of 7.34 when determined within 10 minutes after death.

Olson (1940) determined the pH of 473 samples of ingesta from the rumens of slaughtered animals. The average pH of the fresh material was 6.89. In general, the pH increased with the age of the sample so that at the end of two days it was 7.02, after 4 days 7.54 and at the end of 10 days 7.96. It seems likely that this rise in pH could be

accounted for by loss of CO_2.

Smith (1941) carried out *in vivo* studies of hydrogen ion concentrations in the rumen of the dairy cow in order to eliminate pH changes that might occur when the ingesta were exposed to the air and CO_2 was lost. Using animals with open fistulas he consistently found lower pH values by *in vivo* determinations than by *in vitro* studies. An average pH of 6.27 was obtained for alfalfa hay and 6.00 for beet pulp and alfalfa when checked 3 times a day over a 5 day period. Variations in pH were encountered throughout the day and also in different regions of the rumen. A specially constructed glass electrode assembly, 23.5 inches long connected by a ten foot lead to a Beckman pH meter, was employed in these studies.

Myburgh and Quin (1943) found that the pH of the ruminal ingesta in Merino sheep, determined immediately after withdrawal through rumen fistula, varied between pH 5.5 and 6.8 depending on the carbohydrate content of the diet. They also observed that the feeding of alfalfa hay produced slightly acid ingesta (pH 6.45-6.95) whereas fresh green alfalfa and mature veld grass hay yielded slightly alkaline conditions (pH 7.3-7.7). The pH showed slight fluctuations only during the digestion of a single meal, the tendency being towards increased acidity within the first 4-6 hours after which it steadily reverted to its previous level. The drop in pH coincided precisely with the production of volatile fatty acids in the rumen of sheep (Phillipson, 1942) and in cattle (Elsden, Hitchcock, Marshall, and Phillipson, 1946). Previously Phillipson and McAnally (1942) had introduced cellulose, starch, cane

sugar, glucose, and a variety of other sugars in the rumen of a sheep and had obtained clear evidence of fermentation. Their results obtained were complimentary to those changes noticed with normal diets.

In several of the papers reviewed it was noted that there was a tendency to report an average or mean pH for a number of samples. Since pH is defined as the negative log of the hydrogen ion concentration, it is questionable whether one is justified in averaging pH values. If the differences among pH readings are great, considerable error may be involved in a simple arithmetic average. It would seem more appropriate to report the range or variations in pH values obtained.

Role of Bacteria in the Rumen.

Sijpesteijn (1948) points to Haubner as probably the first to demonstrate the decomposition of crude fibers by animals. In 1855 Haubner showed that of the crude fiber in hay fed to an ox, about 61 per cent had disappeared when the feces were analyzed. These findings were confirmed by Henneberg and Strohmann in 1859 and by Hofmeister studying sheep in 1864 and 1869. In 1884 Tappeiner incubated ingesta samples from the rumen, small intestine, cecum, and colon and after observing the remaining crude fiber concluded that cellulose digestion occurred in all of the compartments except the small intestine. Other workers subsequently confirmed these findings. The next question that arose pertained to the agents causing the decomposition of cellulose. It was found possible to destroy the cellulytic activity of the ingesta

by boiling and this led to the consideration of the animal secreting digestive enzymes into the rumen or of the activity of the microorganisms which were observed in the rumen in great numbers.

Tappeiner incubated rumen contents in vitro with and without chloroform. Since 23 per cent of the crude fiber was decomposed in the absence of the antiseptic and none in its presence, and since bacterial growth was observed and CO_2 and CH_4 were evolved in the non-treated sample, he concluded that microorganisms were responsible. In 1906 Scheunert passed the liquid contents from the cecum of a horse through a Berkefeld filter to retain the microorganisms and found that cellulytic activity was greatly, although not completely, inhibited. From the results of this experiment he believed that microorganisms were the responsible cellulytic agents. Subsequently Scheunert and Grimmer presented convincing evidence that cellulytic enzymes were not present in a large number of the foods ingested by herbivora. In 1915 Scheunert and co-workers definitely proved that no cellulases were secreted in the forestomachs and cecum of animals, but that cellulose digestion in these organs was due to microbial activity. (Sijpesteijn, 1948).

Ankersmit (1905) was able to obtain only anaerobic cellulytic rods with terminal spores when attempting to isolate cellulose digesting bacteria from the rumen by employing enrichment media. However, he concluded that these organisms were of little significance in the rumen because they were seldom observed in direct microscopic examinations of rumen fluid, they developed too slowly, and they were found to about

the same extent in hay.

Hopffe (1919a) was unsuccessful in attempting to isolate organisms from the rumen of cattle that she felt played an important role in cellulose digestion. Later she was consistently able to isolate a fungus which she called Aspergillus cellulosae from the rumen of cattle and sheep as well as from a number of other sources (Hopffe, 1919b). Thom and Church (1926) report that a culture under this name received from Neuberg proved to be Aspergillus fumigatus Fresenus. The aerobic nature of her culture makes it very doubtful that this organism was any more than a transient in the digestive tract.

Khouvine (1923) described an anaerobic cellulose digesting gram negative sporeforming rod which she isolated from the intestinal tract of man and named Bacillus cellulosae dissolvens. Later (1926) she isolated similar organisms from the rumen and ceca of herbivora and concluded that they were extremely important in cellulose decomposition. Pochon (1935) employed enrichment media containing rumen fluid and isolated a cellulose digesting bacterium from the rumen of sheep and cattle. He believed that this organism which he named Plectridium cellulyticum represented the truly digestive bacteria of ruminants.

While carrying out his studies on cellulose digestion by ciliates in cattle, Hungate (1944) isolated an anaerobic, mesophilic, cellulytic bacterium which he named Clostridium cellobioparus n. sp. on the basis of its anaerobic nature, peritrichic flagellation, and spore formation. Hungate (1946a) also isolated an anaerobic strain of Micromonospora from a culture of the protozoa from the rumen of cattle that appeared

identical to an anaerobic cellulose-decomposing actinomycete isolated from a worker termite (Amitermes minimus) that he named Micromonospora propionici n. sp. Stanier and van Niel (1941) have discussed the main outlines of bacterial classification based largely on morphology and have postulated that the actinomycetes originated from the propionic bacteria. Hungate's M. propionici furnishes physiological evidence for this hypothesis.

Other pertinent literature regarding the isolation and cultivation of cellulose decomposing bacteria from the rumen has been well reviewed by Sijpesteijn (1948). In many cases there has been a question as to the purity of the cultures described. Hungate (1950a) discusses in detail an isolation technic by which he and his students have been able to obtain numerous pure cultures of cellulytic organisms including actinomycetes, thermophilic sporeformers, nonsporeforming rods and cocci, and mesophilic sporeformers. His criteria of culture purity are:

> "The organism must grow in solid cellulose medium and give isolated spots in which the cellulose is digested and in which the cellulose-digesting colony (or diffuse growth if no colony is formed) may be identified and picked. Picked colonies must give growth in solid cellulose medium when inoculated into dilution series and the numbers of colonies obtained must be approximately proportional to the number expected. From cellulose, the organism must be inoculated into a solid sugar or other clear medium in which all types of colonies which develop can be seen. A single colony in a high dilution must be subcultured in a second clear dilution series and this must be continued until two successive series give colonies of only one sort, the uniformity being determined not only macroscopically but also by microscopic examination. Any colony from this second sugar series must, upon reinoculation into a solid cellulose medium, give rise to cellulose-digesting colonies present in approximately the expected numbers in the various dilutions and containing cells microscopically similar to those in the sugar series. Cultures which

> successfully pass these tests under critical scrutiny are considered pure. In no case have any phenomena been observed which would suggest any impurity of cultures thus obtained, except that occasional chance contaminants have been encountered. These were eliminated by the procedure of the initial isolation."

After studying approximately 5000 bacterial cultures isolated from the rumens of 350 cattle and sheep from several herds in three states, Gall and Huhtanen (1951) proposed the following five criteria for judging whether a bacterium isolated from the rumen is a true rumen organism: (a) anaerobiosis, (b) presence in numbers of at least 1 million per gram of fresh rumen contents, (c) at least 10 isolations of similar type bacteria in at least 2 geographical locations, and (d) production by the organism of end-products found in the rumen from substrates found in the rumen. These authors also described but did not name 5 gram positive organisms including 1 coccus and 4 rods from a group of 20 that they employed in establishing their criteria. However they point out that,

> "These bacteria were found typically in the rumens of animals fed large amounts of available carbohydrate, and, therefore should not be considered to be typical of rumen bacteria in general."

Although new organisms are continually being isolated from the rumen and their properties studied, it is still a fertile and controversial field for investigation.

Role of Protozoa in the Rumen.

The significance of protozoa in the rumen has been studied by numerous workers. Becker, Schulz, and Emmerson (1929) and Winogradow,

Winogradowa-Fedorowa, and Wereninow (1930) employed starvation and copper sulfate to defaunate the rumen. Subsequently the cellulytic ability of the defaunated animals was found to be the same as when the animals had their normal fauna, indicating to them that the protozoa were unable to digest cellulose. Others, however, including Schuberg, Braune, Schulze, and Trier, employed microscopic observations in concluding that cellulose was digested, while Weineck used microchemical tests to indicate that sugar was present around cellulose particles in the digestive sack of certain rumen protozoa (Hungate, 1942). In addition Baker and Martin (1939) and Baker (1943) call attention to the fact that of the structural cellulose present in the rumen, only a small amount is made up of a size small enough to permit ingestion by even the largest ciliates. They also stress that particles ingested are always accompanied by bacteria and are usually already in the process of disintegration. Finally van der Wath and Myburgh (1941) emphasize that claims for the significance of the protozoa must depend for acceptance upon the demonstration that the enzymes concerned originate in the protozoan itself and not in its ingested or symbiotic bacteria. These workers found that the bacterial population important in starch digestion was restored after defaunation by inoculation of an iodophilic coccus isolated by van der Wath.

Many of these differences of results appear to be explained quite satisfactorily by the work of Hungate (1942, 1943, 1946b) since he was able to culture a cattle ciliate Diplodinium neglectum employing a medium containing grass, cellulose, and organic salts and to extract a

cellulase preparation. Additional studies revealed that all species of Diplodinum digest cellulose but the members studied of Entodinium, Isotrichia, Dasytricha, and Bütschlia did not. He then concluded that when cellulose was digested at the same rate in the normal as in the defaunated animal, the number of cellulose digesting protozoa was probably few in number and hence of little significance quantitatively. Or, on the other hand, the removal of the protozoa might throw the entire burden on to other agents present.

Baker and Harriss (1947-48) also concluded from the available data that protozoa play a minor role in the digestion of starch in the rumen as compared to bacteria.

Uzzell, Becker, and Jones (1949) studied the rumen contents from 12 calves and 1 cow after slaughtering. They observed 8 genera of protozoa including 18 species but were unable to find any protozoa in the digestive tract of 2 new-born calves that had not nursed prior to killing. These workers call attention to the presence of green inclusions in many of the protozoa including Entodinium bursa. Hungate (1943, 1946b) previously had concluded that members of this genus did not produce cellulase. There does not appear to be any great conflict here since food might be ingested but not digested.

Hungate (1950b) in summarizing the literature on mutualisms of protozoa with ruminants presents evidence that cellulose and starch digestion within the protozoa are due to enzymes elaborated within the protozoa rather than to ingested bacteria. As defaunated ruminants appear to function normally, what then is the role of protozoa in the

rumen since they have been found to constitute about 1/20 of the weight of the rumen contents. Also the nitrogen of the protozoa composes about 20 per cent of the total nitrogen in the sheep rumen. A satisfactory answer appears to be given by Hungate (1950b):

> "The successful utilization of fibrous foods by defaunated ruminants shows that the rumen bacteria alone can successfully accomplish the microbial role in the mutualistic relationship. But the equally satisfactory utilization when protozoa are an important microbic component shows that their presence does not diminish the successful utilization of ruminant food. Since they are so numerous, it must be concluded that in addition to the action of some of them in digesting starch and cellulose they play a significant role in the food chain leading to the host."

Role of Direct Microscopy in Rumen Studies.

Another approach to the study of rumen microbiology has been that of direct microscopic observation. This method is of value in determining whether isolated organisms are present in significant numbers to be important or whether they may only be transient travelers through the digestive tract.

Henneberg (1922) appears to be one of the first to employ direct microscopy in his study of cellulose disintegration. He observed areas of erosion surrounding microorganisms in plant structures. He also employed chlorzinciodine to distinguish iodophiles (organisms that appeared blue from the reaction with iodine) and structural cellulose.

Baker (1931, 1939) and Baker and Martin (1937, 1938) extended such studies and used polarized light and microchemical tests. Cellulose and hemicellulose components of plant walls are birefringent, a property that is lost upon disintegration. Under crossed nicols the affected

areas are indicated by black patches, whereas the unaffected areas appear as a luminous background. The presence of cellulose and hemicellulose is also shown by a blue reaction with chlorzinciodine and by a strong affinity for tetrazonium dyes. These reactions do not occur within the zones of erosion. When staining with iodine and congo red, the iodophiles appear blue while structural cellulose is red.

Baker (1931) observed cocci appearing to play an important role in the disintegration of cell wall substances obtained from the cecum of the guinea pig as well as from decaying leaves present in a pool at a river's edge. Baker and Martin (1937) studied the morphology of a number of iodophilic organisms indigenous to the cecum of a rabbit and found giant streptococci, diplobacilli, vibrios, sporeforming rods, small micrococci and yeasts. Subsequently Baker and Martin (1938) found similar organisms in the rumen of sheep. To the indigenous protozoa present they assigned only a minor role because plant fragments were too large for ingestion and smaller detached fragments had already been attacked by the iodophilic microflora.

In a review on this subject, Baker (1939) emphasizes the importance of iodophilic microorganisms in cellulose disintegration, whether occurring in the rumen of ruminants or in the cecum of non-ruminants. However, these organisms are greatly reduced in numbers by the time the food residues have reached the lower regions of the intestinal tract. Baker (1942a, 1942b) investigated the factors responsible for this decrease and concluded that the following were important: (a) ingestion and digestion by ciliates, (b) action of digestive enzymes, and

(c) bacterial autolysis. He noted that free-living iodiphiles and those attached to fragments were taken in and assimilated by protozoa which were in turn digested in the abomasum. Since he had demonstrated that the ciliates were more susceptible to enzymatic attack than iodophilic organisms, this conclusion seems justified. On the other hand another provisional conclusion, namely, "That it is the substances synthesized, such as microbial protein and polysaccharide, rather than the initial products of decomposition, such as organic acids, that are utilized by the host animal," may be challenged by the more recent work of Gray (1947, 1948) concerning the absorption of volatile fatty acids from the rumen. This research will be discussed at a later point.

Smith and Baker (1944) found that the blue reaction of iodophiles with iodine was due to the endocellular bacterial synthesis of a polysaccharide resembling starch.

Baker (1943) also studied the rumen population of the ox by direct microscopy and found the three main groups to be comprised of iodophilic and non-iodophilic bacteria and protozoa. The protozoa digested starch but not cellulose, while the iodophilic microorganisms attacked both. The microbial population was stable and persistent and qualitatively independent of the range of diet, the locality of the herd, and the breed.

Since present cultural methods are inadequate for studying the microflora of the rumen, Baker and Nasr (1947) have described in detail useful microscopic technics for the study of cellulose and starch decomposition in the digestive tract. Their technics include histo-

chemical staining and optical methods, the latter including the polarizing microscope and fluorescence microscopy.

The importance of direct microscopy is again emphasized in the review by Baker and Harriss (1947-48) in which they discuss microbial digestion in the rumen and cecum with particular emphasis on the breakdown of structural cellulose. Baker, Nasr, Morrice and Bruce (1950) investigated microscopically the locations and agents responsible for the decomposition of various starches and starch products in the digestive tract of various animals. In ruminants this disintegration was brought about by bacterial action in the rumen.

Gall (1946) in her thesis pertaining to studies on the microorganisms in the rumen of sheep and cattle has reviewed the literature on several methods of cultural and microscopical investigations of the population. In addition she developed a direct microscopic technic in which she employed nigrosine as a negative stain. Rumen ingesta were diluted and shaken vigorously for 5 minutes to free bacteria from food particles and to break clumps. The sample was transferred with a 0.01 ml loop to a clean slide where it was mixed with a similar loopful of one-half saturated methanol solution of nigrosine covering an area of 4 square cm. The slide was quickly dried on a hot electric plate and the representative fields counted. Employing this technic, Gall obtained bacterial counts of about 100 billion per gram of solid material from a fistulated animal. She emphasized the importance of vigorous shaking in comparing her counts with those of Köhler (1940) who obtained about 13 billion per gram of fresh rumen contents. In one

series of experiments employing rumen ingesta from fistulated lambs on normal diets, she obtained an average slide count of 62 billion bacteria on the liquid fraction and 93 billion organisms on the solid fraction. In another investigation she obtained counts of about 30 billion bacteria per ml from a sheep with a catheter inserted through a rumen fistula and fed a normal diet. In the experience of the writer and others in his laboratory, this procedure was not found satisfactory and hence was not used extensively. However counts similar to those reported in her last investigation were obtained using a method described later.

Bortree, Dunn, Ely, and Huffman (1946) employed direct microscopy in studying factors affecting the rumen microflora. Samples collected before feeding and at 2-hour intervals for 10 to 12 hours after feeding were preserved in formalin, stained with Gram's iodine, and counted in a Petroff-Hauser chamber. They admit that since only the larger organisms which stained deep blue with iodine were counted, their results do not reflect changes in the total population. In the light of more recent studies which indicate the importance of the smaller organisms, this appears to be a very true supposition. Although no data were presented, they noted a rapid increase in the number of rumen organisms within 2 hours after feeding and this number was maintained for several hours before returning to the level obtained prior to feeding. They found little fluctuation in counts when the animals studied were changed from hay to pasture or when the quality of the roughage fed was varied. On the other hand, addition of 3 pounds of

glucose to a hay diet increased the counts about 100 fold while the administration of starch caused little increase above the number observed with hay alone.

Van der Wath (1948b) employed a Petroff-Hauser counting chamber for studying ruminal populations. Five per cent carbolic acid or 30 per cent hydrogen peroxide was added to the sample followed by staining with nile blue sulfate. Although no counts were included in his report, he admitted that those obtained were not true counts since many organisms adhere to food particles and hence would not be revealed by his technic. It would also appear that many ruminal organisms present in the specimen were not observed since van der Wath used Zeiss Huygen 7X oculars and a Zeiss Achromate 40X dry lens. In work to be reported later it was noted that many organisms in the fields which were being counted, employing the oil immersion objective and 10X oculars, were near the resolving power of the microscope in size and hence would not be seen with van der Wath's optical system.

Moir and Williams (1950) employed a stomach tube to take a series of samples from a number of sheep which were then immediately killed and further representative whole-rumen samples obtained. A quantity of the ingesta was immediately diluted with half its volume of 50 per cent formalin. A Petroff-Hauser counting chamber was employed in enumerating the organisms in a 1 to 500 dilution stained by a 0.1 per cent aniline blue solution. They felt that the relatively close comparison of the total count of microorganisms in whole-rumen and stomach tube samples justified the latter method of sampling when comparisons of total counts

were being made. These workers then employed this technic in determining the effects of 3, 6, 9, 10, and 12 per cent protein (casein and oaten hay) in the dry matter of the ration on the number of ruminal microorganisms. They obtained counts of 25 billion bacteria per ml on a nitrogen intake of 1.53 g per day to 64 billion bacteria per ml on an intake of 13.3 g per day. Admittedly these counts represent the "free" organisms and are not total counts. However, their results compare favorably with those of Gall, Stark, and Loosli (1947), and Gall et al. (1949b) as well as those obtained in this work. Moir and Williams concluded that the number of organisms was definitely influenced by the protein intake, and that approximately 50 per cent of the food protein was converted to bacterial protein. Earlier van der Wath (1942) had found no increase in bacterial count when dextrinized starch was added to a basal ration of poor wheat straw but on the further addition of urea there was a 74 per cent increase. Louw and van der Wath (1943) claimed a highly significant increase in the numbers of ruminal bacteria by adding meatmeal containing 3 per cent protein to a poor quality hay ration. Minor increases in counts were obtained when corn replaced varying amounts of meatmeal up to 150 g. When 300 g of corn were fed in the absence of meatmeal, there was a decline in bacterial numbers.

Gall, Burroughs, Gerlaugh, and Edgington (1949a) developed special methods for culturing and studying rumen populations. Employing these technics Gall et al. (1949b) obtained rumen samples, using stomach tubes, from 21 cattle and 12 sheep on practical winter rations and

from 11 cattle and 6 sheep on pasture. Slide counts from the animals on winter rations gave averages of about 50 billion organisms per gram of fresh rumen contents while the counts were considerably higher for the animals on pasture, being about 96 billion organisms for cattle and 85 billion bacteria for sheep. In general the counts were comparable for cattle and sheep on similar diets, and likewise there appeared to be little difference in the types of organisms observed by gram staining on the different animals or on different diets. Cultural findings from both species on all rations were also quite similar although addition of grain to the diet led to a marked increase in the number of fast-growing organisms observed.

