A STUDY OF THE ACTIVITIES OF RUMEN BACTERIA

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To Mary, whose
sacrifices and encouragement
made possible the completion
of this work.

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INTRODUCTION

Ruminant digestion and microbiology have been studied by various means and devices for nearly a century. The goal of higher production per animal with low cost rations has increased the interest in rumen microbiology during the past decade. Numerous methods have been developed for studying the microbial digestion that occurs in the rumen, yet the fundamentals that many of the investigators seek still elude them because of the paucity of knowledge concerning the individual functions and requirements of the rumen microorganism.

The purpose of this work was to use the best of the available methods of studying rumen bacteria with the desire to establish a method of routinely culturing rumen organisms that indicate the efficiency of digestion. While this was not completely accomplished the results of this investigation will aid further research.

LITERATURE REVIEW

A number of review articles on rumen microbiology, physiology and ruminant digestion have appeared in the literature over the years; of particular interest may be those by Marston (1939), Goss (1943), McAnally and Phillipson (1944), Hastings (1944), Cole et al. (1945), Hungate (1950), Baker (1947-48), Owen (1947), Elsden (1945), Thorton et al. (1952), and Doetch and Robinson (1953). The reader is referred to these articles for a more complete review of the field. Below appears a review of the literature and statements that appear pertinent to this study.

Function of the Rumen and Rumen Microbiology

The ruminant occupies a rather unique place in the animal kingdom by virtue of its polygastric type of digestive system as compared to that of the more common monogastric animals. This characteristic has allowed it to survive by utilizing a foodstuff that is unfit for the monogastric digestive tract. The ruminant has achieved a high degree of symbiosis with the bacteria and protozoa in its digestive tract, allowing it to consume large quantities of cellulosic material. After mastication, the microorganisms of the rumen break down these materials, converting them to byproducts that may be absorbed directly from the rumen or pass on further along the alimentary canal to the abomasum and from there to be acted on as in the normal monogastric digestive tract.

According to Hungate (1946) and Sijpesteijn (1948) the first recorded study of a controlled feeding experiment was carried on with an ox by Haubner in 1855. This work showed that part of the cellulose disappeared in its passage through the digestive tract of ruminants and that this

material had a nutritional value equivalent to that of starch. Later work by Henneberg and Stohman in 1864, Hofmeister in 1864 and 1869 and Stohman in 1870 confirmed this early investigation and they extended these studies to sheep and goats. Tappeiner in 1884 showed that the site of cellulolytic activity was in the region of the rumen, reticulum and cecum. This work was the first case of in vitro incubation of rumen contents. The following years brought similar experiments and in vivo studies such as the one by Hofmeister in 1881 when a capsule containing grass was placed in the rumen and allowed to remain there for three days. Analysis at the termination of this period showed that 78.4 percent of the crude fiber of the grass had disappeared.

Methods of Studying Rumen Microbiology

Following the establishment of the site of cellulolytic action in the ruminant, various methods of study were applied to the microorganisms found in the rumen. Henneberg (1922) was one of the first to employ direct microscopy to study the disintegration of plant material. He used chlorozinciodine to stain the organisms and observed areas of erosion around the bacteria that were attached to this material. These cavities enlarged as the organism was allowed to act over a period of time. Later, Baker (1931) studied the role of coccoid microorganisms in the disintegration of cell wall substances in material collected from the cecum and natural decaying vegetation. Baker (1939), Baker and Martin (1937, 1938), Baker and Nasr (1947) and Baker and Harriss (1947-48) used various microchemical tests, phase contrast microscopy and polarized light to observe the breakdown of the plant material in the digestive tract of ruminants and other animals. A large portion of their work deals with the iodophilic population of the rumen and cecum. Baker

(1939) stressed the importance of these organisms in relation to cellulose digestion.

Gall, Burroughs, Gerlaugh and Edgington (1949) published a technique using a gram stain and a negative stain, water soluble nigrosine, to determine the morphological types and enumerate the number of bacteria found in the rumen. Smears of the 1-10 and 1-1000 dilutions of the rumen contents were made. The smear of the 1-10 dilution was used for the gram staining procedure. Using a Breed pipette, 0.01 ml. of the 1-1000 dilution was placed on the slide, and mixed with a loopful of nigrosine. This was spread over an area of 4 sq. cm. and dried rapidly on a hot plate. Moir (1941) and Williams and Moir (1951) also used the negative staining technique to determine the total numbers of rumen microorganisms. They varied the procedure slightly by using formalized samples and using a circle with an area of 4 sq. cm. instead of the square as described by Gall et al. (1949). These techniques, though satisfactory for comparative work have difficulties that seriously reflect on the accuracy of the results. These criticisms are deemed sufficently important to quote, Gall et al. (1949) who stated:

> "Excess fluid often causes uneven drying. Too much or too concentrated dye causes cracking, while too slow drying causes a large area of shrinkage. An uneven plate causes ridges of dye, and any foreign particle causes an unstained area which tends to be round."

> "The slide was counted only after a gram stain of the original material had been examined. This acquaints the examiner with the types of organisms which are present and during counting any questionable-looking spots on the nigrosine slide were examined carefully unless they appeared to have the morphology of a bacterium seen on the gram stain. For example, a perfectly spherical white spot would probably be an artifact, if no such perfectly round organism were seen on the gram stain. A spherical area with cracks around it is usually caused by an air bubble."

William and Moir (1951) stated:

> "Some comment on the accuracy of the counts for total free bacteria and of the extent of diurnal and day to day variations in individual animals on a fixed dietary regime is appropriate at this point, ..."
>
> ". . . . It is exceedingly difficult to distinguish with certainty between artifacts and bacteria when their size is less than 0.05 μ even though the presence of more minute organisms can be demonstrated in stained preparations. . . . The value of phase contrast microscopy in overcoming this difficulty is being investigated at the present time."

Van der Wath (1948) and Bortree, Smith, Sarkar and Huffman (1948) used a Petroff-Hauser counting chamber to determine the total numbers of rumen microorganisms. The staining solutions employed were nile blue sulfate and crystal violet respectively. While this technique involves positive staining of the organisms, it has the disadvantages of brownian movement and a delicate technique. Thus it does not lend itself to routine use in a laboratory.

Pounden and Hibbs (1948) used the gram stain to determine the morphological types, but no counts were given. Hungate (1946) employed a dilute solution of Ziehl-Nielsens carbol fuchsin to count rumen organisms, however the counts given are lower than those achieved by using either negative stains or the Petroff-Hauser counting chamber.

Cultural Studies

Margolin (1930) devised four media which utilized agar, peptone, Lemco meat extract and Locke's solution as a base. These media were altered by adding various amounts of hay infusion, rice starch, pulped filter paper, magnesium oxide and saline citrate. Although he was primarily interested in the cultivation of ciliates from the rumen, the bacteria that were present in the inoculum grew very readily. It was

also noted that ciliate subculture was more successful after the filter paper had been partially digested by cellulose digesting organisms introduced from earlier cultures.

Kohler (1940) used two cultural procedures to determine the numbers of rumen bacteria. The plate counts were carried out with a weakly alkaline bouillon agar, incubated aerobically at 37 C from one to five days. The average of the counts after one day of incubation was 20,000 per gm. while after five days of incubation the number had increased to 137,000 per gm. He cited Ankersmits, who in 1905 and 1906 found a 70,000 - 900,000 count on gelatin plates and 2,160,000 - 15,000,000 on dextrose agar.

Tube dilutions made by Kohler, using dilutions up to 10^{-12} showed growth up to 10^{-6}. An average count of 550,000 was obtained by this method. Colonies picked from plates showed only sporeforming rods on the surface while cocci were obtained from the depths. Dextrose bouillon agar was needed to grow cocci and these cultures lived for only three transfers. He was unable to culture cellulose digesting bacteria.

Elsden (1945) used Stephenson's inorganic medium pH 7.4 and supplemented it with 0.4% Difco yeast extract w/v and 1% w/v sodium lactate. This was inoculated with one drop of rumen liquor and incubated for 10 days under an atmosphere of N_2 containing 5% CO_2. After repeated subcultures on liquid and solid media a pure culture of Propionibacterium was obtained. These organisms, small gram negative cocci, developed colonies 1-2 mm. diameter dome shaped and cream colored. They were strictly anaerobic, with good growth in the bottom of a yeast extract lactate agar stab, and fermented glucose and lactic acid with the production of propionic and acetic acids and CO_2.

Van der Wath (1948) isolated an iodophilic streptococcus and studied it in detail. The technique he employed was to place a small silk bag filled with crushed and shelled sterilized maize kernels into the rumen. This was suspended there for 48 hours by a string through a rumen fistula. After this period of digestion the bag was removed to a sterile petri dish, and a few of the partially digested kernels were dropped into sterile saline. From this suspension surface cultures were made on dextrose agar or starch agar slants. The small dew-drop type colony usually proved to be the streptococcus associated with the maize kernels. This organism fermented glucose, lactose, sucrose, maltose, levulose, raffinose, starch and inulin to produce acid but no gas. It did not ferment mannitol, galactose, salicin, sorbitol, rhamnose, inositol and dulcitol. It grew poorly at room temperature but grew well at 37 C. Plain agar supported only feeble growth while dextrose or starch agar gave more profuse growth. Serum also supported growth but there was no liquefaction of the gelatin.