In another series of papers, Pounden and Hibbs (1948a, 1948b, 1949a), Hibbs and Pounden (1948), Pounden, Ferguson, and Hibbs (1950), and Conrad, Hibbs, Pounden, and Sutton (1950) employed the gram stain and direct microscopy in studying the microflora of dairy calves. Although their technic was not described and no counts were presented, they established a "hay" and a "grain" flora which appeared to be characteristically associated with the ingestion of these feeds. The "hay" flora consisted of 2 groups. In the first were large gram positive cocci in closely knit pairs and in the second were large gram positive, thick fairly square-ended rods, very large gram negative, cigar-shaped rods, and small gram negative, short rods in tetrads and multiples of 4 in shapes suggestive of window panes. The "grain" flora included medium sized, comparatively thin, gram positive rods sometimes staining in a granular manner and also gram negative rods

resembling organisms of the coliform group.

The significance of these hay and grain organisms remains to be determined but on the basis of cultural studies by other workers it appears doubtful that these bacteria play an important role in the rumen. On the other hand, it is possible that these indicator organisms may prove of value in studying variations in concentration of different feeds in the diet. Also Pounden and Hibbs (1948a, 1949a, 1949b) were able to establish characteristic rumen microorganisms in young calves on pasture when they were inoculated with cud materials from older cattle and when grain was not fed in excessive amounts. These animals also did not suffer from digestive tract disturbances as did uninoculated groups.

Recently Moir and Masson (1952) proposed a scheme for the microscopic identification of organisms (ciliate protozoa were not included) commonly encountered in the rumens of sheep. Their plan is based on direct microscopy and the terms employed to describe the 33 forms presented fall into 3 categories, namely, (a) morphological, (b) histochemical and staining reactions, and (c) ecological and functional. Twenty-five micrographs illustrating the different forms in their present scheme are included in the report. Their plan has merit since the study of rumen microbiology has advanced to the stage where some system of comparing organisms from the rumen is necessary in order that different workers have some common ground for discussion. These investigators admit that their scheme is not final and hence should provide a starting point for continued research in the microscopic examination

and description of the rumen microflora.

Fermentation Studies on Rumen Ingesta.

Much of the early literature pertaining to various phases of fermentation processes in ruminants has been reviewed by Washburn and Brody (1937). These investigators also developed a mask-spirometer method for obtaining directly expired air uncontaminated by chamber or outdoor air from cattle. This technic permitted the analysis at short intervals of O_2 consumption, CO_2 and CH_4 expiration and allowed accurate mapping of the time course after feeding. Employing the same animal at the same time, parallel time curves on the composition of rumen gas and on the CO_2 to CH_4 ratios were also made. They found that on an alfalfa ration the ratio of CO_2 to CH_4 declined from 2.6 immediately after feeding to 0.77, 15 hours later. When an alfalfa-grain ration was fed, the CO_2/CH_4 ratio declined from 2.6 to 0.97, at 15 hours and to 0.48, 23 hours after feeding. On grass the ratio was very high, 4.1, dropping to a minimum of 0.9 after 18 hours. It was observed that the rumen CH_4 level was relatively constant on all diets at about 30 per cent whereas on the alfalfa and alfalfa-grain diet the CO_2 level was between 60 and 70 per cent for the first 7 hours following feeding and then declined to 30 per cent 15 hours after feeding, and to 20 per cent after 20 hours. When the grass was fed, the CO_2 level rapidly rose to 80 per cent but soon started declining so that 12 and 23 hours after feeding the percentages were 40 and 10 respectively. Small quantities of H_2 were generally encountered in the rumen gas and the N_2 and O_2

time trends were opposite those of CO_2.

Washburn and Brody (1937) also determined the volumes and composition of gases in the rumen as well as other parts of the digestive tract of slaughtered animals. Data obtained on 20 animals including sheep and goats indicated that 75 to 100 per cent of the alimentary gas was contained in the rumen. With animals slaughtered within 24 hours after feeding, 96 per cent of the gas was found in the rumen, while 100 per cent was obtained in the rumens of goats killed 48 to 72 hours after feeding. As observed in living animals, the time course of the CO_2 to CH_4 ratio in slaughtered animals indicated variations in CO_2 percentages and not CH_4. These workers also found a relatively low CO_2/CH_4 ratio of about 0.5 in the intestine as compared to that in the rumen. This might be attributed to greater diffusibility of CO_2 than CH_4 from the intestine to the blood or possibly variations in bacterial flora or substrates fermented bringing about different relationships between CO_2 and CH_4 (or combustible gases).

Quin (1943) studied the influence of diet on gas production in the rumen of sheep. The volume of gas was measured by connecting the rumen through a fistula tube to a large graduated water manometer. The water level in the manometer arms was equalized thereby permitting the liberated gas to be recorded directly at constant atmospheric pressure. Sheep were fed a number of basic rations supplemented by test meals. Animals fed lucerne as a basic diet caused a rapid fermentation of a test meal of lucerne while a dosing through the fistula of wheat straw or maize samp (coarse hominy) failed to bring about any gas production

in the 90-minute test period. Dosing the rumen with 50 g of cane sugar or glucose brought about rapid fermentation and gas production similar to that noted with lucerne. On the other hand, most animals completely starved or on a ration of poor quality hay or straw lost the power to ferment glucose.

In vitro fermentation studies were performed by adding 50 ml of ruminal fluid stained through muslin to 250 ml Erlenmeyer flasks constantly agitated in a 39 C water bath. These flasks were connected to individual manometers which permitted direct readings of the gas volumes at atmospheric pressure. The gas produced from the rumen fluid itself was recorded at the end of 10 minutes after which 1 ml of 20 per cent glucose was added and subsequent readings taken at 10-minute intervals for 30 minutes. Practically no gas liberation was noted from rumen fluid obtained before the early morning feeding of sheep on a good diet whereas the addition of glucose caused a prompt fermentation and maximum gas evolution within the first 10 minutes, followed by a decrease so that in the third 10-minute interval the volume of gas produced did not greatly exceed the original basal rate. When 8 sheep were studied simultaneously in this manner there was a close relationship in the results obtained in 7 of the animals.

Quin varied the amount of glucose and found that the gas production was closely related to the concentration up to a level 0.4 per cent. With amounts from 0.8 per cent to 10.0 per cent there was no pronounced difference, whereas 15.0 per cent glucose caused a depression of the fermentation rate. When a number of different carbohydrates

were investigated, glucose and fructose gave similar results while sucrose showed a slower rate but exhibited a definite peak in the first 10 minutes. Maltose and mannite did not show any peak in the first 10 minutes and were fermented slowly. Lactose, galactose, arabinose, xylose, and rhamnose were fermented very feebly, while raw and boiled tapioca starch were not attacked in 30 minutes. However, at the end of 4 hours, 3 times as much gas was evolved from the boiled as from the raw starch. This volume from the soluble starch was approximately equivalent to that produced in the 30-minute period by the glucose and fructose.

Quin (1943) starved a sheep for 65 hours and found that the animal did not regain its normal daily consumption until after the third day, and did not return to its glucose fermentation ability as determined in vitro until the 10th day. Additional studies led to the conclusion that the rapid glucose fermentation in the rumen and in vitro tests was not associated with a free enzyme system but with cellular elements of relatively large size, viz., a yeast which he named Schizosaccharomyces ovis. He found that when ruminal fluid was mixed with glucose and agitated in the presence of air at 39 C, part of the sugar underwent extremely rapid oxidation with the evolution of large volumes of gas. Simultaneously another fraction was assimilated by the yeast and synthesized and stored as glycogen. If the animal was starved the yeasts disappeared and upon resumption of feeding a strongly iodophilic coccus supplanted the yeast cells. The cocci were responsible for the active synthesis of starch. Gas production was at a lower level but

was maintained for a longer period. As the yeast population increased, starch synthesis by the iodophilic cocci diminished. However, since ruminal activity occurs in an anaerobic environment, one would question whether this yeast, which yielded large volumes of gas under aerobic conditions, was important in ruminant digestion.

Later van der Westhuizen, Oxford, and Quin (1950) failed to isolate Schizosaccharomyces ovis from the rumen although other yeast-like organisms were obtained. However, none had the properties of S. ovis as observed in ingesta viz. the power of quick fermentation of added glucose with simultaneous storage of massive amounts of glycogen in the cells. They also showed the rumen "yeasts" not to be Mucor spores, nor oidia or yeast-like forms of Monilia, which they resemble in size and shape. Therefore they concluded that S. ovis is misnamed and is probably not a member of the Eumycetes.

McAnally (1943) considered the rate of gas evolution as a useful index in studying the rate of fermentation of different carbohydrates in vitro. In her procedure rumen ingesta were withdrawn by aspiration through the fistula before the morning feed from a sheep which received 400 g of lucerne hay twice daily, and was dosed with 3 liters of water daily in 2 portions through the rumen fistula. The ingesta were poured onto muslin and the fluid portion squeezed through. Following this, 8 ml portions of the filtrate were introduced into fermentation tubes which were then allowed to stand in the incubator at 37 C until their contents had reached this temperature. The substrate was then added in solution in an amount which was never more than 2 ml. The ingesta

and substrate were then mixed by shaking and the fermentation was observed in the incubator at 37 C. She employed glucose and noted considerable gas was formed in the first 10 to 15 minutes followed by a slower rate. She also observed that when the ratio of weight of glucose to ingesta volume was increased above a value which was approximately 0.2 g/8 ml there was a diminution in the rate of gas evolution. Comparing a number of carbohydrates, McAnally found glucose to be fermented most rapidly. Sucrose fermentation, though not starting as rapidly, soon attained a rate similar to glucose, while lactose was fermented only slightly. At the end of one hour about one-fourth the volume of gas was produced from maltose as from glucose, or from cellobiose as from sucrose. In other trials maltose fermentation rates compared favorably with those of glucose after a lag period during which the disaccharide was probably hydrolyzed to glucose. Cellulose digestion was studied by placing a number of bags of fine natural silk containing weighed mashed filter paper in the rumen of a sheep having a fistula. Periodically a bag was removed, washed, dried, and weighed. A few such experiments indicated that considerable digestion of cellulose occurred between the 20th and 26th hours.

The fermentation of carbohydrates in the rumen of sheep is known to lead to the formation of large amounts of the lower fatty acids including acetic, propionic, and butyric acid. These acids are readily absorbed directly into the blood from the rumen, as well as from the reticulum, omasum, and large intestine. The rate of absorption increases as the pH falls (Gray, 1947, 1948). Danielli, Hitchcock,

Marshall, and Phillipson (1945) observed that at pH 5.8 large amounts of free acids as well as fatty acid ion were lost from the rumen. Part of the free acid was absorbed through water-filled pores, but the greater part was absorbed through the lipoid membranes of the epithelial cells. They found that the permeability of the rumen epithelium was such that the pH of the ingesta tended to move towards neutrality, independently of the neutralizing action of the saliva.

Phillipson (1947-48) reviewed the literature pertaining to fermentation in the alimentary tract and the metabolism of the derived fatty acids and estimated that sufficient quantities of the lower homologues are produced in the rumen to furnish at least 40 per cent of the fasting energy requirements.

Recently Gray, Pilgrim, and Weller (1951) measured the amounts of volatile fatty acids and methane produced during fermentation of wheaten hay and lucerne hay in vitro by microorganisms from the rumen of sheep. They found it necessary to employ a large inoculum of rumen fluid in order to obtain results similar to those known to occur in the natural process. Their data indicated the products per kilogram of wheaten hay to be 200-250 g fatty acids with 41 per cent acetic acid, 43 per cent propionic acid, and 16 per cent butyric acid (by weight). Fifteen liters of methane were formed but no hydrogen. Employing lucerne hay they obtained 250-300 g fatty acids, with 53 per cent acetic acid, 29 per cent propionic acid, and 18 per cent butyric acid (by weight); and 20 liters methane but no hydrogen. They concluded that because of the more rapid absorption of propionic acid from the

rumen than of the other acids, the proportion of this acid remaining in the rumen fluid was considerably less than the proportion actually formed in the fermentation.

Gray and Pilgrim (1951) criticized the work of Barcroft, McAnally, and Phillipson (1944), Danielli, Hitchcock, Marshall, and Phillipson (1945), and Elsden and Phillipson (1948) who concluded that acetic is more rapidly absorbed by the rumen than either propionic or butyric acid. The criticisms were based on the alkaline pH at which some of the work was conducted and also because the experimental conditions differed greatly from the normal conditions of the rumen. Gray and Pilgrim (1951) fed sheep with permanent rumen fistulas a constant ration of either wheaten hay or lucerne hay. Samples of rumen fluid were withdrawn at intervals after feeding and analyses of the fatty acids were made. It was found that characteristic changes in the composition of the mixture of volatile fatty acids in the rumen take place throughout the day. The data presented support their view that propionic acid is relatively more rapidly absorbed than either acetic acid or butyric acid, and that the fermentation of wheaten and lucerne hays in the rumen produces a mixture of fatty acids in which propionic acids constitutes a larger proportion than it does under similar conditions in vitro.

Johnson, Hamilton, Robinson, and Garey (1944) studied the utilization of non-protein nitrogen in two lambs on urea and casein rations. Maximum methane production occurred during the first hour following feeding regardless of the ration. A gradual fall in gas formation was

observed until at the end of 9 hours there was about a 50 per cent decrease.

Sapiro, Hoflund, Clark, and Quin (1949) carried out studies in vitro to determine the fate of nitrate in ruminal ingestion. They found that the variation of nitrate disappearance followed the same trend as glucose fermentation and cellulose breakdown in any particular sheep. These investigators concluded that the disappearance of nitrate from rumen contents established another test for ruminal activity.

This review of the literature indicates that numerous approaches have been taken in studying ruminants digestion but many problems pertinent to this subject remain unsolved. The fact that ruminants rely on microorganisms for a phase of their digestion before their own digestive enzymes have an opportunity to act makes them an interesting group for study particularly because of their economic importance to man.

The present investigation was undertaken in an effort to determine whether a manometric method employed in preliminary experiments by McBee (1950) for studying the activity of the rumen microflora was suitable for evaluating other aspects of ruminant digestion. Other manometric methods have been employed in studies of this type, but in general they have not been carried out under conditions closely approximating those in the rumen. The present method measures the rate of gas evolution when an excess of a known substrate is added to rumen ingesta in a constant volume Warburg apparatus. At the start it was recognized that each phase of the technic would have to be checked a number of

times in order to become familiar with the details and to determine the optimum conditions for operation. Following this it was planned to investigate the effect of adding different substrates to rumen ingesta obtained from sheep at different periods after feeding different rations. Since conflicting reports were found in the literature regarding the pH of rumen contents and the numbers of bacteria therein, it was planned to gather such information on the samples collected whenever possible and to note whether any correlations in different aspects of the work existed.

MATERIALS AND METHODS

Experimental Animals.

An ewe of the Targhee breed furnished by the Animal Industry Department at Montana State College and identified as T6359 was used in the first part of these studies. In December of 1949, members of the Veterinary Research staff at this institution provided a permanent rumen fistula in the 4-year-old sheep following the general procedure recommended by Quin, van der Wath, and Myburgh (1938). The rumen fistula was closed with a lucite plug made by the Nittany Scientific Service, State College, Pennsylvania. This plug had an inside flange 60 mm in diameter attached to a tube 60 mm in length and having an inside diameter of 20 mm. The threaded tube permitted a lock washer to be turned down to the desired tightness as well as allowing a cap to be screwed on to prevent loss of rumen contents. Previously 2 different plugs made at this institution from plexiglass and very similar in design to the one described had been placed in a ram and an ewe. Both had "lost" these plugs from the fistulas on several occasions. Forcing the plugs back into place or enlarging the openings by cutting and then suturing the plug in position eventuated in the death of both animals.

The manufactures of plexiglass, Rohm and Haas Co., felt that their product was not toxic to tissues since this material had proven entirely compatible with human body tissues and had been used in surgical implants. It was possible that the plugs employed in some of the earlier work were not as smooth as the one used most recently and that this was

responsible for the animal ejecting the tube. However, from correspondence and conversations with others working in this field, it appears that loss of plugs and even death of such experimental animals is not too uncommon in the early stages of such work. In the light of later experiences, it now appears likely that both animals might have been saved if their rumens had been inoculated with rumen contents from healthy animals and plenty of water administered through the fistula.

The experimental animal had free access to alfalfa hay and water at all times unless otherwise specified. She was isolated in a stall having a heavy one-inch mesh wire screen flooring six inches above the main floor, through which excretory wastes dropped and could be washed into the sewer.

A second sheep used in later studies is described in experiment 10.

Collection of Samples.

Samples of rumen ingesta were generally collected before the morning feeding after the animal had been deprived of food and water for 12 to 15 hours. After a feeding period, all rations and water were removed and additional samples were obtained at various intervals.

Clean milk dilution bottles previously flushed with CO_2 and closed with rubber stoppers were filled by inserting a 55 cm plastic tube having 6 mm inside and 8 mm outside diameters into the rumen through the fistula and withdrawing a portion of the contents. The practice of squeezing and releasing the rubber bulb on the end of the tube several

times in the upper regions of the rumen allowed the bulb and tube to be filled with rumen gases and possibly aided in maintaining more normal conditions for rumen microorganisms during collection and until tested. Minor changes in the collection of samples were made and are described later.

Direct Microscopic Counts.

Samples were immediately taken to the laboratory and shaken vigorously by hand 50 times. Serial dilutions of 10^2 and 10^4 were prepared in tap water and a calibrated loop was used to transfer and spread 0.01 ml of each dilution onto duplicate clean microscope slides over an area of 1 cm^2. The smears were air-dried and fixed by passing through a flame 3 times. Staining was for 10 minutes employing Ziehl's carbol fuchsin diluted 1:10. Since the smear of the 10^2 dilution generally gave a more representative picture of the rumen population, between 12 and 20 fields were counted using the oil immersion objective (95X) on a Spencer binocular microscope. One of the 10X oculars contained a disc with crossed lines dividing the field into squares. Only the organisms in the four center squares were counted. Representative areas were chosen by moving the mechanical stage in a definite pattern, although any field having much debris was not counted. This seldom occurred as most of the fields could be counted rather easily, even though the majority of organisms observed were very small.

The following data were used in calibrating the microscope: The diameter of the microscopic field obtained by using a stage micrometer

was 140 microns, giving a radius of 0.007 cm and an area of 0.00015 cm^2. The area of the smear was 1 cm^2 making 6,666 fields over which 0.01 ml of the sample was spread. This gave a factor of 666,600 for the sample. However, the area of the four center squares used in counting was 0.000019 cm^2. This then gave a factor of 5,262,633 or when using the 10^2 dilution of the sample and rounding off the numbers, a factor of 526,000,000.

pH Determinations.

Determinations of pH were made in the laboratory after the sample for the manometric studies had been removed. A Beckman glass electrode pH meter, model H2, was employed and the entire contents (60-80 ml) of the collection bottle were tested. Less than 0.1 of a pH unit change was noted when the sample was returned to the bottle, stoppered, and allowed to stand at room temperature for 1-2 hr before rechecking. This slight change in pH indicated that the material was well buffered and that pH determinations made within 20-30 min of collection were probably quite representative of conditions in the rumen.

Manometric Procedures.

Umbreit, Burris, and Stauffer (1947) point out that if one employs a bicarbonate buffer in an atmosphere of CO_2, any CO_2 released by the cells will escape into the atmosphere and can be measured manometrically. Likewise any acid produced under these conditions will combine with the metallic ions formerly associated with bicarbonate thus

releasing one mole of CO_2 into the atmosphere for each equivalent of acid formed. Hence a measure of acid and gas production should give some indication of the fermentation capacity of the ruminants.

The Warburg manometers were calibrated using mercury according to the method of Loomis (1949) and the total volume of reactants per flask was 2.5 ml. Brodie's solution was employed in the manometers, unless otherwise mentioned. All trials were run at 37 C, which closely approximated the normal temperature of about 39 C for sheep. Since the rumen functions in an anaerobic atmosphere, all reactions were carried out in the presence of CO_2. The details of the flask reactants and the amounts employed are described with each experiment performed.

EXPERIMENTS AND RESULTS

Experiment 1. Trials conducted in developing the manometric procedure.

The first phase of the problem undertaken was to find whether the fermentation results obtained on 3 different samples of rumen ingesta were comparable. In each case the basal rate (no substrate added) and the glucose fermentation rate were studied. One ml of 1.0 per cent $NaHCO_3$ and 1.0 ml of rumen fluid were added to each vessel. In the basal runs 0.5 ml of 0.5 per cent $NaHCO_3$ was placed in the sidearms, whereas 0.5 ml of 0.5 per cent $NaHCO_3$ containing an excess of glucose was added to the other sidearms. Equilibration was for 10 min before dumping. Readings were made at 5-min intervals for 45 min. The findings are given in table 1.

TABLE 1. Basal and glucose fermentation of rumen samples obtained consecutively from ewe.

Sample	Microliters CO_2 produced		Glucose/Basal ratio
	Basal	Glucose	
1	75	831	11.1
2	80	950	11.8
3	67	767	11.4

Total reaction volume, 2.5 ml including: 1 ml rumen ingesta plus 1.0 ml 1.0 per cent $NaHCO_3$ in vessel and 0.5 ml 0.5 per cent $NaHCO_3$ with or without glucose in sidearms. Atmosphere, CO_2; temperature, 37° C; duration, 45 min; manometers, Brodie's solution.

The results are recorded as microliters CO_2 which were obtained by multiplying the respective flask constants by the total change

recorded on the manometers during the experimental period. Although other gases may have been liberated, it was assumed that CO_2 constituted most of the volume. It was found that the basal rates varied between 6 and 16 per cent while the glucose varied between 8 and 20 per cent. However the glucose-basal ratio showed a variation among experimental vessels of less than 7 per cent. Since the rumen is such a dynamic organ, these variations were not considered excessive at this point.

Treatment of Rumen Samples.

The next point considered was the effect of treating the rumen samples in different ways before adding the material to the Warburg vessels for making basal runs. The sample was divided into 3 parts and handled as follows: (a) no treatment, (b) disintegrated in Waring blendor for 1 min in an atmosphere of CO_2, (c) filtered through 2 layers of cheesecloth and filtrate used.

One ml of each part of rumen sample plus 1.5 ml 1.0 per cent $NaHCO_3$ were added to respective flasks. Three different basal runs were made on rumen ingesta obtained from the ewe at different intervals after feeding, viz, 16.0, 1.3 and 5.3 hr. The results are shown in table 2.

In general the amount of CO_2 evolved was lowest (48-56 ul) from the sample collected prior to the morning feeding or about 16 hr after the ewe had received food and water. However 5.3 hr after the morning meal the fermentation activity (41-66 ul) had dropped considerably

TABLE 2. Basal fermentation capacity of rumen samples collected at 3 intervals following feeding and treated in different ways.

Treatment of sample	Microliters CO_2 produced Hours after eating		
	16.0	1.3	5.3
None	48	96	66
Waring blendor	56	101	63
Filtrate of original	52	87	41

Total reaction volume, 2.5 ml including: 1.0 ml rumen ingesta plus 1.5 ml 1.0 $NaHCO_3$. Atmosphere, CO_2; temperature, 37 C; duration, 35 min; manometers, Brodie's solution.

below the high point (87-101 ul) obtained about 1.3 hr following feeding. From this study there did not appear to be any definite advantage to disintegrating the rumen contents in the Waring blendor whereas the rumen mixture of liquid and solid (no treatment or Waring blendor treatment) generally resulted in greater gas production than the filtrate. It seems probable that many organisms were retained in the residue during the filtration and hence were not present to contribute to the fermentation activity in the flask.

Additional studies were then performed to note the effect of treating rumen samples in different ways prior to studying basal as well as glucose fermentation. Samples of rumen contents were obtained on 3 occasions, viz, 16.0, 1.3 and 5.0 hr following feeding. Each sample was divided and treated as follows: (a) no treatment, (b) disintegrated for 1 min in a Waring blendor continually flushed with CO_2, (c) half of (b) poured through 2 layers of cheesecloth and filtrate used. The vessels contained 1 ml of rumen material and 1 ml 1.0

per cent $NaHCO_3$. For the basal runs the sidearms contained 0.5 ml 0.5 per cent $NaHCO_3$ whereas the same amount of buffer containing 10.0 per cent glucose was added to the other sidearms. The runs were for 35 min and the findings are given in table 3.

TABLE 3. Basal and glucose fermentation capacity of rumen samples collected at 3 intervals following feeding and treated in different ways.

Treatment of sample	Microliters CO_2 produced					
	Hours after eating					
	16.0		1.3		5.0	
	Basal	Glucose	Basal	Glucose	Basal	Glucose
None	55	1140 (20.7)*	163	895 (5.5)*	55	657 (11.9)*
Waring blendor	69	1117 (16.2)	179	817 (4.6)	64	638 (10.0)
Filtrate from blendor	48	994 (20.7)	141	659 (4.7)	52	610 (11.7)

*Times basal rate exceeded.

Total reaction volume, 2.5 ml including: 1.0 ml rumen material plus 1.0 ml 1.0 per cent $NaHCO_3$ in vessel and 0.5 ml 0.5 per cent $NaHCO_3$ with or without 10.0 per cent glucose in sidearms. Atmosphere, CO_2; temperature, 37 C; duration, 35 min; manometers, Brodie's solution.

In general the basal rates were similar to those observed in table 2 except that in the present trials the amount of CO_2 liberated from the samples obtained 1.3 hr after the ewe had eaten was nearly twice as great. The basal samples disintegrated in the Waring blendor yielded 10 to 20 per cent more gas than those not treated. This difference was further accentuated when the filtrate obtained from the disintegrated material was compared.

The glucose fermentation rates of the untreated samples were

about 3 to 10 per cent greater than the disintegrated samples and about 7 to 14 per cent greater than the filtered materials.

It is interesting to note that the glucose rate exceeded the basal rate from 16 to 20 times after the ewe had been fasted for about 16 hr but dropped to about 5 times when the rumen contents were studied approximately 1.3 hr after feeding. This was caused by a greater basal activity and a lower glucose activity than encountered at the 16 hr period. Five hours after eating the spread between the glucose and basal rates had increased 10 to 12 times largely because of the decreased basal activity.

As a result of these runs, it did not appear that the differences obtained by various treatments warranted disintegrating the sample in the Waring blendor or employing a filtrate of the ruminal contents. Hence the sample was used as obtained from the rumen, unless indicated.

Different Concentrations of Substrate.

At this point various concentrations of glucose were added to the rumen ingesta and the fermentation activities determined.

A sample of rumen ingesta was obtained after the ewe had been without food for 36 hr. The material was quite liquid and very few solid particles were present. One ml 1.0 per cent $NaHCO_3$ and 1.0 ml rumen fluid were added to the vessels. One half ml 0.5 per cent $NaHCO_3$ solution containing 5, 10, and 20 per cent glucose and giving a final sugar concentration in the dumped flasks of 1, 2, and 4 per cent respectively was employed in the sidearms. The run was for 35 min and the

results and shown in table 4. The microliters of gas produced for 0, 1, 2, and 4 per cent glucose were 29, 508-519, 534, and 456-496 respectively. Thus the maximum volume of gas was formed with 2 per cent glucose while 4 per cent of the carbohydrate seemed to have an inhibitory effect.

TABLE 4. Effect of varying the glucose concentration on the fermentation capacity of rumen ingesta obtained from ewe after fasting 36 hr.

Per cent glucose	Microliters CO_2 produced
0	29
1	508
1	519
2	534
4	496
4	456

Total reaction volume, 2.5 ml including: 1.0 ml rumen ingesta plus 1.0 ml 1.0 per cent $NaHCO_3$ in vessel and 0.5 ml 0.5 per cent $NaHCO_3$ with or without glucose in sidearms. Atmosphere, CO_2; temperature, 37 C; duration, 35 min; manometers, Brodie's solution.

When the data from this run are compared with those given in table 3 in which the animal had been fasted for 16 hr instead of 36, it will be noted that the basal and glucose (2.0 per cent final concentration) fermentation rates were approximately 50 per cent less. However, the glucose-basal ratios were about the same, namely, 18.4 in the present run and 20.7 in the previous trial. This might indicate that there were fewer organisms in the rumen after prolonged fasting but that those remaining had a fermenting capacity similar to those present after 16 hr of fasting.

The results of this run indicated that a 10.0 per cent concentration of glucose in the sidearms, giving a 2.0 per cent final concentration in the vessels, appeared satisfactory for future studies.

Different Periods of Equilibration.

It is well established that the microorganisms in the rumen are functioning in an atmosphere devoid of free oxygen. In an effort to maintain anaerobic conditions and to be certain that the CO_2 of the atmosphere in the flasks was in equilibrium with the reactants, the effect of different periods of equilibration, before dumping the substrates, was studied.

A rumen sample was obtained on the same day as in the previous run but 1 hr after the ewe had been fed. The basal flask (no substrate) was equilibrated for 25 min and the other flasks containing glucose in the sidearms were agitated 25, 20, 15, 10, and 5 min respectively before tipping the substrate.

TABLE 5. Effect of equilibration period on fermentation of rumen ingesta from ewe 1 hr after eating.

Equilibration (min)	Substrate	Microliters CO_2 produced
25	none	65
25	glucose	213
20	"	223
15	"	184
10	"	184
5	"	202

Total reaction volume, 2.5 ml including: 1.0 ml rumen ingesta plus 1.0 ml 1.0 per cent $NaHCO_3$ in vessel and 0.5 ml 0.5 per cent $NaHCO_3$ with or without glucose in sidearms. Atmosphere, CO_2; temperature, 37° C; duration, 35 min; manometers, Brodie's solution.

The flasks contained 1.0 ml rumen contents and 1.0 ml 1.0 per cent $NaHCO_3$, while 0.5 ml 0.5 per cent $NaHCO_3$ containing 10.0 per cent glucose was placed in the sidearms. The results (table 5) were not very consistent being 213, 223, 184, 184, and 202 ul CO_2 for the equilibration periods of 25, 20, 15, 10, and 5 min respectively. However the 10 min period was selected for future runs as probably representing sufficient time for the contents to reach equilibrium.

Experiment 2. A comparison of bicarbonate and phosphate buffers on the fermentation rates of rumen ingesta studied manometrically.

Garton (1951) investigated the distribution of inorganic phosphorus and soluble calcium and magnesium in the stomach of sheep. He found that ruminal fluid was a good buffer in which phosphate, along with bicarbonate, played an important role in buffering acid production resulting from bacterial fermentation.

A series of runs was made to determine the merits of these buffers. The $NaHCO_3$ buffer previously mixed with rumen contents and investigated manometrically was compared with a phosphate buffered salt solution. composed of equal parts of solution A (1.5 per cent $Na_2HOP_4 \cdot 12H_2O$); solution B (1.2 per cent NaCl, 0.3 per cent KH_2PO_4, 0.09 per cent $(NH_4)_2SO_4$, 0.3 per cent $MgSO_4$, 0.03 per cent $CaCl_2$); and solution C (0.03 M $NaHCO_3$). The final pH was approximately 7.0. Basal and glucose fermentations were studied employing 1.0 ml of rumen contents with 1.0 ml of each buffer. In addition 0.5 and 0.2 ml of rumen fluid and glucose were investigated with the phosphate buffer. The rumen

ingesta were obtained from the ewe 7.5 hr after eating on the same day that the samples for the previous runs were collected. The contents of all flasks were in an atmosphere of CO_2.

The data given in table 6 indicate that the bicarbonate buffer results exceeded those obtained with the phosphate buffer by 20 per cent in the basal runs and by 10 per cent in the glucose runs when 1.0 ml of rumen sample was employed. Decreasing the volume of rumen inoculum decreased the fermentation activity in about the proportion expected.

TABLE 6. Basal and glucose fermentative capacity of rumen ingesta from ewe 7.5 hr after eating, employing bicarbonate and phosphate buffers and different volumes of sample.

Buffer	Substrate	Rumen sample (ml)	Microliters CO_2 produced
Bicarbonate	none	1.0	75
Phosphate	"	1.0	60
Bicarbonate	glucose	1.0	535
Phosphate	"	1.0	483
"	"	0.5	203
"	"	0.2	75

Total reaction volume, 2.5 ml including: 1.0, 0.5 or 0.2 ml rumen ingesta plus varying amounts of buffer with or without glucose to make 2.5 ml. Atmosphere, CO_2; temperature, 37 C; duration, 35 min; manometers, Brodie's solution.

Another similar run was made employing a rumen sample after the ewe had been deprived of food for 22 hr. The findings are given in table 7. The amount of gas liberated in the basal run (36 ul) employing phosphate buffer exceeded that in the bicarbonate buffer (30 ul) by about 17 per cent. On the other hand, the volume of CO_2

was 13 and 6 per cent greater respectively for 1.0 ml and 0.05 ml of rumen inoculum in the bicarbonate buffer as compared to the phosphate buffer. When the amount of rumen fluid was halved the ul CO_2 formed were decreased by nearly 50 per cent. This was advantageous since with less gas evolved it was not necessary to reset the manometers as often, if at all.

TABLE 7. Basal and glucose fermentative capacities of rumen ingesta from ewe 22 hr after eating, employing bicarbonate and phosphate buffers and different volumes of sample.

Buffer	Substrate	Rumen sample (ml)	Microliters CO_2 produced
Bicarbonate	none	1.0	30
Phosphate	"	1.0	36
Bicarbonate	glucose	1.0	538
Phosphate	"	1.0	469
Bicarbonate	"	0.5	248
Phosphate	"	0.5	234

Total reaction volume, 2.5 ml including: 1.0 or 0.5 ml rumen ingesta plus necessary amounts of buffer, with or without glucose, to make 2.5 ml. Atmosphere, CO_2; temperature, 37 C; duration, 35 min; manometers, Brodie's solution.

Experiment 3. Employment of the phosphate buffer in comparing the fermentation activity, number of bacteria and pH of rumen samples collected for 5 days from ewe at different periods after eating.

In the light of later results, it is rather difficult to interpret the findings obtained when employing the phosphate buffer as compared to the bicarbonate buffer where one can determine the metabolic CO_2 and the CO_2 liberated by acid production under anaerobic conditions. However, a number of experiments were conducted using the phosphate

buffer and since the results appear to give some indication of differences when various substrates were employed or when samples were collected at different hours during the day or when the animal was fed different diets, these experiments and results will be presented at this point. This seems justified in light of the fermentation studies reported by Washburn and Brody (1937), McAnally (1943), Quin (1943), Johnson, Hamilton, Robinson and Garey (1944) and discussed in the literature review.

Several similar runs were made over a period of 5 days. The first samples were collected in the morning after the animal had been without food and water for 15 and 12 hr respectively. Alfalfa and water were then available for 1 hr feeding. Additional samples were then obtained approximately 0.5, 2.0, 4.5, and 6.5 hr thereafter. Basal and glucose fermentation runs were made for a 40 min period employing 0.5 ml rumen fluid, 1.5 ml phosphate buffer and 0.5 ml 10.0 per cent glucose (except in the basal run where 0.5 ml phosphate was added).

The data are shown in table 8. It will be noted that the basal fermentation activity from the samples collected after the ewe had been without food for 15 hr was generally considerably lower than at any other time during the period studied. Exceptions to this were found in the runs on 7-14-50. Generally the basal activity was greatest in the samples obtained 0.5 hr after the ewe had eaten and tended to decrease at later periods. One will also note considerable variation among comparable runs on different days. When glucose was added as a substrate, the amount of CO_2 evolved was always greater than in the

TABLE 8. Basal and glucose fermentation activities of rumen ingesta obtained on 5 days from ewe at different periods after eating.

Sample collected after eating (hr)	Microliters CO_2 produced									
	7-12-50		7-14-50		7-18-50		7-19-50		7-21-50	
	Basal	Glucose	Basal	Glucose	Basal	Glucose	Basal	Glucose	Basal	Glucose
15.0	18	256	53	407	18	260	30	222	30	273
0.5	67	192	87	308	165	278	187	320	239	334
2.0	36	203	40	239	181	292	155	327	169	381
4.5	28	141	95	238	60	223	96	345	176	349
6.5	38	184	24	251	47	262	103	341	118	301

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta, 1.5 ml phosphate buffer and 0.5 ml 10.0 per cent glucose (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

basal studies. There was a very pronounced increase in gas production in the basal runs employing rumen ingesta from the ewe shortly after eating. Sometimes there was a decrease but never as great an increase from the sample to which glucose was added. The extent that the glucose fermentation exceeded the basal activity is depicted in table 9.

TABLE 9. Glucose/basal fermentation ratio of rumen ingesta obtained on 5 days from ewe at different periods after eating.

Hours after eating	7-12-50	7-14-50	7-18-50	7-19-50	7-21-50
15.0	14.2	7.7	14.4	7.4	9.1
0.5	2.9	3.5	1.7	1.7	1.4
2.0	5.6	6.0	1.6	2.1	2.3
4.5	5.0	2.5	3.7	3.6	2.0
6.5	4.8	10.5	5.6	3.3	2.6

In general the greatest difference between the basal and glucose activity was on the morning samples before the ewe was fed and the

least difference on the samples collected 0.5 hr after the 1 hr feeding period. At later intervals there was a gradual increase in the ratio particularly on the last 3 days.

Direct microscopic bacterial counts were made on all of the samples drawn for this experiment. Likewise pH determinations were made soon after the samples were brought to the laboratory in closed bottles. These findings are given in table 10.

TABLE 10. Bacterial counts and pH of rumen samples from ewe at different periods after eating.

Hours after eating	7-12-50		7-14-50		7-18-50		7-19-50		7-21-50	
	Counts*	pH	Counts	pH	Counts	pH	Counts	pH	Counts	pH
15.0	10.0	7.0	32.1	7.4	35.8	7.3	21.0	7.4	22.6	7.5
0.5	22.1	7.0	42.6	7.2	21.0	6.9	27.3	6.9	32.1	7.2
2.0	32.6	6.8	10.0	7.3	17.4	7.3	26.8	6.8	29.5	7.2
4.5	28.4	7.0	21.0	7.1	16.0	6.9	34.2	7.1	23.1	7.2
6.5	30.5	7.2	18.4	7.3	25.2	7.6	22.6	7.6	28.4	7.4

*Bacterial counts in billions per ml.

Some variations in the numbers of bacteria were found and the range for all samples was between 10.0 and 42.6 billion per ml. However no significant trend or pattern was apparent in the counts from samples collected at various intervals or on different days.

The pH of the early morning sample was generally alkaline (7.0-7.5). Shortly after food was ingested and fermentation was active in the rumen, the pH often dropped to 6.9-7.2, but 6.5 hr after eating it had risen to about the level of the first sample of the day. The pattern of pH changes appears quite similar for the different days.

The variations obtained in the fermentation studies and in the bacterial counts might be expected when one considers that it is difficult to influence the amount of food and water consumed by the sheep during the periods allotted for feeding. The next experiment was designed to control one variable, namely, the intake of water.

> Experiment 4. A comparison of fermentation activity, number of bacteria and pH of rumen samples collected from ewe on controlled water intake and at different periods after eating.

The weight of alfalfa hay and water consumed by the ewe at each feeding period was determined for a number of days prior to this experiment. It was found that the animals drank about 3 pounds of water for every pound of hay eaten; hence this amount of liquid was added directly to the rumen via the fistula each day at regular times. It was customary to add a known amount of water at the start of the feeding period and then an additional amount at the end after the weight of hay consumed was determined.

Samples were collected from the rumen on 3 consecutive days, 15 hr after fasting and 1 and 5 hr after the morning feeding period. Manometric studies were made over 40 min periods employing 0.5 ml rumen contents, 1.5 ml phosphate buffer and 0.5 ml 10.0 per cent glucose (except in basal runs where 2.0 ml of buffer was used).

The results are shown in table 11 and substantiate those of the previous run (table 8) in that the basal activity was lowest (27-55 ul) in the early morning samples collected 15 hr after eating and highest (121-246 ul) in the ingesta obtained 1 hr after feeding. Five hours

after eating, the basal fermentation (114-155 ul) was less than that of the previous period. On the other hand, the glucose fermentation capacity was greatest in the early morning samples (275-416 ul) and tended to decrease to 252-347 and 263-390 ul 1 and 5 hr respectively after eating.

TABLE 11. Basal and glucose fermentation activities of rumen ingesta obtained on 3 consecutive days at different periods after eating from ewe on controlled water intake.

Date	Microliters CO_2 produced								
	Hours after eating								
	15			1			5		
	Basal	Glucose	Glucose/ Basal	Basal	Glucose	Glucose/ Basal	Basal	Glucose	Glucose/ Basal
7-25-50	40	301	7.5	164	268	1.6	134	299	2.2
7-26-50	27	275	10.1	121	252	2.1	114	263	2.3
7-27-50	55	416	8.6	246	347	1.4	155	390	2.4

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta, 1.5 ml phosphate buffer and 0.5 ml 10.0 per cent glucose (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

Although variations were still apparent in the ul CO_2 produced in the basal and glucose fermentations at comparable periods on the 3 different days of this experiment, the glucose/basal ratio seemed to give a better comparison (table 11). This was particularly true in the samples collected 5 hr after eating. It seemed quite likely that ruminal activity was approaching an equilibrium at this point, whereas the results in the period shortly after eating may be influenced by the amount of food and water consumed. However, one might expect a better agreement of results in the early morning sample since there had been

ample time for equilibrium to be established. A number of factors might have been responsible for these differences, such as the rate at which food left the rumen, the nature of the sample obtained and the manometric method itself under investigation.

The number of bacteria (table 12) varied somewhat in comparable periods on the different days as well as in the different periods on the same days. In no instance was the difference more than approximately double (16.8-32.1 billion per ml). However, it was questionable at this point whether these variations were particularly significant, and it appeared that the numbers of bacteria remained rather constant within the conditions of the present experiment.

TABLE 12. Bacterial counts and pH of rumen samples obtained on 3 consecutive days at different periods after eating from ewe on controlled water intake.

Date	Hours after eating					
	15		1		5	
	Counts*	pH	Counts	pH	Counts	pH
7-25-50	22.1	7.4	16.8	6.9	31.6	7.1
7-26-50	25.8	7.5	24.7	6.7	22.6	7.0
7-27-50	28.9	7.5	32.1	6.5	23.1	6.8

*Bacterial counts in billions per ml.

The pH findings given in table 12 substantiate those of the previous run (table 10) in that the pH was highest in the morning (7.4-7.5) after the animal had been deprived of food for 15 hr. Shortly after eating, the pH of the ingesta had dropped to 6.5-6.9, and when tested again 5 hr after eating, it had risen to pH 6.8-7.1.

Experiment 5. A comparison of fermentation activity on several carbohydrates, bacterial counts and pH of rumen samples from ewe at different periods after eating alfalfa hay.