Using the same technique Van der Wath suspended chemically pure cellulose and casein in separate silk bags, and studied stained preparations of the organisms. The cellulose encouraged the growth of various types of gram negative bacteria while the casein encouraged predominantely gram positive organisms. He felt that the organism cultured played a significant part in the breakdown of the starch while the other organisms studied assisted in the complete metabolism of the ration.

Gall (1946) and Gall, Stark and Loosli (1947) outlined a procedure for isolation and studying some of the predominanting flora of the rumen in cattle and sheep. They devised a rich organic broth to culture the organisms. The rumen contents were obtained by use of a pipette or a

dipper and care was taken so as not to aerate the sample. Dilutions were prepared in a 1% glucose solution, planted into tubes containing broth that had been recently heated to remove the oxygen and then sealed with a vaspar seal. These tubes were incubated at 38 C. Growth was shown by turbidity or "clumping" of the cellulose. In most cases growth occurred in 10^{-9} dilution in 48 hours but sometimes it was as long as 60 days before growth appeared in the higher dilutions.

The organisms cultured were all capable of anaerobic growth and some of the cultures produced cellulose digestion ranging from 10 to 20 percent. These organisms appeared to be the types commonly found in the higher dilutions.

In a later study, Gall and Huhtanen (1951) described some of the organisms that were isolated from the rumens of about 350 cattle and sheep in three states. They also established five criteria for judging true rumen organisms, they were as follows; "(a) Anaerobiosis; (b) presence in the numbers of one million or more per gram of fresh rumen contents; (c) isolation of a similar type bacterium at least ten times from at least two animals; (d) isolation from animals in at least two geographical locations; and (e) production by the organism of end products found in the rumen from substrates found in the rumen." The organisms described are all carbohydrolytic and produce gas and lactic acid. The authors stated that though these organisms fit the criteria, they should not be considered "typical rumen organisms in general" because they are found in rations high in carbohydrate or in immature ruminants.

Huhtanen, Rodgers and Gall (1952) altered the original medium of Gall et al. (1947) and outlined in detail a procedure for the isolation and purification of cultures of rumen organisms.

Cysteine was added to the media as well as to the dilution blanks of buffered distilled water in order to maintain the proper Eh. The data reported showed that these "new techniques" were more satisfactory and gave growth in higher dilutions than did the techniques used previously.

Hungate, in a series of papers (1944, 1946b, 1947 and 1950) discussed and described various types of cellulolytic bacteria, some of which were isolated from the rumen of cattle. The most recent publication described in detail the complicated isolation procedure used to isolate and purify these cultures. The medium was composed of a complex inorganic salt solution, finely ground cellulose, cysteine, resazurin, agar and rumen fluid. This was sterilized and dispensed in sterile CO_2 washed tubes. The tubes were then cooled and inoculated, flushing the tubes with CO_2 while they were being inoculated. After inoculation the tube is stoppered, inverted, then returned to a horizontal position and rotated to seal the junction between the glass and the stopper. This forms a thin layer of agar throughout the tube that will allow the cellulose digesting colonies to be readily seen. The colonies were subcultured with a Pasteur pipette and the same precautions regarding the maintenance of anaerobiosis were employed. The culture was considered pure after two consecutive subcultures produced single types of colonies macroscopically and similar types of organisms microscopically.

Using these techniques Hungate (1943) isolated nine cellulose-decomposing organisms and determined that they occurred in the rumen in numbers ranging from 18,500 to 1,200,000,000 per ml. By calculation he estimated that the original rumen content contained 40,000,000 cellulose-decomposing bacteria per ml. In (1950) Hungate described two other types of cellulolytic organisms that fermented cellulose to volatile and non-volatile fatty acids.

Sijpesteijn (1948) described in detail her work with cellulose-decomposing cultures from the rumen of cattle. The work was carried on over a period of five years, but only three years actual time was involved in the research. She obtained one pure culture of a cellulose-decomposing organism from the rumen and named it Ruminococcus flavefaciens. Another type that was obtained in "nearly pure culture" was named Ruminococcus parvum. She found that maintenance of anaerobic conditions was important. The decomposition products of the cellulose digestion were largely volatile and non-volatile fatty acids.

Unemzu, Tuda, Katuno, Omori, and Minagaki (1951a, 1951b) studied the biological characteristics of the aerobic bacteria of the rumen. They were unable to culture cellulolytic organisms aerobically, but suggested that the aerobic bacteria may play a part in the cellulose digestion indirectly. The symbiosis postulated was one in which the reducing substances were produced by the aerobes, therefore promoting the growth of the anaerobic organisms.

Bryant (1952), and Bryant and Burkey (1953a, 1953b) isolated and described some rumen microorganisms. He used the technique outlined by Hungate (1950) to obtain in pure culture a spirochete reported by Hungate (1947) but which Hungate was unable to obtain in pure culture. This organism is not a cellulose digesting organism, but lives on the breakdown products of the cellulose digesting organisms. Thus it is usually found in close association with the cellulose decomposing bacteria. The more recent publications deal with the numbers and more numerous groups of bacteria in the rumen. They grouped the organism cultured into thirteen groups according to morphology and biochemical characteristics. Only two

of the 13 groups reported are cellulolytic in pure culture, but eight groups attacked cellobiose.

Heald (1952) used xylose in a basal medium to study anaerobic xylose fermenting organisms of the rumen. He isolated 17 strains of gram-negative rods belonging to the coliform intermediate group. These organisms showed marked variations in the end products of fermentation when the substrate was changed from xylose or glucouronic acid to glucose or cellobiose. Like the other workers, the main end-products of fermentation found were volatile and non-volatile fatty acids.

Many in vitro studies have been made of rumen bacteria using mixed cultures. Wenger, Booth, Bohstedt and Hart (1940) incubated rumen ingesta in CO_2 to maintain anaerobiosis. Louw, Williams and Maynard (1949) improved upon this method by placing the rumen ingesta and the substrate in a visking cellulose casing to allow for dialysis of the byproducts of fermentation. These techniques have become known as the "artifical rumen" and has been used by Burroughs, Arias, Gerlaugh and Bethke (1950), Oyaert, Quin, and Clark (1951), McNaught (1951a), Wasserman, Duncan, Churchill and Huffman (1952) and Gall and Glaws (1951) and Huhtanen and Gall (1953).

Some investigators have used "washed cell" suspensions to study the metabolism of rumen bacteria. The rumen liquor is fractionated using various speeds of a centrifuge and the fraction containing the majority of the organisms is resuspended and used as inoculum on various substrates. This technique has been used by Lewis (1950, 1951) to study nitrate reduction, Elsden and Sijpestijn (1950) to study the decarboxylation of succinic acid and Doetsch, Robinson and Shaw (1952) to investigate keto acid production.

Quinn (1943), McAnally (1943), Walter (1952) and McBee (1950) employed a manometric technique to evaluate the activity of the rumen microflora. This consisted of adding the ruminal liquor to a buffered substrate and measuring the respiration of the cells using a Warburg apparatus.

In vivo Studies

Monroe and Perkins (1939) determined the pH of rumen contents at 2 hour intervals. They found that the pH varied greatly depending upon the time of feeding and the type of ration. Generally speaking, the pH before feeding was near neutrality and decreased to 6.0 within three hours after feeding, then rising slowly to neutrality again. Rations of hay and grain gave the highest readings, pH 7.01, and blue grass pasture gave the lowest reading, pH 6.47. Smith (1941) placed the electrodes of the pH meter in the rumen and found that the pH of the rumen ingesta in situ was 0.6 of a pH unit lower than when the sample was withdrawn and checked. He also found that closing the cover of the fistula lowered the reading 0.3 of a pH unit. When feeding an alfalfa ration the pH of the rumen ingesta was 6.3. Monroe and Perkins (1939) have shown that the pH of rumen ingesta 12-18 hrs. after feeding is about 7.34. This alkaline condition is brought about by the constant flow of saliva that has a pH of about 8.5 to 8.71 (Reid and Huffman 1949).

Patel (1952) investigated chemical and nutritional properties of rumen contents of cattle and found the surface tension of rumen liquor to vary from 54.86 to 69.87 dynes per sq. cm. This is somewhat higher than was found for saliva by Reid and Huffman (1949) when they recorded a range of from 45.54 to 49.20 dynes per sq. cm.

Armsby (1911) stated that non-protein nitrogen could be a partial substitute for protein in the ration of ruminants. Although true protein

was needed, the non-protein nitrogen could be used for maintenance, milk production and growth.

Johnson, Hamilton, Mitchell and Robinson (1942) stated the protein of a ruminants ration is digested in the abomasum in the same manner as in any monogastric animal. The protein produced in the rumen by the bacteria is then available to the host animal and the biological value of the protein would be relatively constant. They found that the biological value of the nitrogen in the ration was about 60 when the rations contained from 10-12% protein.

Loosli and Harris (1945) fed lambs rations containing urea and urea plus methionine. The added methionine caused increased gains and nitrogen retention. They considered urea formed bacterial protein mildly deficient in methionine.

Reid, Moir and Underwood (1949) investigated the value of rumen bacterial protein. They separated the cells by fractionation using various speeds of a centrifuge and concluded that they were low in digestability, high in biological value and mildly deficient in methionine. Bacterial protein formed from green and dry rations had about the same cystine content but the protein from the green feed contained a higher level of methionine. Later, Moir and Williams (1950) found that about 50 percent of the ingested protein was converted to bacterial protein, as the protein in the ration increased, the biological value of the bacterial protein increased.