Since the findings in the last experiment showed a general pattern for the basal and glucose fermentation on 7 different days, this experiment was designed to note the fermentation activity on several other carbohydrates. The pH and the number of bacteria were also determined.

On 2 consecutive days the activity of rumen ingesta on glucose, D-xylose, hemicellulose from corn cobs, Difco's soluble starch and lignin was studied manometrically. The lignin was a by-product from a wood molasses plant in Wisconsin and has been used as an inert filler in a number of experimental diets by the Department of Animal Industry at Montana State College.

Rumen samples were collected in the morning 15 hr after feeding as well as 1 and 5 hr after the ewe had eaten and had received water through the fistula as in the previous experiment. The first sample of each day was thick and rather difficult to collect. This was not the case when the other samples were withdrawn within a few hours after eating. Because of the hot weather and the apparent need for more liquid, the water supplied through the fistula was increased to 3.5 pounds per pound of hay consumed.

Manometric procedures were the same as in the previous experiment, and 0.5 ml of a 10.0 per cent concentration of each substrate was employed in the sidearms. The results obtained after 40 min runs are

shown in table 13. Variations were found between the amount of gas formed on the two consecutive days. However, the fermentation ratio of the different substrates and the basal indicated a close agreement in many instances. The basal and glucose activities are in general similar to those shown in tables 9 and 11.

TABLE 13. Basal and various carbohydrate fermentation activities of rumen ingesta obtained on 2 consecutive days at different periods after eating from ewe on controlled water intake.

Substrate	Microliters CO_2 produced					
	8-1-50			8-2-50		
	Hours after eating					
	15	1	5	15	1	5
Basal	50	96	88	45	139	114
Glucose	640 (12.8)*	157 (1.6)	244 (2.8)	334 (7.4)	297 (2.1)	316 (2.8)
D-xylose	127 (2.5)	117 (1.2)	150 (1.7)	168 (3.7)	168 (1.2)	196 (1.7)
Hemicellulose	173 (3.5)	108 (1.1)	181 (2.1)	187 (4.2)	128 (0.9)	160 (1.4)
Soluble starch	151 (3.0)	126 (1.3)	119 (1.4)	138 (3.1)	288 (2.1)	188 (1.7)
Lignin	187 (3.7)	156 (1.6)	175 (2.0)	202 (4.5)	242 (1.7)	- **

*Times basal rate exceeded.
**Laboratory accident.

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta, 1.5 ml phosphate buffer and 0.5 ml 10.0 per cent substrate (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

The glucose fermentation exceeded that of D-xylose, hemicellulose, soluble starch and lignin by several fold in some runs. The differences in the fermentation of these latter substances were not too great, although it was surprising to find the lignin being attacked rather

actively. Subsequently the water soluble fraction of the lignin was extracted, and when the residue was tested manometrically, the results were very similar to the basal. This would explain the findings shown in table 13, but perhaps indicates the need for further investigations on the lignin if it is to be used as an inert material in nutritional diets. However this is beyond the scope of the present study. In all cases the ratio between the fermentation of the substrates and that of the basal was greatest in the early morning samples after the animal had been deprived of food and water for about 15 hr. Likewise the ratio was always lowest with the samples collected approximately 1 hr after the morning feeding. An increase in the ratio was also observed when rumen material was obtained 5 hr after the ewe had eaten. These ratios were influenced considerably by the variations in the basal activity of the different samples studied.

The number of bacteria and the pH of the rumen ingesta are given in table 14. The extremes of the bacterial counts for all samples were between 18.4 and 35.8 billion per ml, and the numbers were similar to those reported in the 2 previous trials.

TABLE 14. Bacterial counts and pH of rumen ingesta obtained on 2 consecutive days at different periods after eating from ewe on controlled water diet.

Date	Hours after eating					
	15		1		5	
	Counts*	pH	Counts	pH	Counts	pH
8-1-50	35.8	7.6	21.0	6.7	26.0	6.8
8-2-50	23.8	7.6	20.5	6.7	18.4	6.7

*Bacterial counts in billions per ml.

The counts differed by 33, 3, and 29 per cent for the samples obtained on the 2 different days at the 15, 1, and 5 hr periods respectively. The pH of the early morning samples were alkaline (7.6) but were acid (pH 6.7-6.8) when samples were collected 1 and 5 hr after eating.

Experiment 6. Attempts to provide more homogeneous samples for Warburg studies of various carbohydrate substrates.

Although the data obtained in the last 3 experiments seemed to reveal a general pattern in regard to the fermentation studies, it was noted that the rumen samples were not uniform, as might well be expected, and often varied considerably from a watery to a thick consistency and frequently contained gross particles of feed. Such conditions sometimes made pipetting difficult and possibly led to variations in the amount of inoculum added to the Warburg flasks. In an effort to obtain a more homogeneous sample, a wide-tipped pipette was used to transfer 5 ml of rumen material to 15 ml of phosphate buffer. The buffer was mixed and kept anaerobic by bubbling CO_2 through a bent Pasteur pipette. A narrow-tipped pipette, filled with CO_2 by gentle suction above the liquid, was then employed to transfer the rumen-phosphate mixture to the vessels.

The same carbohydrates studied in the last experiment were included in this 40-min run except that sucrose, which is frequently encountered in various plants consumed by animals, was substituted for lignin.

The microliters CO_2 produced and the number of times that the carbohydrate fermentations exceeded the basals are shown in table 15. In each run the fermentation of sucrose was greater than that of any of the other carbohydrates. As in the previous experiment, the glucose fermentation was nearly twice that of D-xylose, hemicellulose from corn cobs, or soluble starch.

TABLE 15. Basal and carbohydrate fermentation activities of rumen ingesta from ewe obtained at different periods after eating.

Substrate	Microliters CO_2 produced					
	Hours after eating					
	15		1		5	
Basal	22	—	33	—	105	—
Glucose	256	(11.6)*	234	(7.1)*	351	(3.3)*
D-xylose	83	(3.8)	118	(3.6)	214	(2.0)
Hemicellulose	137	(6.2)	133	(4.0)	169	(1.6)
Soluble starch	103	(4.7)	138	(4.2)	214	(2.0)
Sucrose	343	(15.6)	286	(8.7)	407	(3.9)

*Times basal rate exceeded.

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta and 1.5 ml phosphate buffer mixed previous to adding to vessel and 0.5 ml 10.0 per cent substrate (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

In the trial made on the sample collected 1 hr after eating, the work was interrupted several times and the substrates were not dumped after the 10-min equilibration period. Hence all flasks were unknowingly run as basals for 40 min. It was found that the ul CO_2 evolved from the different vessels varied between 195 and 215 showing a difference between extremes of less than 10 per cent. Subsequently the manometers were reset and the substrates tipped. As a result of

this oversight, the data for this run are not comparable to previous findings but do indicate similar fermentation patterns to those of the other runs in this experiment.

The numbers of bacteria counted by the direct microscopic method were 39.4, 20.0, and 22.6 billions per ml, while the pH of the ingesta were 7.4, 7.3, and 7.0 for the samples collected 15, 1, and 5 hr respectively after eating. These findings agree well with those of previous experiments except that the pH of the rumen contents collected 1 hr after the ewe had eaten was generally more acid.

As a result of this experiment, it was felt that a more uniform sample was obtained by mixing a larger volume of rumen material than was normally added to individual flasks with the amount of phosphate buffer needed to give the desired dilution when transferred to the Warburg vessels. Hence this procedure was employed in a number of future runs.

At this point it should be mentioned that McAnally (1943) and Quin (1943) studied the fermentation of various carbohydrates by rumen ingesta in vitro. In both cases the rumen material was strained through muslin and the liquid portion used. McAnally mixed 7.5 ml of ingesta with 2.0 ml of 8 per cent carbohydrate in fermentation tubes and noted gas production at different intervals in a 37 C incubator. At the end of 40 min, which is the time for which the data in tables 13 and 15 were recorded in the present work, she obtained the following ml gas for the different substrates: glucose, 10.4; sucrose, 6.5; maltose, 1.2; cellobiose, 1.1; and lactose, 0.2.

Quin (1943) added 50 ml of rumen liquid and 0.2 g carbohydrate to 250 ml Erlenmeyer flasks closed by air tight connections attached to individual manometers. The entire apparatus was so constructed that the flasks could be agitated constantly and maintained at 39 C. He found that in the absence of shaking, the volume of gas recorded decreased about 50 per cent. A series of carbohydrates was studied, and the following ml gas were formed in 30 min: glucose, 22.2; fructose, 22.4; sucrose, 16.8; maltose, 9.4; lactose, 5.1; xylose, 3.2; raw tapioca starch, 2.4; and boiled tapioca starch, 2.7. When the experiment was allowed to continue for 4 hr, the raw starch produced 8.5 ml gas while the soluble starch yielded a total of 24.5 ml. Quin stated that the starches underwent no fermentative disintegration within the 30-min test period; hence this might be considered the basal as designated in the present work. Since the rumen samples were collected before feeding, the carbohydrate/basal ratio may be compared with the similar ratios given in table 15. The ratios for comparable substrates obtained by Quin and in the present work were respectively: glucose, 9.3-11.6; sucrose, 7.0-15.6; xylose, 1.3-3.8; and soluble starch, 1.0-4.7. If the data of McAnally were treated in the same manner, the ratios for glucose and sucrose would be 52.0 and 32.7 respectively.

It is apparent that the method of Quin gave results somewhat similar to the present findings. This is not surprising since his technic was more comparable than was that of McAnally. It appears that the Warburg method has certain advantages over those discussed in that

an atmosphere of CO_2 can be maintained in equilibrium with the reactants more readily, and the amounts of gases produced recorded more accurately.

Fermentation Studies on Glucose and Starches.

After these studies were discontinued for 2 months, during which time the ewe received alfalfa and water ad libitum, a number of runs were made to check the previous findings. A sample was collected on the morning of October 20, 1950, after the animal had been deprived of hay and water for 16 hr. Following this, 0.5 pounds of hay was consumed, and 5.3 pounds of water was added to the rumen via the fistula. Other samples were obtained after 4.5 and 23 hr. One week later similar trials were made after 15 hr fasting and 3 and 22 hr after eating 1 pound of hay and receiving 5.8 pounds of water. A third study was made 2 weeks later after fasting the ewe for 15 hr, feeding 0.7 pounds of hay, adding 5.8 pounds of water and subsequently collecting samples after 3 and 21.5 hr.

In the latter manometric runs, corn starch was included as a substrate to compare with the soluble starch, which is a rather indefinite material partially hydrolyzed by dilute HCl and so-called because it readily forms a limpid, clear solution with hot water. The amylopectin of the granule is apparently modified by the acid so that it disintegrates more completely on heating with water. Since this type of starch is not common to plants, corn starch was selected for study, being similar to the starch in some of the common grains. A good

quality product was obtained from the National Starch Products Company, Inc., 270 Madison Avenue, New York 16. The analysis furnished indicated it to be approximately 22-24 per cent amylose and 76-78 per cent amylopectin.

In this experiment the basal, glucose, soluble starch and corn starch fermentations were studied employing 0.5 ml of rumen ingesta and 1.5 ml phosphate buffer in the vessel and 0.5 ml of 10.0 per cent of the substrates in the sidearms. The results are given in table 16.

TABLE 16. Basal and carbohydrate fermentations by rumen ingesta from ewe at different periods after eating alfalfa.

Date 1950	Microliters CO_2 produced											
	Hours after eating											
	15-16				3-4				22-23			
	Basal	Glucose	Sol. starch	Corn starch	Basal	Glucose	Sol. starch	Corn starch	Basal	Glucose	Sol. starch	Corn starch
10-20	60	585 (9.8)*	194 (3.2)	—	45	369 (8.2)	144 (3.2)	—	42	287 (6.8)	113 (2.7)	—
10-27	43	582 (13.5)	150 (3.5)	—	14	121 (8.6)	66 (4.7)	—	38	722 (19.0)	187 (4.9)	—
11-10	80	841 (10.5)	231 (2.9)	107 (1.3)	106	651 (6.1)	254 (2.4)	115 (1.1)	25	494 (19.8)	127 (5.1)	39 (1.6)

*Times basal rate exceeded.

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta and 1.5 ml phosphate buffer mixed prior to adding to vessel and 0.5 ml 10.0 per cent substrate (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

The pattern of the glucose and soluble starch fermentation as compared to the basal activity was similar to earlier findings although variations were encountered. The differences between results obtained on

the early morning samples of 2 consecutive days might be due to the ewe having had all the food and water desired the previous evening and then fasting for 15-16 hr before a sample was taken while subsequently an hour feeding was allowed and none then permitted till the next morning (22-23 hr later).

It will be noted that on 11-10-50 the fermentation of corn starch was less than half that of the soluble starch and generally not much greater than the basal rate in the 40-min periods investigated. When the last run shown in table 16 was allowed to continue for 5 hr, the microliters CO_2 produced from the basal and corn starch trials was 151 and 313 respectively. This indicated that whereas the corn starch fermentation exceeded the basal by 1.6 times after 40 mins, at the end of 5 hr the ratio had increased slightly to 2.0, possibly indicating the need for extended periods of observation. The data presented (table 16) indicate that the partially hydrolyzed soluble starch was attacked by the rumen microorganisms more readily than was the corn starch. Hence one would expect that it would take some time for the microbial enzymes to ferment the corn starch at a rate comparable to the more readily fermentable carbohydrates.

The bacterial counts and the pH of the ingesta are given in table 17. One will note that there was not a great variation in the number of organisms counted after any period of fasting, especially those shown in the first column (15-16 hr), where on 3 different days the variation was between 35.8 and 37.3 billion per ml. There was a decrease in numbers found in the samples collected about 4 hr after the morning

feeding. The counts rose again the following morning to 31.0-49.4 x 10^9. In general these counts were slightly higher than those given in tables 12 and 14, although the times were not comparable in all cases.

TABLE 17. Bacterial counts and pH of rumen ingesta from ewe at different periods after eating alfalfa.

Date	Hours after eating					
	15-16		3-4		22-23	
	Counts*	pH	Counts	pH	Counts	pH
10-20-50	36.3	6.75	27.4	6.70	49.4	7.40
10-27-50	37.3	7.00	15.3	6.50	44.7	7.38
11-10-50	35.8	7.10	21.6	6.80	31.0	7.51

*Bacterial counts in billions per ml.

There was a slight drop in pH in the first morning samples from 6.75-7.10 to 6.50-6.80 after feeding. This was not as great a difference as noted in tables 12 and 14. After a 22-hr fasting period, the pH of the ingesta became slightly alkaline (7.38-7.51) probably due to the decrease of readily-fermentable materials in the rumen and the continual swallowing of saliva containing sodium bicarbonate.

Experiment 7. A comparison of basal, glucose and starch fermentation, bacterial counts, and pH of rumen samples from ewe at different periods after eating alfalfa hay and barley grain.

Since the fermentation rates for glucose, D-xylose, hemicellulose from corn cobs, soluble starch and sucrose as well as the basal rate remained fairly constant over a period of about a month on an alfalfa and water diet, it was decided to note the effect on the fermentation rates as well as any fluctuation in the number of microorganisms present

and the pH when grain was added to the diet. As barley is fed extensively in Montana as a grain supplement, 0.3 pound was included with the morning and evening feedings. The ewe exhibited considerable relish for the barley and always ate the entire portion before turning to the alfalfa. A partial analysis of the cracked barley follows: Dry matter, 89.8 per cent; crude protein, 10.5 per cent; ether extract, 2.0 per cent; crude fiber, 4.8 per cent; ash, 2.6 per cent; and N-free extract, 69.9 per cent.

The first sample in this series of runs was taken August 15, 1950, after the animal had been without alfalfa and water for 15 hr. Subsequently barley was fed with the regular diet and other samples were obtained at intervals. Manometric studies were performed as in the last experiment and basal and glucose runs were for 40 min. Since grains are high in carbohydrates, especially starches, Difco's soluble starch was included on the last 2 days to note the fermentation of this substrate.

The results (table 18) were interesting in that they were relatively similar on the first day to those obtained previously, especially when the glucose/basal ratio was considered. The basal rate was exceeded 20.4, 2.7 and 5.7 times for the samples collected 15, 1, and 5 hr respectively after the ewe had eaten. However on the second and fifth days following the feeding of barley, the basal and glucose rates had at least doubled in nearly all cases from the first day. As barley was continued in the diet, the spread between the fermentation of glucose and soluble starch decreased until at the time when the last 2

samples were collected on the fifth day, there was no significant difference between the activity on these 2 substrates. It is possible that a starch-digesting flora had been enhanced by the grain in the diet.

TABLE 18. Basal, glucose and soluble starch fermentation by rumen ingesta from ewe at different periods after eating alfalfa and barley.

Date	Microliters CO_2 produced								
	Hours after eating								
	15			1			5		
	Basal	Glucose	Soluble starch	Basal	Glucose	Soluble starch	Basal	Glucose	Soluble starch
8-15-50	18	368 (20.4)*	—	67	183 (2.7)	—	56	321 (5.7)	—
8-17-50	56	637 (11.4)	291 (5.2)	137	397 (2.9)	269 (2.0)	178	787 (4.4)	488 (2.7)
8-20-50	63	651 (10.3)	363 (5.8)	134	297 (4.7)	294 (4.7)	174	465 (2.8)	461 (2.7)

*Times basal rate exceeded.

Total reaction volume, 2.5 ml including: mixture of 0.5 ml rumen ingesta and 1.5 ml phosphate buffer in vessel and 0.5 ml 10.0 per cent substrate in sidearms (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

The numbers of bacteria (table 19) counted in the samples investigated were similar to those previously found, although the extremes of all the samples were greater than heretofore encountered, namely 12.1 to 51.0 billion per ml. The average of the counts for the 2 test days after barley had been included in the diet was 36.8, 23.4, and 33.7 billion for the 15, 1, and 5-hr periods respectively, as compared to 31.5, 12.1, and 20.0 billion per ml for the first day of

this series. On the basis of the greatly-increased fermentation activity encountered after barley was fed, it seems rather surprising that there was not a greater difference noted in the bacterial population. It is possible that while certain types of organisms increased under favorable nutritional conditions other groups decreased until conditions became advantageous for their multiplication.

TABLE 19. Bacterial counts and pH of rumen ingesta from ewe at different periods after eating alfalfa and barley.

Date	Hours after eating					
	15		1		5	
	Counts*	pH	Counts	pH	Counts	pH
8-15-50	31.5	7.4	12.1	6.9	20.0	6.9
8-17-50	22.6	7.0	19.5	6.0	35.8	6.2
8-20-50	51.0	6.9	27.4	6.0	21.6	6.4

*Bacterial counts in billions per ml.

A pronounced drop in pH was obtained on all samples on the second and fifth days following the addition of barley to the diet. At these times the pH was 6.9-7.0 on the early-morning samples which generally were more alkaline. One hour after eating, the pH was 6.0 and rose only to 6.2-6.4, 4 hr later. Likewise, there was a notable change in the character of the rumen contents. Whereas previously it was rather easy to obtain a rumen sample which soon separated into a solid and liquid layer shortly after standing, now it proved to be a laborious task to draw the ingesta into the plastic collection tube because of the thick consistency.

Second Series of Fermentation Studies with Barley in Sheep's Diet.

Another series of runs was made about 2 months after those just described in order to determine whether the addition of barley to the regular alfalfa diet would have a similar effect on the fermentation capacity of the rumen ingesta. Glucose and corn starch were employed as substrates in the manometric studies. Runs were made on 3 different days with barley being incorporated in the diet after the collection of the first sample on November 22, 1950.

The results are presented in table 20. In both periods (12-15 hr and 4-6 hr) where comparable data are given for particular periods on the 3 different days, there was a gradual build-up in the fermentation activity, especially with corn starch where a 3-to 5-fold increase was noted. As observed in the previous series, the activity on glucose was 12 to 15 times greater than the basal on the early morning samples but exceeded it only about 3 to 4 times several hours after eating because of the increased basal activity and decreased glucose fermentation. In the 40-min period investigated, the activity on corn starch exceeded the basal by only 1.5 to 2.5 times on the early morning samples and was less than this, 0.4 to 1.5, with the ingesta obtained at the 4-6-hr period. An indication that the corn starch was fermented more rapidly after an extended period in the Warburg apparatus was obtained on 2 occasions. On November 24, in the first morning run, the corn starch fermentation exceeded the basal by 2.2 times after 40 mins and 6.0 times after 5 hr. The November 27 sample, obtained 22.5 hr after feeding the ewe, gave a corn starch activity 1.1 and 7.5 times

greater than the basal after 40 mins and 8.5 hr respectively. This increased fermentation rate of starch after an extended incubation period is in agreement with the results reported by Quin (1943) and discussed in experiment 6.

TABLE 20. Basal and carbohydrate fermentations by rumen ingesta from ewe at different periods after eating alfalfa and barley.

Date 1950	Microliters CO_2 produced											
	Hours after eating											
	12-15			1			4-6			22		
	Basal	Glucose	Corn starch	Basal	Glucose	Corn starch	Basal	Glucose	Corn starch	Basal	Glucose	Corn starch
11-22	32	410 (12.8)*	44 (1.4)	—	—	—	99	308 (3.1)	42 (0.4)	—	—	—
11-24	43	660 (15.3)	96 (2.2)	—	—	—	121	532 (4.4)	130 (1.1)	—	—	—
11-26/27	48	706 (14.7)	121 (2.5)	160	306 (5.1)	168 (1.1)	128	551 (4.3)	191 (1.5)	33	397 (12.0)	52 (1.6)

*Times basal rate exceeded.