Block and Stekol (1950) and Block, Stekol and Loosli (1951) fed radioactive sodium sulfate (S^{35}) to ruminants and found that the cystine and methionine formed contained radioactive sulfur in appreciable amounts.

In case of the goat, the radioactivity was the same for the methionine and cystine in the milk, serum albumin, and rumen. These results indicate that methionine and cystine were synthesized at approximately the same rate as were used by the tissues to make protein in the quanities needed.

The synthesis of lysine by bacteria during incubation of rumen contents in vitro has been shown by McNaught (1951), McDonald (1948) fed a lysine free ration to sheep that had both rumen and duodenal fistulas. The organisms recovered contained 7 percent lysine in their mixed proteins. They estimated that about 40 percent of the zein had been converted to microbial protein.

Agrawala, Duncan and Huffman (1953) and Duncan, Agrawala, Huffman and Luecke (1953) studied rumen synthesis and passage in steers fed natural and purified rations. They concluded that about 60 percent of the dry matter was removed from the rumen in six hours irrespective of the ration fed and that "true" protein synthesized varied from 33 to 109 gm. depending upon the ration fed. Considerable amino acid synthesis was demonstrated, and with the exception of histidine, the amino acid pattern found in the mixed protein of the purified diet was essentially the same as found in the natural ration. The synthesis of lysine was proportionally greater than that of methionine.

Chance (1952) studied rumen synthesis and passage in steers on natural and purified diets supplemented with aureomycin. The passage noted here was somewhat slower than reported by Agrawala et al. (1953) and could be attributed to the feeding of the aureomycin. The majority of the passage had occurred by 12 hours. The amount of amino acids present in the rumen ingesta was less when aureomycin was fed as compared to the control period.

He noted that the rate of removal of amino acids was increased when 0.5 gm. of aureomycin was added but reduced again when the amount of aureomycin was increased to 1.0 gm per day.

Many methods have been used to study the activities of the rumen microorganisms. This study was undertaken to determine if a combination of several of these techniques could be employed to determine the activity of the rumen bacteria on a particular ration.

PROCEDURE

Animals used in the Study

The animals used in this study were two three-year old steers 707, a Guernsey and 714, a Holstein which were fitted with lucite fistula plugs. These plugs were fitted with caps that could be easily removed when a sample was desired, or could be disassembled and removed to empty the rumen contents.

Previous to this study the animals were maintained on a ration of hay alone for 30 days. A ration of hay and corn was fed during the first two week period and a ration of hay and distillers solubles was fed during the second two week period of the experiment.

Method of Feeding

The steers were fed the total ration of 4 pounds of corn and 13 pounds of hay once daily. The same was true during the second period when the corn was replaced with 4 pounds of distillers solubles. The corn was placed in the manger and usually consumed in 30 minutes at which time the hay was fed. The hay was eaten readily, usually being consumed in less than three hours. The distillers solubles (containing 20.5% rye solubles) were refused periodically. In these cases the solubles were emptied directly into the rumen through the fistula for two or three days after which time the animals would again eat the ration. Chemical analysis of ingredients in the ration are given in Table I.

Water was fed ad libitum.

TABLE I

Chemical Analyses of Corn, Hay and Distillers Solubles
Used in the Natural Ration

Ration	Dry Matter %	Crude Protein %	Methionine %*	Lysine %*
Hay	91.9	10.00	0.026	0.080
Corn	89.6	11.00	0.155	0.111
Distillers solubles	86.2	30.25	0.500	0.711

* Based on dry matter

Sampling Procedure

Chemical analysis. The rumen contents were removed through the fistula opening after disassembling and removing the plastic plug. The ingesta was thoroughly mixed, weighed and composite samples were taken for chemical analysis. Approximately a 1000 gm sample was taken for amino acid, total nitrogen and dry matter determinations. These operations were done quickly so as to prevent excessive aeration and cooling of the ingesta that was returned to the rumen. The sample taken for chemical analysis was placed in a brown glass bottle and one liter of 95% ethyl alcohol was added to stop bacterial action and facilitate later handling and drying. These samples were taken on the 14th day of the experimental period.

Bacteriological and physical chemical analysis. These samples were taken four hours after feeding at four or five day intervals throughout the experimental periods and three times daily on the thirteenth day (the day preceding the sampling for chemical analysis). These samples taken on the thirteenth day were taken before feeding, four hours and

nine hours after feeding. Rumen organisms can likely withstand the treatment accorded them when the ingesta is weighed and then returned to its natural enviroment, but attempting to culture these organisms on an artifical medium at that time may be more difficult. These samples were taken with a sterile 10 ml dipper so that a representative sample of the ingesta could be obtained. The ingesta was placed in a screw cap sample bottle and transported to the laboratory. Care was taken to fill the bottle to capacity to prevent excessive aeration.

Method of Analysis

Chemical analyses. The samples of rumen contents and feeds taken for chemical analyses were dried in a forced-hot air oven at 60 C. The dried samples were ground in a Wiley mill to pass a 20 mesh screen and stored in air tight glass containers until analyzed. An extended period of time passed between drying and the actual analyzing, so the samples were redried and all determinations were carried out on oven dried samples.

The total nitrogen determinations were carried out using the Kjeldahl method as outlined by the AOAC (1950).

Hydrolyzates for the amino acid determinations were prepared according to the procedure of Stokes, Gunness, Dwyer and Caswell (1945). One gram of the material was dispersed in 25 ml of 6N HCl and autoclaved for eight hours at 121 C. The hydrolyzates were cooled, neutralized with NaOH, made up to 100 ml volume, filtered, covered with a few drops of toluene and stored in the refrigerator until analyzed. The concentration of this solution was 10 milligrams per ml.

The microbiological method was used to determine the amino acids. The composition of the media used for determining the amino acids is

TABLE II

COMPOSITION OF THE MEDIA USED IN AMINO ACID ASSAY
(Per 500 milliliters of double-strength medium)

Composition	I	II
H_2O_2 treated peptone (gm)	—	7.5
DL (-)-Alanine (mg)	200	—
L (+)-Arginine-HCl (mg)	100	—
L-Asparagine (mg)	200	—
L(-)-Cystine (mg)	200	100
L(+)-Glutamic Acid (mg)	400	—
Glycine (mg)	100	—
L(+)-Histidine•HCl•H_2O (mg)	100	—
DL-Isoleucine (mg)	200	—
DL-Leucine (mg)	200	—
DL-Methionine (mg)	200	—
DL-Phenylalanine (mg)	100	—
L(-)-Proline (mg)	50	—
DL-Serine (mg)	200	—
DL-Threonine (mg)	200	—
DL-Tryptophan (mg)	100	100
L(-)-Tyrosine (mg)	100	100
DL-Valine (mg)	200	—
Glucose (gm)	20	20
Na acetate (anhyd.) (gm)	20	12
NH_4Cl (gm)	—	6
KH_2PO_4 (mg)	500	500

TABLE II (concluded)

Composition	I	II
K_2HPO_4 (mg)	500	500
$MgSO_4 \cdot 7H_2O$ (mg)	200	200
$FeSO_4 \cdot 7H_2O$ (mg)	10	10
$MnSO_4 \cdot 4H_2O$ (mg)	10	10
NaCl (mg)	10	10
Adenine $SO_4 \cdot 2H_2O$ (mg)	10	10
Guanine $HCl \cdot 2H_2O$ (mg)	10	10
Uracil (mg)	10	10
Xanthine (mg)	10	—
Thiamine·HCl (mg)	0.5	1.0
Pyridoxine·HCl (mg)	1.0	2.0
DL-Ca pantothenate (mg)	0.5	2.0
Riboflavin (mg)	0.5	2.0
Nicotinic acid (mg)	1.0	2.0
p-Aminobenzoic acid (mg)	0.1	0.01
Biotin (ug)	1.0	5.0
Folic acid (mg)	0.01	0.0015
pH before autoclaving	6.8	7.0

given in Table II. Medium I, McMahan and Snell (1944) was used for Leuconostoc mesentroides P-60 (8042) to determine lysine. Medium II, Lyman, Mosely, Butler, Wood and Hale (1946) was used for Lactobacillus mesentroides for the determination of methionine.

The assays were carried out according to the procedure of preparing a standard in triplicate of the amino acid being assayed. Duplicate 1.0 ml, 2.0 ml and 3.0 ml portions of the diluted hydrolyzate were used to determine the amount of amino acids in the sample. The tubes were incubated for 72 hours at 37 C to permit development of the lactic acid. This acid was adjusted electrometrically with 0.1 N NaOH to pH 7.0.

Bacteriological analyses. The methods of Huhtanen, Rodgers and Gall (1952) were followed to culture the rumen bacteria. The rumen ingesta was weighed directly into a wide mouthed glass stoppered bottle containing 90 ml of sterile buffered distilled water. This dilution blank had been saturated by bubbling with CO_2 for two minutes and immediately before use 0.1 ml of sterile 10% cysteine solution in 2.3% $NaHCO_3$ was added. All dilution blanks used were treated in this manner. Dilutions of 10^{-1}, 10^{-3} and 10^{-5} were prepared and a 1.0 ml portion was inoculated into a tube containing 9ml of broth, outlined in Table III. Dilutions of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} were prepared by planting 1.0 ml from the preceding tube to the tube containing the dilution being made. Vaspar seals were used in the first part of this work and later it was found that a neutral oil would be as satisfactory (Mallmann, 1953). Any tube showing turbidity within a 12 day period was considered positive. One loopful of this culture was transferred to an agar deep (Table III) and maintained there by subsequent transfers until it could be purified.