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta and 1.5 ml phosphate buffer mixed prior to adding to vessel and 0.5 ml 10.0 per cent substrate in sidearms (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

On the November 26 and 27 runs it is interesting to note the basal findings of 48, 160, 128, and 33 ul CO_2 for the fasting periods of 15, 1, 5, and 22 hr respectively. This appears to indicate the amounts of readily-fermentable substrates present. The pH of the ingesta at these times were 7.01, 5.98, 6.00, and 7.30 respectively.

The bacterial counts and the pH of the rumen samples are given in table 21. Similar counts are noted on the 3 different days, at respec-

tive times when samples were collected. The counts of the early-morning contents varied between 31.0 and 37.3 billion, and the ingesta collected 4–6 hr after eating showed a variation of 16.8–23.8 billion per ml. The pH of the first samples was between 6.98 and 7.18 but dropped to 6.00–6.40 several hours after the animal had eaten. These findings substantiate those given in tables 17 and 19.

TABLE 21. Bacterial counts and pH of rumen ingesta from ewe at different periods after eating alfalfa and barley.

Date 1950	Hours after eating							
	12–15		1		4–6		22	
	Counts*	pH	Counts	pH	Counts	pH	Counts	pH
11-22	31.0	7.18	—	—	23.8	6.40	—	—
11-24	35.2	6.98	—	—	16.8	6.08	—	—
11-26/27	37.3	7.01	15.8	5.98	23.7	6.00	34.8	7.30

*Bacterial counts in billions per ml.

Third Series of Fermentation Studies with Barley in Sheep's Diet.

After a lapse of about 1 month, during which time alfalfa and water were always available, a series of runs similar to the previous trials were made. However in addition to the substrate of glucose and corn starch, barley was included in the manometric studies. The barley, similar to that fed the ewe was ground in a Wiley mill, and 0.5 ml of a 10.0 per cent suspension was added to the flask sidearms.

Samples were obtained in the morning after the animal was deprived of food and water for 15 hr and again 5 hr after eating 1.6 pounds of hay and 3.0 pounds of water. For the next 3 feedings 0.3 pounds of barley was included in the diet. Additional samples were collected

after fasting 15 hr as well as 4.5 and 22 hr after consuming 0.3 pounds barley, 1.5 pounds hay and 2.6 pounds water.

The data are presented in table 22. It will be noted, as previously, that after 15 hr fasting, the glucose fermentation exceeded the basal about 10 to 15 times; whereas after 5 hr it was approximately 5 to 7 times as great, largely because of the increased activity in the basal flasks a few hours after the animal had eaten. However when the ewe was without food for 22 hr, the basal activity dropped to 20 ul CO_2, while the glucose fermentation remained quite active and exceeded the basal by 20.6 times.

TABLE 22. Basal, glucose and barley fermentations by rumen ingesta from ewe at different periods after eating alfalfa and barley.

Date 1950	Microliters CO_2 produced											
	Hours after eating											
	15				5				22			
	Basal	Glucose	Corn starch	Barley*	Basal	Glucose	Corn starch	Barley	Basal	Glucose	Corn starch	Barley
12-28	37	556	41	233	68	500	93	324	--	--	--	--
		15.0**	1.1	6.3		7.4	1.4	4.8				
12-30/31	60***	644	130	324	110	531	156	304	20	412	57	180
		10.7**	2.2	5.4		4.8	1.4	2.8		20.6	2.9	9.0

*Barley ground in Wiley mill.
**Times basal rate exceeded.
***Barley supplemented at 3 feedings prior to sampling.

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta and 1.5 ml phosphate buffer mixed prior to adding to vessel, plus 0.5 ml 10.0 per cent substrate in the sidearm (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

The corn starch fermentation after a 40-min run was not a great

deal above the basal even on samples collected 5 hr after the feeding period. With one exception (barley tested at the 5-hr period) the fermentation of all substrates was increased following the addition of barley to the alfalfa diet for 3 feedings prior to sampling for the second series of trials on 12-30/31-50.

The bacterial counts of the rumen samples are given in table 23. The variation was between 23.7 and 35.2 billion per ml. The pH of the ingesta was alkaline (7.07-7.30) after 15 to 22 hr fasting but became acid (6.00-6.62) especially when the animal was fed barley and samples were checked 5 hr after eating.

TABLE 23. Bacterial counts and pH of rumen ingesta from ewe at different periods after eating alfalfa and barley.

Date	Hours after eating					
	15		5		22	
	Counts*	pH	Counts	pH	Counts	pH
12-28-50	35.2	7.28	23.7	6.62	—	—
12-30/31-50**	29.5	7.07	29.5	6.00	35.2	7.30

*Bacterial counts in billions per ml.
**Barley supplemented at 3 feedings prior to sampling.

In general the results of this trial follow a similar pattern to those of the previous ones in this experiment (tables 18 to 21) and indicate that the fermentation of different substrates and the pH were affected by the addition of barley to the diet but that there was no pronounced effect on the number of organisms in the rumen samples. There was a marked change in the consistency of the rumen ingesta, which became thicker as the ewe continued to receive barley. Evidences

of gas evolution from the fistula were apparent, particularly a few hours after eating, and a characteristic odor was noted.

Experiment 8. The fermentation capacity of rumen ingesta on various substrates, including barley and alfalfa, after the ewe had been placed on a poor diet of oat straw and later supplemented with corn starch.

Van der Wath (1948a) felt that he could control the starch-digesting bacteria of the rumen of sheep by placing them on a wheat straw diet devoid of chemically-detectable starch. His sheep were also freed of infusoria by previous treatment with copper sulfate in order that any starch added later would be available to the bacterial population only. When no starch granules could be observed in the rumen contents, 5 g of corn starch were added through the fistula and 2 ml samples removed periodically and observed in a wet preparation to which Lugol's iodine had been added. Photographs taken at intervals showed the progressive disintegration of the starch granules which in most instances seemed to be due to streptococci. Additional *in vitro* experiments led him to conclude that, in the absence of diastatic enzymes in the sheep saliva, or in the absence of minute amounts in the ruminal fluid, rumen bacteria were then responsible for the entire disintegration observed.

Van der Wath (1948a) then promoted the development of a starch-digesting flora by administering 100 g of corn starch to the rumen daily for 10 days. Two days prior to performing his experiments, the starch was omitted so that on the day of testing the rumen was devoid of starch granules and all organisms had lost their iodophilic reactions.

At this time 100 g of corn starch was added through the fistula and at 30-min intervals, 2 to 3 ml rumen contents were withdrawn and observed as previously. From these studies van der Wath concluded that in sheep receiving a regular supply of starch, disintegration started after 5 hr and was completed within 18-20 hr. On the other hand, when starch had been deleted in the diet, disintegration did not begin until after 7 hr and required 26-30 hr for completion.

The present experiment was patterned somewhat after that of van der Wath's in that the ewe was fed only oat straw and water for 15 days and then 100 g corn starch was mixed with 300 ml tap water and added to the rumen via the fistula each morning for 9 days. Whereas van der Wath examined the rumen contents and noted starch disintegrating, in this work the fermentation activity of rumen ingesta was studied manometrically as described in the previous experiments. At intervals of 12, 15, 22, and 26 days respectively samples were obtained in the morning after the animal had been without food and water for approximately 15 hr.

The substrates in the Warburg vessels included glucose, corn starch, ground barley, ground barley (autoclaved), ground alfalfa and ground alfalfa minus the water-soluble fraction. The grinding of all substrates was performed in a Wiley mill. Autoclaving of the ground barley, spread thinly in a Florence flask, was at 121 C for 35 min to inactivate any enzymes present that might contribute to the fermentation. The water-soluble fraction of the alfalfa was removed by soaking 100 g of the ground hay in 500 ml cold water for about 3 hr, filtering through filter paper and drying the residue at 95 C.

The trials were continued for several hours, but the results at the end of 40 min are presented in table 24. The basal activity for the 4 different runs was similar and about 50 per cent below that generally observed in previous experiments when the ewe was fed alfalfa. In the first trial the fermentation of glucose was approximately half that measured in recent work. A decrease in activity was noted in each of the 2 subsequent runs with an increase in the last, which gave 226, 163, 69, and 91 ul CO_2 respectively. The fermentation of corn starch decreased from 46, 35, to 25 ul CO_2 respectively in the first 3 trials and then rose to 69 ul CO_2 after corn starch had been administered to the ewe for 9 days. The ground barley and ground alfalfa were also fermented less actively in each succeeding test, but the fourth run was nearly twice that of the third. Whether the increase in fermentation in the fourth run was associated with the development of a starch digesting flora following administration of corn starch to the rumen is difficult to state on the basis of this one run.

It will be noted (table 24) that the difference between the gas formation from glucose and from corn starch decreased with the increase in the time that starch was provided the ewe. The same situation occurred with the ground barley to the point that after starch was administered for 9 days, the fermentation of this substrate was greater than that of glucose. It is interesting to note that throughout this experiment the activity on alfalfa always exceeded that of glucose even when the water-soluble fraction was removed from the hay.

The first 3 runs of this experiment were extended for 11-12 hr. The corn starch activity was then found to exceed the basal by 8.9,

TABLE 24. Comparison of basal and different substrate fermentations by rumen ingesta collected periodically 15 hr after eating from ewe on oat straw and corn starch diet.

Straw fed (days)	Microliters CO_2 produced					
	Basal	Glucose	Starch	Barley	Barley (autoclaved)	Alfalfa
12	27	226 (8.4)*	46 (1.7)	125 (4.6)	132 (4.9)	283 (10.5)
15	20	163 (8.2)	35 (1.8)	94 (4.7)	81 (4.1)	201 (10.0)
Straw and starch						
5	23	69 (3.0)	26 (1.1)	63 (2.7)	78** (3.4)	99 (4.3)
9	23	91 (4.0)	69 (3.0)	118 (5.1)	130** (5.7)	188 (8.2)

*Times basal rate exceeded.
**Ground alfalfa minus water-soluble fraction.

Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta and 1.5 ml phosphate buffer mixed prior to adding to vessel, plus 0.5 ml 10.0 per cent substrate (except in basal runs). Atmosphere, CO_2; temperature, 37 C; duration, 40 min; manometers, Brodie's solution.

4.5, and 2.5, whereas at the end of 40 min it exceeded the basal 1.7, 1.8, and 1.1 times respectively. The results seem to indicate that although the ewe had been fed oat straw for 12 days, the rumen microflora were able to attack the corn starch at a faster rate after an extended incubation. However this fermenting capacity was about 50 per cent less 3 days later and further decreased approximately 45 per cent even though starch had been added to the diet for 5 days.

A progressive decrease in the number of bacteria in the 4 rumen samples studied was found (table 25), viz., 23.7, 21.6, 12.1, and 10.5

billion per ml respectively. In the 11 previous runs made on samples collected early in the morning after a 15-hr fasting period, the bacterial counts were between 22.6 and 51.0 with an average of 33.9 billion. Hence the number of bacteria in the rumen decreased about 50 to 80 per cent after the animal was changed from an alfalfa or alfalfa and barley diet to a straw or straw and corn starch diet.

TABLE 25. Bacterial counts and pH of rumen ingesta collected periodically 15 hr after eating from ewe on oat straw and corn starch diet.

Straw fed (days)	Counts*	pH
12	23.7	7.05
15	21.6	6.86
Straw and starch		
5	12.1	6.85
9	10.5	6.89

*Bacterial counts in billions per ml.

The pH of the samples employed in the present work varied between 6.85 and 7.05 (table 25). These values were slightly lower than those usually obtained with samples collected in the morning before feeding alfalfa.

Apparently the oat straw fed for 15 days followed by oat straw and corn starch for 14 days was not an adequate maintenance diet, since the animal's weight dropped from about 152 to 132 pounds during the test period. Whether there was any connection between the loss of approximately 20 pounds by the ewe and the loss of the fistula plug,

which had remained in place for more than a year is difficult to determine, but 5 days before this work was discontinued the plug fell out. It had been planned to start supplementing the oat straw and corn starch diet with Du Pont 262 to note the effect of this non-protein nitrogen source on the fermentation rates of different substrates as well as on the number of bacteria. However an operation was performed on the ewe to replace the rumen fistula plug and further experiments were discontinued at this time.

The data presented in this experiment seem to indicate that a diet of oat straw even when supplemented with corn starch markedly reduces the number of bacteria in the rumen as well as their fermenting capacity.

Experiment 9. A comparison of ruminal fermentation activity employing phosphate and bicarbonate buffers.

Up to this point most of the manometric experiments had been performed in the presence of a phosphate buffer. However, by employing a bicarbonate buffer, both acid and metabolic CO_2 can be measured, whereas there may be a question as to what is being measured with the phosphate buffer. Therefore it seemed necessary to compare these buffers in order to gather information regarding their relative merits for this particular type of investigation involving complex rumen material.

Optimum Bicarbonate Concentration.

Before starting the comparative studies, a series of runs was made

to determine the concentration of sodium bicarbonate buffer which would be optimum for the fermentation of glucose. The Warburg vessels contained 1.0 ml of rumen ingesta collected 14, 2, and 6 hours after the ewe had eaten alfalfa, and 1.0 ml of 0.5, 1.0, 2.0, and 4.0 per cent $NaHCO_3$ respectively. The sidearms held 0.5 ml 10.0 per cent glucose. Mercury was employed in a different set of manometers (described in experiment 11), since it was planned to determine the metabolic and acid CO_2 produced in 2 of the flasks which contained 0.5 ml $3NH_2SO_4$ instead of glucose. However the results obtained were not considered valid, since in the first run the amount of CO_2 released at the end, indicating residual bicarbonate, was greater than the initial available bicarbonate. It appeared likely that such determinations would be more significant if made during the study of glucose fermentation when greater volumes of gas were produced. Such an experiment is described in experiment 11.

The results given in table 26 indicate that the 1.0 per cent concentration of the bicarbonate buffer was optimum for the production of gas in each of the 3 runs. It appears from this experiment that 4.0 per cent bicarbonate and possibly even the 2.0 per cent buffer inhibited glucose fermentation. This confirms the work of Clark and Lombard (1951) who studied the effect of pH on ruminal motility and noted that sugar fermentation was inhibited in an alkaline medium.

It will be observed that the basal rate was relatively lower in the early-morning sample as well as in the one obtained 6 hr after eating than when the rumen contents were obtained 2 hours after the

feeding period. Likewise, as found previously, glucose fermentation was greatest on the first sample of the day regardless of the concentration of buffer.

TABLE 26. Effect of different concentrations of sodium bicarbonate buffer on glucose fermentation of rumen ingesta from ewe T6359 at different periods after eating alfalfa.

Hours after eating	Microliters CO_2				
	Per cent sodium bicarbonate				
	0.5	0.5	1.0	2.0	4.0
14	47*	766	859	593	452
2	94	580	610	570	428
6	47	603	655	570	428

*Basal, no glucose.

Total reaction volume, 2.5 ml including: 1 ml rumen ingesta, 1 ml bicarbonate buffer in vessels and 0.5 ml 10.0 per cent glucose in sidearms (except basal). Atmosphere, CO_2; temperature, 37 C; duration, 45 min; manometers, mercury.

Optimum Glucose Concentration.

After determining that the optimum concentration of sodium bicarbonate was 1.0 per cent for these studies, 2 series of runs were made in order to determine the most suitable concentration of glucose for these fermentation investigations employing the 2 buffers.

One ml of rumen contents obtained on 2 different days was employed in the vessels. On each occasion a sample was collected early in the morning after the ewe had been without food and water for 14.5 hr. An additional sample was also studied on each day, one obtained 1.5 hr after feeding and on the other day 6.5 hr after the food and water were removed. One ml of the respective buffers was used, and 0.5 ml of

5, 10, or 20 per cent glucose was added to the sidearms. All runs were made in an atmosphere of CO_2 at 37 C for 45 min using mercury in the manometers.

The results are given in table 27. One will note that in 8 of the 12 comparable runs more gas was evolved when the bicarbonate buffer was employed. In some instances the difference between the findings is probably not significant, while in others the spread is marked. These data also indicate that there was no definite advantage to employing 5, 10, or 20 per cent glucose. The highest gas production in the 4 different runs was found once with each of the 5 and 10 per cent concentrations and twice with the 20 per cent. The data obtained with the phosphate buffer show that the 5 per cent sugar yielded the most gas in 3 cases, while the 20 per cent was at the peak once. With the

TABLE 27. Fermentation activity of rumen ingesta obtained from ewe after different periods of fasting while employing phosphate and bicarbonate buffers and different concentrations of glucose.

Hours after eating	Microliters CO_2					
	Phosphate buffer			Bicarbonate buffer		
	Per cent glucose			Per cent glucose		
	5	10	20	5	10	20
Series A						
14.5	470	515	603	588	547	476
1.5	432	400	348	429	365	381
Series B						
14.5	635	632	580	678	684	690
6.5	611	562	510	565	730	619

Total reaction volume, 2.5 ml including: 1 ml rumen ingesta plus 1 ml phosphate buffer or 1 ml 1.0 per cent $NaHCO_3$ in vessels and 0.5 ml of 5, 10, or 20 per cent glucose in sidearms. Atmosphere, CO_2; temperature, 37 C; duration, 45 min; manometers, mercury.

bicarbonate buffer the following were found to rank at the top: 5 per cent, once; 10 per cent, once; and 20 per cent, twice. Again the differences among some of these findings are probably not significant, and hence one seems justified in employing a 10 per cent concentration of glucose as a substrate.

Comparative Buffer Studies Including Starch as a Substrate and Brodie's Solution in Manometers.

Since the constants for the flasks used with the manometers containing mercury were found to be approximately 15 times greater than those employed with the manometers having Brodie's solution, it was sometimes difficult to detect small differences in gas production as perhaps was the case in the data just presented. With this in mind, the phosphate and bicarbonate buffers were again compared using Brodie's solution and including corn starch as a substrate.

One ml of phosphate or 1.0 per cent $NaHCO_3$ was employed in the flasks as well as 0.5 or 1.0 ml (starch flask) water and 0.5 ml rumen ingesta. Rumen material was obtained from ewe K1111 (information regarding this animal is given in experiment 10) on 2 different days after food and water had been removed for 12, 2, and 6 hours. The sidearms contained 0.5 ml water, 0.5 ml 10 per cent glucose or corn starch powder respectively. The 45-min trials were made in an atmosphere of CO_2 at 37 C.

The results given in table 28 show that in these runs the microliters CO_2 obtained with the bicarbonate buffer exceeded those with the phosphate buffer in 15 of the 18 comparable points. In general the

differences between comparable runs were greater where the substrate was actively fermented. This might indicate that both metabolic and acid CO_2 were being measured by the use of the bicarbonate buffer. Trials in which metabolic and acid CO_2 were determined employing these 2 buffers are described in experiment 11.

TABLE 28. Fermentation activity of rumen ingesta obtained from ewe K1111 at different periods after eating while comparing phosphate and bicarbonate buffers in Warburg vessels.

Hours after feeding	Microliters CO_2					
	Phosphate buffer			Bicarbonate buffer		
	Basal	Glucose	Starch	Basal	Glucose	Starch
Series A						
12	18	323 (17.9)*	60 (3.3)*	20	348 (17.4)*	53 (2.7)*
2	38	207 (5.4)	70 (1.8)	32	340 (10.6)	102 (3.2)
6	37	662 (17.9)	81 (2.2)	48	706 (14.7)	91 (1.9)
Series B						
12	13	315 (24.2)	50 (3.8)	17	351 (20.6)	55 (3.2)
2	90	153 (1.7)	106 (1.2)	94	253 (2.7)	123 (1.3)
6	43	385 (9.0)	86 (2.0)	53	526 (9.9)	82 (1.6)

*Times basal exceeded.

Total reaction volume, 2.5 ml including: 1 ml phosphate or 1.0 per cent $NaHCO_3$ buffer, 0.5 or 1.0 ml (starch flask) water, and 0.5 ml rumen ingesta in vessels and 0.5 ml water, 10.0 per cent glucose or corn starch powder in sidearms. Atmosphere, CO_2; temperature, 37 C; duration, 45 min; manometers, Brodie's solution.

If one considers the number of times that the microliters CO_2 produced in the glucose and corn starch fermentations exceeded the basal when the 2 different buffers were employed (table 28), one will note a general trend or pattern. An exception is found in series A with the 2-hr sample where the difference between the substrate/basal

fermentation ratios of the 2 buffers is approximately 50 per cent, whereas in all other cases it is about 15 per cent or less. This would seem to indicate that either buffer might be useful in comparing the activity of a rumen sample on different substrates.

This series of runs confirms previous work in which it was found that, in the periods investigated, the greatest spread between the basal and glucose fermentation occurred with rumen ingesta obtained 12 to 14 hr after the animal was deprived of food and water. Likewise the narrowest spread was shortly (1-2 hr) after eating when the basal activity was often highest and the glucose fermentation lowest. Six hours after the feeding period the basal activity had decreased in some cases, but the ability to ferment glucose had markedly increased in each instance.

In an attempt to explain these results, the pH of the ingesta, the amount of sediment thrown down by centrifuging 15 ml of the rumen material for 1 min at 2600 r.p.m. in an International centrifuge, size 1, having an 11.5-inch conical head, and the number of bacteria counted by direct microscopy were recorded (table 29). One will note a

TABLE 29. Bacterial counts, pH and sediment of rumen ingesta from ewe K1111 at different periods after eating alfalfa.