TABLE III

COMPOSITION OF MEDIA USED FOR CULTURAL STUDIES

	Broth	Agar	Carbohydrate fermentation Base [3]
Bacto Beef Extract	10 gm[1]	10 gm	—
Bacto Peptone	10 gm	10 gm	10 gm
Bacto Tryptone	10 gm	10 gm	10 gm
Bacto Yeast Extract	10 gm	10 gm	10 gm
Glucose	1 gm	1 gm	—
K_2HPO_4	1 gm	1 gm	1 gm
KH_2PO_4	1 gm	1 gm	1 gm
Agar		15 gm	—
Cysteine (10% solution in 2.3% $NaHCO_3$) [2]	0.1 ml	0.1 ml	0.05 ml
Alfalfa meal [2]	trace	trace	—
Carbohydrate being tested	—	—	10 gm

1. Ingredients sufficient to make 1000 ml.

2. Added directly to the tubes after dispensing.

3. Usually dispensed in 5 ml amounts.

Media were sterilized at 121 C for 10 minutes.

The purification of the cultures was carried out by using a transfer from the agar deep to a broth to establish a 48 hour culture. One loopful of the mixed culture was placed in an agar shake tube, this was mixed well, a transfer was made from this tube to a second shake tube and so on until three tubes had been inoculated. These were poured into petri dishes and incubated in Brewer anaerobic jars. The cultures that were pure microscopically and gave only one colony type upon subsequent transfer as well as maintained their purity microscopically were transferred to carbohydrate fermentation broths (Table III). The cultures that were not pure were again replated using the shake tube method in attempts to purify them.

The bacteria-free filtrates were obtained by filtering the materials through an ultra fine fritted glass filter.

Physical Chemical Analyses. The rumen liquor was expressed from the remainder of the sample and used for these determinations. The pH determinations were carried out using a Cenco electrometric titration pH meter. The surface tension was measured using a Cenco DuNouy tensiometer. The method followed was that described by Central Scientific Company, Bulletin 101.

Triplicate readings were carried out on each sample.

RESULTS

Composition of Rumen Ingesta of Cattle Fed Natural Rations

The weights and a partial analysis of the constituents of the rumen ingesta obtained at 0-, 4- and 9- hour collections are presented in Table IV. These data are arranged by hour of collection and ration fed so that the data pretaining to the two rations fed a given animal may be compared. It was felt that there was considerable individual variation in the animals used in this experiment. The consistency of the rumen ingesta of steer 707 was consistently lower in dry matter and more finely mascerated than that of steer 741. This increased breakdown of the fibers would allow for greater bacterial action.

Rate of Removal of Dry Matter and Crude Protein from the Rumen

The amount of dry matter and crude protein present in the rumen at 0-, 4- and 9- hours, as well as the dry matter and crude protein fed were calculated. From the amount present in the rumen and the amount fed, calculations were made to determine the rate of removal of dry matter and crude protein during the 0-4 hour and 0-9 hour periods (Tables V and VI). The rate of removal was based on the intake of the component.

Calculations were also made to determine the amount and rate of removal of dry matter and crude protein from the rumen during the 4-9 hour periods. The 4- hour collection was used as a base, and the rate of removal was based upon the intake of the constituent.

TABLE IV

COMPOSITION OF RUMEN INGESTA OF CATTLE
FED NATURAL RATIONS

Ration Fed	Animal No.	Time Hrs.	Weight of Rumen Contents gm	Dry Matter gm	Dry Matter %	Crude Protein gm	Crude Protein %
		0	57,885	5152	8.82	676	13.13
Corn and Hay	707	4	67,816	6510	9.55	879	13.50
		9	63,986	6655	10.37	952	14.31
		0	53,852	6307	11.71	788	12.50
Corn and Hay	741	4	54,508	8296	15.22	1021	12.31
		9	53,600	7563	14.11	1025	13.56
		0	58,420	5374	9.20	775	14.06
Distillers Solubles and Hay	707	4	65,728	6571	10.10	1035	15.75
		9	60,890	5541	9.10	883	15.94
		0	60,354	8268	13.60	1142	13.81
Distillers Solubles and Hay	741	4	73,262	11282	15.40	1812	16.06
		9	60,208	7766	12.80	1320	17.00

TABLE V

REMOVAL OF DRY MATTER FROM THE RUMEN
OF CATTLE FED NATURAL RATIONS

Ration Fed	Animal No.	Sampling Time	Dry Matter In Rumen	Fed	Total	Removed*	
			gm	gm	gm	gm	%
		0	5152	7050	12202	0	0
Corn and Hay	707	4	6510	0	6510	5692	81
		9	6655	0	6655	5547	78
		4-9 hr				-145	
		0	6307	7050	13357	0	0
Corn and Hay	741	4	8296	0	8296	5061	72
		9	7563	0	7563	5794	82
		4-9 hr				733	4
		0	5374	6988	12362	0	0
Distillers Solubles and Hay	707	4	6571	0	6571	5791	83
		9	5541	0	5541	6821	98
		4-9 hr				1030	15
		0	8268	6988	15256	0	0
Distillers Solubles and Hay	741	4	11282	0	11282	3974	57
		9	7766	0	7766	7518	98
		4-9 hr				3544	51

* Based on dry matter intake.