Hours after eating	Series A			Series B		
	Counts*	pH	Sediment**	Counts	pH	Sediment
12	15.2	7.29	1.0	14.2	7.43	1.0
2	10.5	6.38	0.8	7.9	6.20	0.8
6	15.3	7.10	1.4	14.2	7.00	2.0

*Bacterial counts in billions per ml.
**Ml sediment per 15 ml ingesta.

rather close agreement between the results obtained on the 2 different days (series A and B). As found in earlier experiments, the pH of the samples obtained after the animal had been deprived of food for about 12 hr was alkaline. This dropped about 1 pH unit 2 hr after eating when ruminal fermentation was active and then rose to the neutral or slightly-alkaline point about 4 hr later.

The findings obtained when the ruminal material was centrifuged seem logical, since the tube inserted through the fistula was perforated for 80 mm near the end with 50 holes about 3 mm in diameter. These openings permitted only fine material and liquid to seep into the tube. This material withdrawn by a suction tube constituted the sample in experiments 5 through 11. When the early-morning sample was collected, much of the fine material had undoubtedly passed on into the reticulum, omasum and abomasum. Two hours after eating, considerable coarse material, which was not small enough to pass through the tube, was present, whereas 4 hr later when considerable portions had been regurgitated and masticated, the amount of sediment had approximately doubled. Gall (1946) has advocated the vigorous agitation of rumen samples prior to making counts in order to free organisms attached to fibers. It is possible that the material obtained 6 hr after feeding contained many organisms, which were not freed by the manual agitation employed and hence were not in the microscopic fields which were counted but were present and accounted for the increased fermentation of glucose in the Warburg studies. However, one might wonder why a similar increase was not observed with the corn starch substrate.

The number of bacteria (7.9 to 15.3 billion per ml) counted in the 0.01 ml sample of a 10^2 dilution stained by carbol fuchsin (1:10) were similar 12 and 6 hr after the animal had eaten but decreased about 30 per cent at the 2-hr sampling. This decrease was possibly due to dilution by recently-ingested food and water so that the actual number may not have varied appreciably. There is no question but that the microflora is multiplying rapidly particularly when a suitable diet is furnished, but also large numbers of these organisms are continually leaving the rumen via the reticulum, omasum and abomasum to the intestinal tract. It is difficult to find a method which will give an accurate estimate of the number of bacteria present, and this situation has led to a study of the microbial activities by manometric methods. The present method of counting the bacteria in the rumen samples seems to give a rather consistent picture of the free organisms from day to day. Occasionally a piece of fiber was encountered in the microscopic field on which one could observe a few or many organisms. It is impossible to predict how many of these would be freed by a more drastic agitation than was given by manual shaking 50 times. In this work it proved more satisfactory to employ the liquid portion of the rumen contents, which were more easily and uniformly obtained, than a sample containing more of the solids which would possibly lead to greater variations when added to Warburg vessels. In preliminary studies, samples of this type were difficult to pipette into the flasks and necessitated diluting the ingesta with buffer to facilitate transferring. No serious attempt has been made to determine how great a spread must

be found between counts to consider the difference significant. One must recognize the weaknesses inherent in any counting method of this type, particularly when dealing with rumen material which contains many organisms near the resolving power of the microscope in size as well as organisms which are embedded rather firmly in plant fibers. It has been suggested that the phase microscope would be an asset in work of this nature, and since such equipment has been acquired recently at Montana State College, this possibility can be investigated in the future.

One must admit that in the present investigations the number of organisms counted in rumen samples does not follow the increases and decreases noted in the basal, glucose or starch fermentations but tends to be rather constant at the different times of day when rumen samples were collected.

Experiment 10. A comparison of the fermentation activity, number of bacteria and pH of rumen ingesta obtained from 2 ewes on 12 consecutive days.

All experiments performed to this point, unless otherwise indicated, were conducted on a Targhee ewe (T6359) with a rumen fistula provided in December, 1949. At that time the opening was closed by a lucite plug, which remained in position until January 28, 1951, when it was ejected. An attempt was made to replace the plug by surgery, but it did not remain in position more than several weeks. Subsequently, no effort was made to close the fistula during the past one and a half years, and the animal appears to have remained healthy,

although rumen contents often leaked through the opening and escaping gas could frequently be heard.

Experimental Ewe K1111.

It was considered important to compare the manometric findings as well as the number of bacteria and pH with those of another sheep. Unfortunately an animal of the same age and breed was not available, but a Rambouillet ewe (K1111) born April 22, 1949, was furnished by the Department of Animal Industry. A rumen fistula was provided by members of the Veterinary Research staff on June 2, 1952, following the technic suggested by Quin, van der Wath, and Myburgh (1938). A lucite plug was modified, as suggested by Phillipson while visiting our laboratory, from the one previously employed so that the inside flange covered approximately twice as much of the rumen wall. It was anticipated that this plug would not be ejected as readily as the one with the smaller flange. The ewe recovered rapidly from the operation, and subsequently a series of runs was made comparing the activities of the rumen contents in the Warburg apparatus, the number of bacteria per ml and the pH of the samples.

Both animals were kept in the same stall with a wire-screened floor but were separated by a partition so that the hay and water consumed by each could be recorded. Rumen samples were collected early each morning for 12 consecutive days after the animals had been without food and water for about 12 hours, since it had been found previously that fewer variations were encountered at this time than a few hours

after eating. After obtaining the samples, alfalfa hay and water were available for 2 hr, removed for 6 hr to allow for the occasional collection of other samples and furnished again for 3 to 4 hr after which these materials were removed for the night.

Myiasis and Ingesta Transfusions with Ewe T6359.

At the start of these runs it was noted that the older ewe T6359 was not eating as usual. Closer observation revealed that her sides and back were infested with maggots. She was shorn in these areas and "Screw Worm Smear 62" was applied. Whether this myiasis was associated with her anorexia is difficult to determine. However the odor and appearance of the ruminal contents were not normal. When one inserted the collection tube into the rumen, a hard mass was encountered and not enough liquid was present for an adequate sample. On June 16, 1952, 800 ml water was added to the rumen via the fistula and about 5 min later a sample was withdrawn.

Numerous reports in the literature indicate the value of inoculating an abnormal rumen with rumen contents from a healthy animal. It was considered worthwhile to try such a procedure at this point. Since the animal had consumed no water for 2 days, another transfusion was made later in the morning consisting of 150 ml rumen fluid from sheep K1111 plus 100 ml of a 50-per cent commercial glucose solution. Four hours later 500 ml water were added to the rumen and subsequently the ewe consumed a little hay.

The next day it was again necessary to add 500 ml water to the

rumen before a sample could be obtained in the morning. Following this, 100 ml rumen fluid from the other sheep, 100 ml glucose solution and 100 ml water were administered via the fistula. At 4:00 p.m. only 0.1 lb alfalfa and no water had been consumed, hence 200 ml rumen fluid, 100 ml glucose and 200 ml water were added to the rumen. At 8:00 p.m. no hay or water had been taken, and since the rumen contents were still rather hard and matted, 2000 ml water was added.

The following morning sufficient liquid was present to permit collecting a sample, but subsequently 200 ml rumen liquid and 1300 ml water were administered. In the afternoon 100 ml rumen fluid, 100 ml glucose solution and 1300 ml water were given via the fistula. During the day the animal ate 0.6 lb hay. On the fourth day the ewe appeared normal, consumed 1.6 lb hay and 8.4 lb water. The rumen contents appeared more characteristic in odor and appearance, and it was not difficult to obtain a sample. The effect of this experience on the ruminal activity, bacterial counts and pH will be discussed at a later point.

Manometric Studies.

The manometric studies were carried out by employing 1.0 ml 1.0 per cent $NaHCO_3$, 0.5 ml water and 0.5 ml rumen fluid in the vessels and 0.5 ml water, 0.5 ml 10 per cent glucose or 0.5 ml 10 per cent corn starch in the sidearms respectively. Since the corn starch settled out during the equilibration making it difficult to tip all of it into the flasks, dry corn starch was added to the sidearms and 1.0 ml water

instead of 0.5 ml was added to the appropriate vessels. In order to be certain that adequate concentrations of substrate were employed, the presence of reducing sugars and starch in the flask contents was verified at the end of several runs by testing with Benedict's solution and iodine respectively. All runs were in an atmosphere of CO_2 at 37 C for 45 min employing Brodie's solution in the manometers.

The results given in table 30 were recorded for 12 consecutive days in order to note any variations over an extended period. Although averages in this case may not be too significant, it will be noted that the basal, glucose and starch fermentation of the younger ewe (K1111) exceeded that of ewe T6359 by approximately 3.1, 2.5, and 1.7 times respectively. The basal rate of ewe K1111 generally doubled or tripled that of the other animal even when the latter appeared to be normal. One factor which might be responsible was that the rumen was closed in ewe K1111 while with sheep T6359 solids and liquid were continuously being lost. It will be noted that the ability to ferment glucose was 3 to 4 times lower than usual during June 16 and 17 when ewe T6359 was not eating normally.

Numbers of Bacteria.

The number of bacteria per ml determined by direct microscopic counts of slides stained with carbol fuchsin varied between 11.1 and 23.6 billion per ml with an average for the 12 days of 18.5 billion for sheep T6359. For ewe K1111 the average was 22.6 billion per ml with variations between 12.6 and 34.7 billion. As a result of this work,

one will observe that there was a greater variation among the daily bacterial counts of a particular ewe than between the average counts from the 2 animals for the 12 days.

TABLE 30. Comparison of fermentation activity, number of bacteria and pH of rumen contents obtained from 2 sheep 12 hours after eating alfalfa.

Date June 1952	Ewe T6359					Ewe K1111				
	Microliters CO_2*			Counts **	pH	Microliters CO_2*			Counts **	pH
	Basal	Glucose	Starch			Basal	Glucose	Starch		
16	-2	66	32	11.1	6.30	39	751	108	34.7	7.18
17	8	74	41	13.7	6.62	35	625	83	23.7	7.29
18	22	285	69	21.0	6.73	27	567	76	23.7	7.61
19	8	301	51	15.8	7.25	17	563	69	20.5	7.70
20	3	294	51	22.1	7.30	35	766	99	25.8	7.50
21	5	229	34	23.6	7.31	32	688	75	24.2	7.40
22	13	323	64	17.9	6.90	20	445	61	27.4	7.39
23	8	221	35	19.5	7.30	35	603	70	26.8	7.31
24	7	257	37	21.0	7.23	20	388	55	22.1	7.42
25	10	251	37	22.6	7.22	20	370	60	14.7	7.28
26	18	219	38	19.5	7.21	36	607	76	14.7	7.32
27	10	218	35	14.2	7.40	19	410	55	12.6	7.32
Avg.	9	228	44	18.5		28	565	74	22.6	

*Total reaction volume, 2.5 ml including: 0.5 ml rumen ingesta, 1.0 ml 1.0 per cent $NaHCO_3$, and 0.5 or 1.0 ml water (sidearms with dry starch) in vessels plus 0.5 ml water 0.5 ml 10.0 per cent glucose and 0.5 ml 10.0 per cent corn starch or dry starch respectively in sidearms. Atmosphere, CO_2; temperature, 37 C; duration, 45 min; Brodie's solution in manometers.

**Bacterial counts in billions per ml.

pH Findings.

In previous experiments the pH of early-morning samples was generally alkaline. Similar results were obtained in this work with the exception of the material obtained from sheep T6359 when she was off feed. It will be observed that pH 6.30, 6.62 and 6.73 were

obtained on consecutive days when there was little liquid present in the rumen, but when the animal appeared normal on June 19, the pH was 7.25 and remained in this range throughout the remainder of the experiment.

One must conclude from this experiment that there were daily variations in the fermentation capacity, number of bacteria and pH of the rumen ingesta. In general these variations in the fermentative ability of the rumen samples were not as great with ewe K1111 as with sheep T6359. The data given in table 31 show the number of times that the glucose and corn starch fermentation rates, taken from table 30, exceeded the basal rate. Undoubtedly some of the extremes with sheep T6359 could be attributed to her myiasis, especially since most of them occurred in the first 6 days. It will be noted that the findings are

TABLE 31. Number of times glucose and corn starch fermentation of rumen ingesta from 2 ewes exceeded the basal activity.

Date June 1952	Ewe T6359		Ewe K1111	
	Glucose	Corn Starch	Glucose	Corn Starch
17	9.3	5.1	17.9	2.4
18	13.0	3.1	21.0	2.8
19	37.6	6.4	33.1	4.1
20	98.0	17.0	21.9	2.8
21	45.8	6.9	21.5	2.3
22	24.5	4.9	22.3	3.1
23	27.6	4.4	17.2	2.0
24	36.7	5.3	19.4	2.8
25	25.1	3.7	18.5	3.0
26	12.2	2.1	16.9	2.1
27	22.0	3.5	21.6	2.9

Date for these calculations taken from table 30.

quite constant for sheep K1111 with the exception of those for June 19. The fermentation of glucose exceeded that of the corn starch about 8 times with ingesta from ewe K1111 and 6 times with sheep T6359.

Food and Water Consumed.

During the course of this experiment, the hours allotted the sheep for eating were followed closely, and the alfalfa hay furnished after the first 5 days was from the same bale. On the other hand, no attempt was made to regulate the amount of hay and water consumed, since it was desired that the animals act normally in this respect. From the variations in the amount of hay and water consumed each day (table 32), it appears likely that these differences would have some effect on the points that have been investigated. Although both sheep consumed about

TABLE 32. Pounds of alfalfa hay and water consumed by the 2 ewes during a daily 6-hour feeding period*.

Date June 1952	Ewe T6359		Ewe K1111	
	Hay	Water	Hay	Water
16	-	3.0**	-	-
17	0.1	5.0**	1.0	4.1
18	0.6	6.0**	1.7	5.2
19	1.6	8.4	2.5	6.9
20	2.0	6.4	2.0	6.5
21	3.1	12.8	2.5	3.6
22	2.9	13.8	3.3	7.9
23	3.0	14.7	2.4	5.4
24	3.5	11.2	3.1	7.6
25	4.0	13.1	3.5	6.2
26	3.4	6.0	2.8	6.1
27	2.6	12.0	2.7	6.7

*Feeding period included 2 hr in morning after obtaining sample and 4 hr in late afternoon. No food or water available at other times.

**Added via the fistula and contained rumen liquid from ewe K1111, glucose solution and water.

the same amount of hay daily after June 20, the older ewe drank about twice as much water when feeling normal. The larger capacity of her rumen and the constant loss of rumen contents through the open fistula were probably responsible for this difference.

> Experiment 11. Studies pertaining to the amount of bicarbonate in ingesta collected from different areas of the rumen and the acid and metabolic CO_2 obtained during glucose fermentation in the presence of phosphate and bicarbonate buffers.

The saliva of the sheep owes its alkalinity and buffering capacity mainly to sodium bicarbonate. Two to 4 liters of saliva may be secreted daily, and as this material passes into the rumen with food, which has been eaten or reguritated, it forms an important buffer in this organ. Since no report was found in the literature indicating the amount of bicarbonate (expressed as CO_2) in the rumen ingesta, the following trials were designed to give information on this point.

Bicarbonate Content in Anterior and Posterior of Rumen.

Preliminary attempts to determine the amount of CO_2 in rumen ingesta by the Warburg apparatus employing Brodie's solution in the manometers were unsuccessful. If 0.5 or 1.0 ml rumen contents was used in the flask as in earlier runs, the addition of 0.5 ml $3NH_2SO_4$ caused the Brodie's fluid to extend beyond the graduated range of the manometer. If the volume of inoculum was decreased sufficiently to keep the gas liberated within the desired limits, the rumen material proved to be too dilute to give satisfactory basal or glucose fermentation rates.

Therefore an additional set of manometers and flasks was purchased and calibrated employing mercury as the manometer fluid.

In the present experiment, samples were collected from the anterior and posterior areas of the rumen of sheep T6359. All samples were run in triplicate in order to become familiar with mercury as the manometer fluid and to check the equipment. This proved to be worthwhile since a leak was discovered in 1 manometer. Rumen ingesta were obtained on 3 occasions for 4 successive days. The first sample was collected each day after the animal had fasted over night (13-15 hr). Additional material was obtained 1.0 to 1.5 hr and 5.5 to 6.0 hr after a morning feeding period of about 1 hr at which time alfalfa hay and water were available.

One ml of rumen material was pipetted directly to each Warburg flask to which 0.5 ml $3NH_2SO_4$ had been added to the sidearm previously. All flasks were equilibrated for 10 mins in an atmosphere of CO_2 at a temperature of 37 C. After reaching equilibrium, the acid was tipped and the amount of gas evolved was recorded. The pH of the rumen ingesta was determined as in earlier experiments shortly after bringing the sample to the laboratory.

The microliters of CO_2 from the material were determined by multiplying the observed change in the manometer by the flask constants as calculated for 2.5 ml of fluid instead of the 1.5 ml employed in the present study. This entailed a slight error of about 5 per cent but was probably not significant for comparative purposes. Again it was assumed that CO_2 was the gas produced in greatest abundance and did not

take into account any methane which might have been formed.

The results of these runs are summarized in table 33. In most instances, the differences among the triplicate flasks were less than 10 per cent. It will be noted that in all cases more gas was evolved from the ingesta in the anterior than in the posterior area of the rumen except from the samples collected in the morning after a 13-15-hr fasting period. It seems probable that 13 to 15 hr after feeding, the rumen contents did not contain as much coarse material, and hence there was a greater opportunity for a more thorough mixing of the ingesta by rumen movement than 1 hr or even 6 hr after eating.

TABLE 33. Microliters CO_2 liberated from rumen ingesta of ewe T6359 by sulfuric acid on different days, various intervals after eating and from different areas of the rumen.

Days	Hours after eating					
	13.0-15.0		1.0-1.5		5.5-6.0	
	Front	Back	Front	Back	Front	Back
1	1591*	1642	1114	783	1348	1062
2	1630	1691	891	645	1314	1305
3	1044**	1092**	680	491	1255	1007
4	1595	1507	751	576	1314***	1245***

*Data are averages of 3 flasks.
**Animal fed by someone before sample collected.
***Both samples collected from front because insufficient liquid in back.

Since the amount of saliva secreted is increased somewhat during the ingestion of food, bicarbonate would enter the rumen and accumulate in the anterior portion. The amount of gas in the material from the fore section seemed to be quite constant for the different days in that the samples varied less than 10 per cent between extremes when

obtained after the animal was fasted for about 15 hr and also about 5.5 hr after eating. The exception was on the third day of the experiment when someone provided the ewe with hay before the morning sample was collected.

In general, approximately half as much gas was obtained from the samples collected within 1.0 to 1.5 hr after the ewe had eaten than after being without food and water for about 15 hr. The difference was not as great when compared with the material examined 5.5 hr after eating. These differences are probably due to the dilution of bicarbonate by the recently ingested food on to a decrease in bicarbonate caused by acid formation during active ruminal fermentation.

pH of Ingesta from Anterior and Posterior of Rumen.

The pH of the material under investigation (table 34) was slightly alkaline in the morning and about 5.5 hr after eating, whereas soon after eating it was acid. This confirms the observations of previous determinations on the pH of rumen samples collected at different times of the day. There does not appear to be any significant difference in pH between samples collected from the front or the back of the rumen.

Although the data are limited, it would seem as though some other material than bicarbonate was contributing to the buffering action in the later afternoon samples as compared to the early morning ones, since the pH findings were similar but approximately 300 ul more CO_2 were freed from the morning ingesta. The same might hold for some of the samples collected 1.0-1.5 hr after eating.

TABLE 34. pH of ingesta collected from front and back of sheep rumen on different days and various hours after eating.

Days	Hours after eating					
	13.0-15.0		1.0-1.5		5.5-6.0	
	Front	Back	Front	Back	Front	Back
1	-	-	6.85	6.82	7.30	7.20
2	7.20	7.30	6.80	6.70	7.30	7.40
3	6.80*	7.00*	6.70	6.51	7.00	6.85
4	7.31	7.48	6.65	6.54	7.10**	7.10**

*Animal fed by someone before sample collected.
**Both samples collected from front because insufficient liquid in back.

Smith (1941) determined the pH in various areas of the bovine rumen by employing a specially adapted electrode, which could be probed into different sections and which was attached to the pH meter by a long lead. On an alfalfa diet, the pH means in the front of 2 animals were 6.32-6.40, whereas in the rear they varied between 5.83 and 6.28, indicating a slightly higher pH in the fore part of the rumen. Samples of ingesta were collected immediately after in vivo readings were taken and in vitro determinations were made within 30 min. The mean pH range was 6.49-6.90 for the samples obtained from the front of the rumen and 6.10-6.57 for those from the back. In general, the pH was about 0.3 higher with the samples measured in vitro. Since he did not describe his technic of collecting or handling the samples before measuring the pH, it is not known how much CO_2 might have been lost thus causing a rise in pH. However these studies emphasize the importance of keeping the sample in an atmosphere of CO_2 until the pH has been measured. This was done in the present study by filling the

bottle with CO_2 before adding the sample and then keeping it closed until the sample was removed. No in vivo tests were made to note how successful these attempts were.

As a result of this experiment, it would appear that more uniform samples could be obtained from the anterior area of the rumen, particularly a few hours after feeding. On a routine diet of alfalfa, it was observed that the amount of gas, expressed as CO_2, was constant approximately 15 hr after eating. Likewise, constant volumes of gas were liberated about 5.5 hr after feeding. Varied results were obtained on samples collected within 1.5 hr after an hour feeding period. Some of these differences may be attributed to the amount of food and water consumed during this time, since the amount of hay eaten varied between 0.4 to 1.0 pound and the water drunk, between 3.2 to 4.0 pounds. Nevertheless the amount of bicarbonate in the ingesta and the pH consistently appear to reach an equilibrium about 6 and 15 hr after eating.

Acid and Metabolic CO_2 Determined with Bicarbonate Buffer.

After finding that approximately 1600, 800 and 1300 ul CO_2 were liberated by the addition of 0.5 ml $3NH_2SO_4$ from 1 ml of rumen ingesta collected from ewe T6359 after being deprived of food and water for about 15, 1, and 6 hr respectively, the metabolic and acid CO_2 from the system were investigated. As pointed out by Umbreit et al. (1947), the CO_2 produced by the cells does not affect the concentration of bicarbonate buffer employed, but any acid formed will produce CO_2 from the bicarbonate. By employing 2 manometers, the metabolic CO_2 can be

determined as well as that liberated by the addition of sufficient acid to bring the pH below 5. If acid is tipped into the first flask after equilibration, a measure of the total initial bicarbonate, expressed as CO_2, is obtained. If acid is tipped into the second system after the test period, the residual bicarbonate can be determined. The amount of CO_2 produced by acid formation is found by the difference between the initial and residual bicarbonate. This amount subtracted from the CO_2 produced during the experimental period gives the metabolic CO_2 or that formed by the enzymes elaborated by the organisms present.

Previous work indicated that more consistent results could be obtained with the sheep having her rumen closed by a plug than with the sheep having the open fistula, hence material from ewe K1111 was employed in the following studies.

In the first series, duplicate runs were made in order to determine the reproducibility of results. Samples were obtained 3.5 hr after the animal had eaten and on another day 12 and 2 hr after eating. The Warburg vessels held 1 ml 1.0 per cent $NaHCO_3$ and 1 ml rumen contents. The sidearms for the basal runs contained 0.5 ml water, while the others held 0.5 ml 10 per cent glucose. The second sidearms of the manometers containing glucose had 0.3 ml $3NH_2SO_4$ to stop the reactions. All tests were made in an atmosphere of CO_2 at 37 C for 45 min. After equilibrating 10 min, the systems were closed and the mercury in each manometer, including the thermobarometer, was pulled down individually by applying a negative pressure to the closed side of

the manometer while carefully opening the stopcock. This procedure was necessary so that the mercury on the open side of the manometer would not go off the scale when acid was tipped into the vessels. An attempt was made to adjust the mercury in all columns to about the same level.

Basal and glucose fermentations were investigated, and the initial and residual bicarbonates were determined by dumping acid at the start into a flask containing glucose and into the second vessel at the finish of the run. From the information obtained, the acid and metabolic CO_2 were calculated. The findings are given in table 35.

TABLE 35. Basal and glucose fermentation, initial and residual bicarbonate and acid and metabolic CO_2 determined by employing bicarbonate buffer and rumen ingesta from ewe K1111 at different hours after eating.

Hours after eating	Microliters CO_2					
	Basal	Initial bicarbonate	Residual bicarbonate	Glucose	Acid CO_2	Metabolic CO_2
Run 1						
3.5	94	3460	2784	742	676	66
	90	2896	2594	881	302	579
Run 2						
12.0	47	4259	3712	812	547	265
	45	4264	3689	857	575	282
Run 3						
2.0	71	3346	2807	673	539	134
	45	3306	2808	785	498	287

Total reaction volume, 2.8 ml including: 1 ml rumen ingesta and 1 ml 1.0 per cent $NaHCO_3$ in the vessels. Five-tenths ml water (basal) or 0.5 ml 10.0 per cent glucose in one sidearm and 0.3 ml $3NH_2SO_4$ in the second sidearms of vessels containing glucose. Atmosphere, CO_2; temperature, 37 C; duration, 45 min; manometers, mercury.

A slight error of approximately 150 ul is involved in the initial and residual bicarbonate findings, since 2.8 ml was employed in the flasks instead of the 2.5 ml for which the constants were calculated. However the difference would hold throughout and would not affect the values for comparative purposes.

The duplicate results of run 1 do not check as well as those of runs 2 and 3. Some of this variance may be attributed to technic, but with mercury in the manometers, the flask constants are about 15 times greater than with Brodie's solution. This large factor increases differences recorded on the manometers appreciably in some cases. It will be noted that the initial bicarbonate (CO_2) of run 2 exceeds that of run 3 by about 1000 ul. The pH of the sample collected after the ewe was deprived of food and water for 12 hr was 7.30 and 6.80 after a 2-hr fasting. It is probable that the higher pH resulted from the bicarbonate accumulation following the cessation of active fermentation and the continual flow of saliva into the rumen.

In 3 of the 4 trials (runs 2 and 3) the acid CO_2 was approximately twice that of the metabolic CO_2, indicating that acid formation was contributing appreciably to the ul CO_2 obtained in other experiments where bicarbonate was used as the buffer. It was now necessary to learn what was being measured when the phosphate buffer was employed. In order to determine this, a second series of runs was made in which the phosphate and bicarbonate buffers were compared.

Acid and Metabolic CO_2 Determined with 2 Buffers.

Three Warburg vessels were used for each buffer to measure the basal, the initial bicarbonate and the glucose fermentation as well as residual bicarbonate. One ml of buffer and 1 ml of rumen ingesta were added to each flask, while 0.5 ml water was placed in the sidearm of the basal flasks and 0.5 ml 10 per cent glucose in the others, which also contained 0.3 ml $3NH_2SO_4$ in the second sidearm. After the first 2 trials of run 1, the glucose was decreased to 0.2 ml in order to have the reaction volume at 2.5 ml for which the flask constants were determined, instead of 2.8 ml. Also the 0.5 ml water was added directly to the basal flasks, thus eliminating a drop in manometric readings encountered with the phosphate buffer after tipping the sidearm. All runs were in an atmosphere of CO_2 at 37 C for 45 min and equilibration was for 10 min. The mercury in most of the manometers was started at less than 70 mm on the open end. This was accomplished by the method described previously. Readings were taken for 10 min at the end of which time differences among flasks were constant. The substrates were then dumped as well as acid to 1 flask of glucose for each buffer. At the end of the run, acid was also tipped into the other flasks containing glucose. The basal and glucose fermentation, the initial and residual bicarbonate and the acid and metabolic CO_2 are expressed as microliters CO_2 in table 36.

Although in some cases the results on different days seem to check rather closely at comparable times, one will also note considerable variation between buffers and among the different runs with a

TABLE 36. Basal and glucose fermentation, initial and residual bicarbonate and acid and metabolic CO_2 determined comparing phosphate and bicarbonate buffers and employing rumen ingesta from ewe K1111 at different hours after eating.

Hours after feeding	Microliters CO_2											
	Phosphate buffer						Bicarbonate buffer					
	Basal	Initial bicarb.	Residual bicarb.	Glucose	Acid CO_2	Metabolic CO_2	Basal	Initial bicarb.	Residual bicarb.	Glucose	Acid CO_2	Metabolic CO_2
Run 1												
12	47	2176	1694	534 (11.4)*	482	52	23	4469	3879	952 (41.4)*	590	362
2	94	983	719	464 (4.9)	264	200	113	3352	2927	666 (5.9)	425	241
6	118	1615	1206	835 (7.1)	409	426	90	4058	3451	904 (10.0)	607	297
Run 2												
12	71	2153	1647	858 (12.1)	506	352	45	4423	3784	928 (20.1)	639	289
2	306	1030	742	580 (1.7)	288	292	136	3443	2927	690 (5.1)	516	174
6	141	1708	1090	1021 (7.2)	618	403	136	3967	3237	1214 (8.9)	730	484
Run 3												
12	71	2059	1624	650 (9.1)	435	215	90	4264	3594	833 (8.3)	670	163
2	235	866	487	742 (3.2)	379	363	158	3101	2451	1142 (7.2)	650	492
6	94	1591	1067	882 (9.4)	524	358	90	3808	3046	1142 (12.7)	762	380

*Times basal rate exceeded.

Total reaction volume, 2.5 ml including: 1 ml rumen ingesta, 1 ml buffer and 0.5 ml H_2O (basal flasks) in vessels. Sidearms contained 0.2 ml 10.0 per cent glucose and 0.3 ml $3NH_2SO_4$ in second sidearms of flasks containing glucose. Atmosphere, CO_2; temperature, 37 C; duration, 45 min; manometers, mercury.

particular buffer. It has been pointed out previously that the rumen is a dynamic organ, and one should not expect to obtain too close checks on different days when the amount of food and water consumed vary as well as other unknown factors. It proved useful in earlier experiments to determine the glucose/basal ratio which somewhat tended to smooth out daily fluctuations. If this is done with the present data from the phosphate buffer, the following are obtained for the 12-, 2-, and 6-hour samplings respectively for the 3 different days: 11.4, 4.9, 7.1; 12.1, 1.7, 7.2; 9.1, 3.2, 9.4. In a similar manner, the ratios with the bicarbonate buffer were 41.4, 5.9, 10.0; 20.1, 5.1, 8.9; 9.3, 7.2, 12.7.

It will be noted that there is a general pattern in each case of a high value for the early morning sample, indicating a relatively wide spread between the basal and glucose fermentation with a lower value at the 2-hr sampling and again a higher ratio at the 6-hr period. However, the findings between the 2 buffers varied considerably in some cases. Although there was some bicarbonate in the buffered phosphate salt solution employed and also in the rumen ingesta sample, it is possible that there was not sufficient present to permit the liberation of a mole of CO_2 for each mole of acid formed. This is suggested since in the 9 trials 1.2 to 1.7 times more acid CO_2 was produced with the bicarbonate buffer than with the phosphate, and in each run this figure was greatest at the 2-hr sampling when the bicarbonate in the rumen material was lowest.

The pH is another factor which should be considered in discussing the variations in the results obtained with the different buffers. The

pH was determined, employing Accutint papers, at the start of run 3 after the flask contents had been equilibrated in an atmosphere of CO_2 for 8 min. The flasks were removed from the manometers and in each vessel the pH was found to be about 7. At the end of the run, the pH in the basal flasks was still 7, and since acid had been dumped in the other vessels, they were not tested. The pH was also 7 when tested at the beginning of the 6-hr trial (run 3) but when determined at the end of the 2- and 6-hr trials, the pH was about 7 in the basal flasks with the bicarbonate buffer and about 6.4 in those with the phosphate. The pH of the 12-, 2-, and 6-hr rumen samples, as determined by the Beckman pH meter, were 7.40, 6.65, and 7.15 respectively. The initial bicarbonate values (ul CO_2) for the same samples in the phosphate buffer were 2059, 866, and 1591 ul respectively (table 36, run 3). The alkaline pH (7.40), the high initial bicarbonate (2059 ul), as well as the lower basal rate in the 12-hr sample may explain why the pH was still about 7 at the end of the 12-hr trial but lower in the 2- and 6-hr studies when fermentation was more active and bicarbonate was lower. Similar findings (pH 6.2-6.4) in phosphate buffer and 6.8-7.0 in bicarbonate) at the end of several other runs were observed previously when the 2 buffers were compared employing 0.5 ml rumen samples and Brodie's solution in the manometers. Since it is known that various enzyme systems have different optimum pH zones with different buffers, it is possible that the shift in pH to the acid side during the course of the experiment was responsible for the variations in the results obtained with the bicarbonate and phosphate buffers.

If the data of table 36 are again considered, it will be noted that in every trial the glucose fermentation and the acid CO_2 determined by employing bicarbonate buffer exceeded the ul CO_2 obtained with the phosphate. On the other hand, in only 5 of the 9 trials did the metabolic CO_2 measured in the presence of bicarbonate exceed those in the phosphate buffer and no consistency in differences was apparent.

The CO_2 liberated by acid exceeded the metabolic CO_2 between 1.3 and 4.1 times with an average of about 2 times for the 9 trials. This in general confirms the findings of runs 2 and 3 given in table 35 in which the acid CO_2 was about twice the metabolic CO_2. These results indicate the importance of the CO_2 liberated by acid during the experimental period and thus give evidence of an advantage of the bicarbonate over the phosphate buffer in studies of this type.

In all of the experiments performed, the gas liberated in the reactions was expressed as CO_2, although methane may have been included as suggested by the reports of others. Washburn and Brody (1937) used a mask-spirometer to measure the gases expired by cattle. They found the CO_2/CH_4 ratio to be 2.6 soon after feeding and 0.77, 15 hr later when the animal received alfalfa. On an alfalfa-grain ration, the ratio was 2.6 immediately after eating, 0.97, 15 hr later and 0.48 at 23 hr. The rumen methane level held fairly constant (30 per cent) on all diets, whereas on an alfalfa or alfalfa-grain diet the CO_2 level was between 60 to 70 per cent during the first 7 hr after feeding, fell to about 30 per cent 15 hr later and to 20 per cent 20 hr after eating. Johnson *et al.* (1944) found that the maximum methane production occurred

during the first hour after eating regardless of the diet. They observed a rapid drop in gas production for several hours and then a slower decline. Nine hours after feeding an urea or casein ration to growing lambs, the methane production had decreased 50 per cent or more. There appears to be a conflict between these reports in regard to the constancy of the methane level. On the other hand, Gray, Pilgrim and Weller (1951) measured the amounts of short chain fatty acids and methane formed during the in vitro fermentation of hay by rumen fluid. They found that about 15 liters of methane was produced per kilogram of wheaten hay and 20 liters from a similar amount of alfalfa.

These studies show that methane may be an important product of fermentation in the rumen and indicate the need for further investigation regarding the amount which may be present in the gas liberated in the manometric experiments reported in this work.

GENERAL DISCUSSION

Numerous workers have emphasized that the first step in ruminant nutrition is microbial nutrition. The host is dependent upon the activity of microorganisms for the preparation of its food. Hence any information regarding microbial activity in the rumen increases the horizon of our knowledge pertaining to this important process. The present study has been an attempt to contribute to this perplexing and controversial problem.

A manometric method employing a constant volume Warburg apparatus has been used in evaluating the fermentation activity of ingesta from sheep with permanent rumen fistulas under varying conditions of time and diet. Such a procedure has been investigated, since direct microscopy, cultural methods or other manometric technics have not proved entirely satisfactory in answering many of the questions pertaining to rumen microbiology.

A basal fermentation was determined by measuring the gas produced when a mixture of rumen ingesta and bicarbonate or phosphate buffer were allowed to react in an anaerobic atmosphere (CO_2) at 37 C. Glucose and other carbohydrate fermentations were obtained when an excess of substrate was added to the mixture. Studies have been continued over a period of 2 years, and a number of points not previously described have been investigated.

It was found consistently that the basal fermentation of the early morning rumen sample was relatively low but that of glucose exceeded the basal by 10 to 20 times. Samples obtained 1 to 2 hr after eating

generally had a basal rate several times greater than the first, but the activity on glucose was lower. Five to 6 hr after the animal was fed, the ingesta exhibited a lower basal and an increased glucose fermentation over that observed shortly after eating. In other words, the glucose/basal ratio was high in the morning, dropped considerably a short time after eating and rose again several hours later.

Undoubtedly many factors are involved in attempting to explain these differences in ratios. It is possible that when the early morning samples were collected, most of the readily fermentable materials in the rumen had been utilized, and the gas liberated indicated low or endogenous activity. However being in a favorable environment, organisms capable of fermenting glucose readily attacked this substrate when it was dumped into the vessels. Shortly after the ingestion of food, many bacteria found suitable substrates and contributed to an increased basal activity. Several workers have shown that the volatile acids, acetic, propionic and butyric found in the rumen under anaerobic conditions are involved in reactions which could well account for considerable CO_2 and methane and thus increase the basal rate. The ingestion of food might dilute the numbers of glucose fermenters, and hence glucose fermentation would decrease at this time. As the food was utilized, many organisms would find conditions less satisfactory and the basal rate would drop. The glucose fermenters might have increased by this time or products of glucose fermentation such as acetic acid would be available for other organisms and hence contribute to the accelerated rate shown with the glucose substrate several hours after

the animal was fed.

In a limited number of studies, it was found that the glucose fermentation was exceeded slightly by sucrose, but the former was generally at least twice as great as D-xylose, hemicellulose, soluble starch and corn starch, although this varied according to the time after eating. These findings agreed in general with Quin (1943) who studied the fermentation of various carbohydrates in vitro employing a different manometric method.

It was found that the ration influenced the fermentation activity. Barley was added to the diet and caused an increase in the basal as well as the glucose and corn starch fermentation. There was no appreciable change in the number of bacteria counted as compared to an alfalfa diet, but the pH of all samples was lower. The addition of this readily fermentable substrate was responsible for a drop in pH of the ingesta from about 6.8 when on alfalfa to about 6.0 shortly after eating with only a slight rise in the next 4 to 6 hr (6.2-6.3), whereas with alfalfa it was often slightly alkaline.

On the other hand, when the sheep was fed oat straw, the basal rate remained constant at about the level frequently noted in the samples when the ewe had been deprived of alfalfa for 12 to 15 hr. However there was a decline in the fermentation of glucose, corn starch, ground barley and alfalfa employed as substrates in the Warburg vessels. Addition of corn starch for 9 days directly to the rumen after 15 days on an oat straw diet caused some increase in the fermentation of the

substrates but no increase in the basal. Plans to supplement the oat straw-corn starch ration with urea as a nitrogen source did not eventuate, since the ewe lost 20 pounds during this period and the fistula plug which had remained in place for more than a year came out. Since considerable effort had been expended in obtaining this ewe and in providing the rumen fistula, it did not seem wise to risk losing the animal by continuing this experiment. However it would be interesting to repeat this work to determine whether some protein or non-protein supplement would increase the fermentation activity of the rumen microflora, depressed by the poor quality ration.

The numbers of organisms were found to decline from an average of about 30 billion bacteria per ml when the animal was fed alfalfa to about 10 billion per ml on the straw-starch diet.

In these studies addition of ground alfalfa as a substrate consistently was responsible for a greater rate of fermentation than glucose or any other substrate investigated. This would indicate that alfalfa contained some component or components which could be utilized by a larger variety or number of rumen organisms than glucose, starch or barley. It would seem that this phase should be further investigated with the possibility that various test rations might be screened by this method in order to gain some idea regarding their suitability for the rumen microflora.

The rumen fluid is well buffered by phosphates and bicarbonates; hence a number of experiments were performed to determine the suitability of each for studying rumen fermentation <u>in vitro</u> by the Warburg

apparatus. Although the pattern of the glucose/basal ratio was similar for the 2 buffers, the vessels containing bicarbonate in general showed greater volumes of gas being produced. This agrees with what might be expected, since both acid and metabolic CO_2 can be measured under anaerobic conditions by employing a bicarbonate buffer.

The amount of bicarbonate in 1 ml of rumen ingesta was found to be approximately 1600, 800, and 1300 ul CO_2 when collected 15, 1, and 6 hr respectively after eating. This indicated that rumen material contained about twice as much bicarbonate after fasting 15 hr as shortly after eating. Dilution by recently ingested food and the release of CO_2 in the rumen by acid formation would probably account for this. Little difference was found between the amount of bicarbonate in the anterior or the posterior areas of the rumen in the early morning samples when the bicarbonate was well mixed throughout the contents, whereas shortly after eating, there was a greater amount in the front section brought in by the saliva but not yet mixed by the constant churning motion of the normal rumen. This experiment showed that the rumen ingesta contained considerable bicarbonate and hence would be a contributing factor to CO_2 evolution when phosphate buffer was employed and acid was formed in the fermentation.

In a series of comparative runs (table 36) employing the 2 buffers and rumen ingesta, obtained 12 hr after eating, in an atmosphere of CO_2 with mercury in the Warburg manometers, the initial and residual bicarbonates were approximately twice as great with bicarbonate as with the phosphate buffer. These values were decreased about one-half

with the phosphate buffer 2 hr after eating but only about one-third with the bicarbonate. These differences in the bicarbonate level appeared to affect the amount of glucose fermentation as well as acid CO_2, since without exception the results were higher when the bicarbonate buffer was employed. On the other hand, there was no consistent difference when the metabolic CO_2 was considered.

In all of these studies a 3-year-old Rambouillet ewe (K1111) and a 6-year-old Targhee ewe (T6359) with permanent rumen fistulas were used. The rumen ingesta of each were compared for 12 consecutive days. At the start, ewe T6359 was infested with maggots, which may have caused anorexia for several days. A compact mass of ingesta was encountered in the rumen making it impossible to obtain a representative sample. The addition of water, glucose and rumen contents from the healthy ewe directly to the rumen via the fistula resulted in her appearing normal within 4 days. The fermentation capacity of samples from this animal was affected for several days, but even after this point all activity was 25 to 50 per cent below that of sheep K1111. It is possible that some of this difference might be attributed to the rumen of ewe K1111 being closed by a lucite plug whereas that of T6359 was open and rumen contents were continually being lost. On the other hand, there was no significant difference between the numbers of bacteria found in the rumen samples during this period. With sheep T6359 the average count was 18.5 billion bacteria varying between 11.1 and 23.6 billion per ml, and with ewe K1111 the average was 22.6 billion ranging between 12.6 and 34.7 billion bacteria per ml.

Throughout this work there was little correlation between the number of bacteria counted in the rumen samples and the fermentation activity of the ingesta.

The results of all of the fermentation studies have been reported as CO_2, although it is admitted that methane may be included. The literature reports are conflicting regarding the presence and amount of methane in the rumen and this matter bears further investigation.

The manometric method employed in these studies appears to show promise as a procedure for determining the fermentation capacity of the rumen microflora. This would be advantageous, since direct microscopy gives little indication of microbial activity. Cultural methods have been employed rather successfully in recent years, but undoubtedly only a few of the important bacteria have been isolated and their properties studied in detail. Fermentation studies have been carried out in several ways, but none seems to show the potentialities of the present method, which unquestionably can be made more useful by further study. The possibilities of using this technic for evaluating rations, for determining the effect of antibiotics and other therapeutic agents on the rumen microflora and for gathering additional information on ruminant digestion open a road to a phase of this extensive problem that has received little attention.

SUMMARY

A manometric method employing a constant volume Warburg apparatus has been used in evaluating the fermentation activity *in vitro* of ingesta from sheep with permanent rumen fistulas at various intervals after eating different rations.

A basal fermentation was determined by measuring the gas produced when mixtures of rumen ingesta and bicarbonate or phosphate buffer were allowed to react under anaerobic conditions at 37 C. Glucose and other fermentations were investigated when an excess of substrate was added.

The glucose fermentation exceeded the basal by 10 to 20 times with samples obtained 12 to 15 hours after eating. Shortly after consuming hay and water, the basal rate increased and glucose fermentation decreased. As the period following eating lengthened, the basal rate dropped and glucose rose.

Sucrose fermentation exceeded slightly the activity on glucose which was 2 to several times greater than D-xylose, hemicellulose, soluble starch and corn starch, although this varied with the time after eating.

When barley supplemented the alfalfa diet, an increase in the fermentation of all substrates was noted although there was no appreciable change in the number of bacteria counted in the rumen fluid by direct microscopy employing a diluted carbol fuchsin stain. The pH of all samples was lower, dropping to about 6.0 shortly after eating, than when only alfalfa was fed.

An oat straw diet for 15 days resulted in little change in the basal rate of samples collected 12 to 15 hours after eating, but a decrease in the fermentation of glucose, corn starch, ground barley and alfalfa substrates was noted. When corn starch was added to the rumen for 9 days, there was some increase in the fermentation of the substrates but not the basal. The number of organisms declined from about 30 billion bacteria per ml on an alfalfa diet to about 10 billion per ml on the straw-starch ration.

Phosphate and bicarbonate were employed as buffers in the Warburg flasks. In general, greater volumes of gas were recorded with the bicarbonate.

One ml of rumen ingesta treated with acid released about 1600, 800, and 1300 ul CO_2 when obtained 15, 1, and 6 hours respectively after eating. The amount of bicarbonate in the anterior and posterior of the rumen varied with the time in which the saliva had been mixed with the ingesta.

The initial and residual bicarbonates determined in the flasks with early morning samples were about twice as great with the bicarbonate buffer as with the phosphate. These values varied at different times of the day. Without exception, the glucose fermentation and acid CO_2 were higher with the bicarbonate buffer. However, there was no consistent difference between the metabolic CO_2 values.

The fermentation capacity of a 6-year-old sheep with an open fistula was about 50 per cent less than that of a 3-year-old ewe with a fistula closed by a lucite plug. The average number of

bacteria in the rumen fluid for 12 consecutive days on an alfalfa diet were 18.5 billion for the sheep with the open fistula and 22.6 billion bacteria per ml for the other.

The number of bacteria per ml of ingesta from 2 ewes under a variety of conditions over a period of 2 years were between 5 and 50 billion with an average of about 30 billion. In general the counts showed little correlation with the fermentation capacity of the ingesta.

The manometric method warrants further investigation for possible uses in evaluating rations, in determining the effects of therapeutic agents on the rumen microflora, and in obtaining additional information on ruminant digestion.

LITERATURE CITED

Ankersmit, P. 1905 Untersuchungen uber die Bakterien im Verdauungskanal des Rindes. Zentr. Bakt. Parasitenk., I, Orig., 39, 359-369, 574-584, 687-695; 40, 100-118.

Baker, F. 1931 Preliminary note on the role of coccoid microorganisms in the disintegration of cell wall substances. Zentr. Bakt. Parasitenk., II, 84, 452-454.

Baker, F. 1939 The disintegration of cellulose in the alimentary canal of herbivora. Science Progress, 134, 287-301.

Baker, F. 1942a Microbial synthesis and autolysis in the digestive tract of herbivora. Nature, 149, 582-583.

Baker, F. 1942b Microbial factors in the digestive assimilation of starch and cellulose in herbivora. Nature, 150, 479-481.

Baker, F. 1943 Direct microscopical observations upon the rumen population of the ox. I. Qualitative characteristics of the rumen population. Ann. Applied Biol., 30, 230-239.

Baker, F., and Harriss, S. T. 1947-1948 Microbial digestion in the rumen (and caecum), with special reference to the decomposition of structural cellulose. Nutr. Abs. and Revs., 17, 3-12.

Baker, F., and Martin, R. 1937 Some observations on the iodophile microflora of the caecum of the rabbit; with special regard to the disintegration of cell wall substances. The significance of the iodophile population. Zentr. Bakt. Parasitenk, II, 96, 18-34.

Baker, F., and Martin, R. 1938 Disintegration of cell wall substances in the gastro-intestinal tract of herbivora. Nature, 141, 877-878.

Baker, F., and Martin, R. 1939 Studies in the microbiology of the caecum of the horse. Zentr. Bakt. Parasitenk, II, 99, 400-424.

Baker, F., and Nasr, H. 1947 Microscopy in the investigation of starch and cellulose breakdown in the digestive tract. J. Royal Microscopical Soc., 67, 27-42.

Baker, F., Harriss, S. T., Phillipson, A. T., McNaught, M. L., Smith, J. A. B., Kon, S. K., and Porter, J. W. G. 1947-1948 The role of the microflora of the alimentary tract of herbivora with special reference to ruminants. Nutr. Abs. and Revs., 17, 1-37.

Baker, F., Nasr, H., Morrice, F., and Bruce, J. 1950 Bacterial breakdown of structural starches and starch products in the digestive tract of ruminant and non-ruminant mammals. J. Path. and Bact., 62, 617-638.

Barcroft, J., McAnally, R. A., and Phillipson, A. T. 1944 Absorption of volatile acids from the alimentary tract of the sheep and other animals. J. Exptl. Biol., 28, 120-129.

Becker, E. R., Schulz, J. A., and Emmerson, M. A. 1929 Experiments on the physiological relationships between the stomach infusoria of ruminants and their hosts. Iowa State Coll. J. Sci., 5, 215-241.

Bortree, A. L., Dunn, K. M., Ely, R. E., and Huffman, C. F. 1946 A preliminary report on the study of factors influencing rumen microflora. J. Dairy Sci., 29, 542-543. Abs. paper presented at 41st Ann. Meeting of Amer. Dairy Sci. Assoc.

Clark, R., and Lombard, W. A. 1951 Studies on the alimentary tract of the Merino sheep in South Africa. XXII. The effect of the pH of the ruminal contents on ruminal motility. Onderstepoort J. Vet. Res., 25, 78-92.

Cole, H. H., Huffman, C. F., Kleiber, M., Olson, T. M., and Schalk, A. F. 1945 A review of bloat in ruminants. J. Anim. Sci., 4, 183-236.

Conrad, H. R., Hibbs, J. W., Pounden, W. D., and Sutton, T. S. 1950 The effect of rumen inoculations on the digestibility of roughages in young dairy calves. J. Dairy Sci., 33, 585-592.

Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A., and Phillipson, A. T. 1945 The mechanism of absorption from the rumen as exemplified by the behaviour of acetic, propionic and butyric acids. J. Exptl. Biol., 22, 75-84.

Elsden, S. R., Hitchcock, M. W. S., Marshall, R. A., and Phillipson, A. T. 1946 Volatile acid in the digesta of ruminants and other animals. J. Exptl. Biol., 22, 191-202.

Elsden, S. R., and Phillipson, A. T. 1948 Ruminant digestion. Ann. Rev. Biochem., 17, 705-726.

Gall, L. S. 1946 Some studies on the rumen microorganisms of sheep and cattle. Thesis, Cornell Univ. 87 p.

Gall, L. S., and Huhtanen, C. N. 1951 Criteria for judging a true rumen organism and a description of five rumen bacteria. J. Dairy Sci., 34, 353-362.

Gall, L. S., Burroughs, W., Gerlaugh, P., and Edington, B. H. 1949a Special methods for rumen bacterial studies in the field. J. Anim. Sci., 8, 433-440.

Gall, L. S., Burroughs, W., Gerlaugh, P., and Edington, B. H. 1949b Rumen bacteria in cattle and sheep on practical farm rations. J. Anim. Sci., 8, 441-449.

Gall, L. S., Stark, C. N., and Loosli, J. K. 1947 The isolation and preliminary study of some physiological characteristics of the predominating flora from the rumen of cattle and sheep. J. Dairy Sci., 30, 891-899.

Garton, G. A. 1951 Observations on the distribution of inorganic phosphorus, soluble calcium and soluble magnesium in the stomach of the sheep. J. Exptl. Biol., 28, 358-368.

Goss, H. 1943 Some peculiarities of ruminant nutrition. Nutr. Abs. and Revs., 12, 531-538.

Gray, F. V. 1947 The absorption of volatile fatty acids from the rumen. J. Exptl. Biol., 24, 1-10.

Gray, F. V. 1948 The absorption of volatile fatty acids from the rumen. II. The influence of pH on absorption. J. Exptl. Biol., 25, 135-144.

Gray, F. V., and Pilgrim, A. F. 1951 Fermentation in the rumen of the sheep. II. The production and absorption of volatile fatty acids during the fermentation of wheaten hay and lucerne hay in the rumen. J. Exptl. Biol., 28, 83-90.

Gray, F. V., Pilgrim, A. F., and Weller, R. A. 1951 Fermentation in the rumen of sheep. I. The production of volatile fatty acids and methane during the fermentation of wheaten hay and lucerne hay *in vitro* by microorganisms from the rumen. J. Exptl. Biol., 28, 74-82.

Hastings, E. G. 1944 The significance of the bacteria and the protozoa of the rumen of the bovine. Bact. Revs., 8, 235-254.

Henneberg, W. 1922 Untersuchungen über die Darmflora des Menschen mit besonderer Berücksichtigung der „jodophilen Bacterien im Menschen - und Tierdarm sowie im Kompost-dünger. Zentr. Bakt. Parasitenk, II, Orig., 55, 242-281.

Hibbs, J. W., and Pounden, W. D. 1948 The influence of the ration and early rumen development on the changes in the plasma carotenoids, vitamin A and ascorbic acid of young dairy calves. J. Dairy Sci., 31, 1055-1061.

Hopffe, Anna 1919a Bakteriologische Untersuchungen uber die Cellulose-verdauung. Zentr. Bakt. Parasitenk., I, Orig. 83, 374-386.

Hcpffe, Anna 1919b Ueber einen bisher unbekannten cellulöselosenden, im Verdauungs-traktus verkommenden Aspergillus, "Aspergillus cellulosae," seine Züchtung and Eigenschaften. Zentr. Bakt. Parasitenk., I, Orig., 83, 531-537.

Hungate, R. E. 1942 The culture of Eudiplodinium neglectum, with experiments on the digestion of cellulose. Biol. Bull., 83, 303-319.

Hungate, R. E. 1943 Further experiments on cellulose digestion by the protozoa in the rumen of cattle. Biol. Bull., 84, 157-163.

Hungate, R. E. 1944 Studies on cellulose fermentation. I. The culture and physiology of an anaerobic cellulose-digesting bacterium. J. Bact., 48, 499-513.

Hungate, R. E. 1946a Studies on cellulose fermentation. II. An anaerobic, cellulose-decomposing actinomycete, Micromonospora propionici, N. Sp. J. Bact., 51, 51-56.

Hungate, R. E. 1946b The symbiotic utilization of cellulose. J. Elisha Mitchell Scientific Soc., 62, 9-24.

Hungate, R. E. 1950a The anaerobic mesophilic cellulolytic bacteria. Bact. Revs., 14, 1-49.

Hungate, R. E. 1950b Mutualisms in protozoa. Ann. Rev. Microbiology, 4, 53-66.

Johnson, B. C., Hamilton, T. S., Robinson, W. B., and Garey, J. C. 1944 On the mechanism of non-protein nitrogen utilization by ruminants. J. Anim. Sci., 3, 287-298.

Khouvine, Y. 1923 Digestion de la cellulose par la flore intestinale del'homme. B. cellulosae dissolvens, N. Sp. Ann. inst. Pasteur, 37, 711-752.

Khouvine, Y. 1926 Le Bacillus cellulosae dissolvens et la fermentation de la cellulose. Compt. rend. soc. biol., 94, 1072-1074.

Kick, C. H., Gerlaugh, P., Schalk, A. F., and Silver, E. A. 1938 Digestion in cattle. pH of the ingesta. Ohio Agr. Exp. Sta. Bull. 592, 105.

Köhler, W. 1940 Versuche über die zahlenmässige Veränderung der natürlichen Bakterienflora in den Verdauungsorganen der Wiederkäuer. Arch. Mikrobiol., 11, 432-469.

Loomis, W. F. 1949 A convenient and rapid method of calibrating Warburg manometers. Science, 109, 491-492.

Louw, J. G., and Wath, J. G. van der 1943 The influence of varying maize supplements on the digestibility of the cellulose in a poor veld hay in relation to the bacterial population of the rumen of sheep with a note on the nitrogen metabolism. Onderstepoort J. Vet. Sci. and Anim. Ind., 18, 177-190.

Marston, H. R. 1939 Ruminant nutrition. Ann. Revs. Biochem., 8, 557-578.

McAnally, Rachel A. 1943 Studies on the alimentary tract of Merino sheep in South Africa. X. Notes on the digestion of some sugars in the rumen of sheep. Onderstepoort J. Vet. Sci. and Anim. Ind., 18, 131-138.

McAnally, R. A., and Phillipson, A. T. 1944 Digestion in the ruminant. Biol. Revs. Cambridge Phil. Soc., 19, 41-54.

McBee, R. H. 1950 Manometric evaluation of the rumen microflora. Proc. Mont. Acad. Sci., 10, 15.

McDonald, I. W. 1948 The absorption of ammonia from the rumen of the sheep. Biochem. J., 42, 584-587.

McDougall, E. I. 1948 Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J., 43, 99-108.

Moir, R. J., and Masson, Marjorie J. 1952 An illustrated scheme for the microscopic identification of the rumen microorganisms of sheep. J. Path. and Bact., 64, 343-350.

Moir, R. J., and Williams, V. J. 1950 Ruminal flora studies in the sheep. II. The effect of the level of nitrogen intake upon the total number of free organisms in the rumen. Australian J. Sci. Res., Series B, Biol. Sci., 3, 381-392.

Monroe, C. F., and Perkins, A. E. 1939 A study of the pH values of the ingesta of the bovine rumen. J. Dairy Sci., 22, 983-991.

Myburgh, S. J., and Quin, J. I. 1943 Studies on the alimentary tract of Merino sheep in South Africa. IX. The H-ion concentration in the forestomachs of fistula sheep under different experimental conditions. Onderstepoort J. Vet. Sci. and Anim. Ind., 18, 119-130.

Olson, T. M. 1941 The pH values of the ingesta of the rumen of slaughtered animals. J. Dairy Sci., 24, 413-416.

Owen, E. C. 1947 The role of microorganisms in the nutrition of farm animals. Proc. Nutr. Soc., 5, 186-199.

Phillipson, A. T. 1942 The fluctuation of pH and organic acids in the rumen of the sheep. J. Exptl. Biol, 19, 186-198.

Phillipson, A. T. 1947-1948 The role of the microflora of the alimentary tract of herbivora with special reference to ruminants. 3. Fermentation in the alimentary tract and the metabolism of the derived fatty acids. Nutr. Abs. and Revs., 17, 12-18.

Phillipson, A. T., and McAnally, R. A. 1942 Studies on the fate of carbohydrates in the rumen of the sheep. J. Exptl. Biol., 19, 199-214.

Pochon, J. 1935 Role d'une bacterie cellulolytique de la panse, Plectridium cellulolyticum, dans la digestion de la cellulose chez les ruminants. Ann. inst. Pasteur, 55, 676-697.

Pounden, W. D., and Hibbs, J. W. 1948a The influence of the ration and rumen inoculation on the establishment of certain microorganisms in the rumens of young calves. J. Dairy Sci., 31, 1041-1050.

Pounden, W. D., and Hibbs, J. W. 1948b The influence of the ratio of grain to hay in the ration of dairy calves on certain rumen microorganisms. J. Dairy Sci., 31, 1051-1054.

Pounden, W. D., and Hibbs, J. W. 1949a The influence of pasture and rumen inoculation on the establishment of certain microorganisms in the rumen of young dairy calves. J. Dairy Sci., 32, 1025-1031.

Pounden, W. D., and Hibbs, J. W. 1949b Rumen inoculations in young calves. J. Amer. Vet. Med. Assoc., 114, 33-35.

Pounden, W. D., Ferguson, L. C., and Hibbs, J. W. 1950 The digestion of rumen microorganisms by the host animals. J. Dairy Sci., 33, 565-572.

Quin, J. I. 1943 Studies on the alimentary tract of Merino sheep in South Africa. VII. Fermentation in the forestomachs of sheep. Onderstepoort J. Vet. Sci. and Anim. Ind., 18, 91-112.

Quin, J. I., Wath, J. G. van der, and Myburgh, S. 1938 Studies on the alimentary tract of Merino sheep in South Africa. IV. Description of experimental technique. Onderstepoort J. Vet. Sci. and Anim. Ind., 11, 341-360.

Reid, J. T., and Huffman, C. F. 1949 Some physical and chemical properties of bovine saliva which may affect rumen digestion and synthesis. J. Dairy Sci., 32, 123-132.

Sapiro, M. L., Hoflund, S., Clark, R., and Quin, J. I. 1949 Studies on the alimentary tract of the Merino sheep in South Africa. XVI. The fate of nitrate in ruminal ingesta. Onderstepoort J. Vet. Sci. and Anim. Ind., 22, 357-372.

Schalk, A. F., and Amadon, R. S. 1928 Physiology of the ruminant stomach (bovine). Study of the dynamic factors. N. Dak. Agr. Exp. Sta. Bull. 216, 1-64.

Sijpesteijn, A. K. 1948 Cellulose-decomposing bacteria from the rumen of cattle. Thesis, Leiden. 184 p.

Smith, V. R. 1941 In vivo studies of hydrogen ion concentrations in the rumen of the dairy cow. J. Dairy Sci., 24, 659-665.

Smith, J. A. B., and Baker, F. 1944 The utilization of urea in the bovine rumen. IV. The isolation of the synthesized material and the correlation between protein synthesis and microbial activities. Biochem. J., 38, 496-505.

Stanier, R. Y., and van Niel, C. B. 1941 The main outlines of bacterial classification. J. Bact., 42, 437-466.

Thom, C., and Church, M. B. 1926 The aspergilli. Williams and Wilkins Co., Baltimore, Md.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. 1947 Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Co., Minneapolis, Minn.

Uzzell, E. M., Becker, R. B., and Jones, Jr., E. R. 1949 Occurrence of protozoa in the bovine stomach. J. Dairy Sci., 32, 806-811.

Washburn, L. E., and Brody, S. 1937 Growth and development with special reference to domestic animals. XLII. Methane, hydrogen and carbon dioxide production in the digestive tract of ruminants in relation to the respiratory exchange. Missouri Agr. Exp. Sta. Res. Bull. 263, 1-40.

Wath, J. G. van der 1942 Studies on the alimentary tract of the Merino sheep with special reference to the role of the ruminal microflora and flora. Thesis, Pretoria, cited by Baker et al., Nutr. Abs. and Revs., 17, 2, 1947-1948.

Wath, J. G. van der 1948a Studies on the alimentary tract of Merino sheep in South Africa. XI. Digestion and synthesis of starch by ruminal bacteria. Onderstepoort J. Vet. Sci. and Anim. Ind., 23, 367-383.

Wath, J. G. van der 1948b Studies on the alimentary tract of the Merino sheep in South Africa. XII. A technique for the counting of ruminal bacteria. Onderstepoort J. Vet. Sci. and Anim. Ind., 23, 385-387.

Wath, J. G. van der, and Myburgh, S. J. 1941 Studies on the alimentary tract of Merino sheep in South Africa. VI. The role of infusoria in ruminal digestion with some remarks on ruminal bacteria. Onderstepoort J. Vet. Sci. and Anim. Ind., 17, 61-88.

Westhuizen, G. C. A. van der, Oxford, A. E., and Quin, J. I. 1950 Studies on the alimentary tract of Merino sheep in South Africa. XVI. On the identity of Schizosaccharomyces ovis. Part I. Some yeast-like organisms isolated from the rumen contents of sheep fed on a lucerne diet. Onderstepoort J. Vet. Sci. and Anim. Ind., 24, 119-124.

Winogradow, M., Winogradowa-Fedorowa, T., and Wereninow, A. 1930 Zur Frage nach der Einwirkung der Panseninfusorien auf die Verdauung der Wiederkaüer. Zentr. Bakt. Parasitenk., II, 81, 230-244.