TABLE VI

THE REMOVAL OF CRUDE PROTEIN FROM THE RUMEN OF CATTLE FED NATURAL RATIONS

Ration Fed	Animal No.	Sampling Time	Crude Protein In Rumen	Fed	Total	Removed*	
			gm	gm	gm	gm	%
		0	676	721	1397	0	0
Corn and Hay	707	4	879	0	879	518	72
		9	952	0	952	445	62
		4-9 hr				-73	
		0	788	721	1509	0	0
Corn and Hay	741	4	1021	0	1021	488	68
		9	1025	0	1025	484	67
		4-9 hr				-4	
		0	775	1015	1770	0	0
Distillers Solubles and Hay	707	4	1035	0	1035	735	72
		9	883	0	883	947	93
		4-9 hr				212	21
		0	1142	1015	2157	0	0
Distillers Solubles and Hay	741	4	1812	0	1812	345	34
		9	1320	0	1320	837	82
		4-9 hr				492	48

* Based on crude protein intake.

TABLE VII

METHIONINE AND LYSINE PRESENT IN RUMEN INGESTA
OF CATTLE FED NATURAL RATIONS

Ration Fed	Animal No.	Sampling Time	Methionine				Lysine			
			In Rumen		Fed	Total	In Rumen		Fed	Total
		hr	%	gm*	gm	gm	%	gm	gm	gm
Corn and Hay	707	0	0.068	3.50	3.27	6.77	0.168	8.70	6.13	14.83
		4	0.093	6.04	0	6.04	0.237	15.40	0	15.40
		9	0.099	6.59	0	6.59	0.356	23.69	0	23.69
Corn and Hay	741	0	0.066	4.16	3.27	7.43	0.282	17.79	6.13	23.92
		4	0.069	5.72	0	5.72	0.319	26.46	0	26.46
		9	0.083	6.27	0	6.27	0.365	27.60	0	27.60
Distillers Solubles and Hay	707	0	0.127	6.82	9.22	16.04	0.395	21.22	15.45	36.67
		4	0.111	7.29	0	7.29	0.361	23.72	0	23.72
		9	0.141	7.81	0	7.81	0.226	12.52	0	12.52
Distillers Solubles and Hay	741	0	0.088	7.27	9.22	16.94	0.437	36.13	15.45	51.58
		4	0.103	11.62	0	11.62	0.505	56.97	0	56.97
		9	0.105	8.15	0	8.15	0.401	31.14	0	31.14

* Based on dry matter.

Methionine and Lysine Present in Rumen Ingesta

The amounts of methionine and lysine present in the rumen ingesta were determined. The percentages and amounts, based on dry matter intake and dry matter present in the rumen (Table V) are recorded in Table VII.

The pH and Surface Tension of Rumen Liquors

The pH and surface tensions of the rumen liquors are recorded in Table VIII. These readings were taken at intervals throughout the experimental periods and at 0-, 4- and 9- hours on the thirteenth day of each period. All other determinations were made on 4- hour samples.

Number of Organisms as Determined by Limiting Dilutions

The number of viable anaerobic bacteria present in the rumen of the animals used are recorded in Table IX. A single series of tubes was inoculated for each animal for each time the rumen ingesta was cultured. There was no significant difference in the number of rumen organisms cultured on the two different rations. The rumen ingesta of steer 707 contained slightly higher numbers of organisms cultured, but it is questionable whether this difference is significant. There was not a significant difference on samples taken at different times on the same day.

Pure Culture Work

Nine different organisms were isolated in pure culture and studied (Table X). All of the cultures studied were isolated using anaerobic cultural techniques and all carbohydrate fermentations were carried out anaerobically. The fermentation broth was made up and used without an indicator. After 96 hours incubation 0.1 ml of 0.008% brom cresol purple solution was added to detect acid production.

Culture 8a. A gram positive diplococcus was frequently found. It fermented all of the carbohydrates tested to acid, gas was not produced. One culture, 34B, had the same biochemical reactions, but was extremely pleomorphic, some cells ranging to 3μ in length. Final pH in glucose broth was 4.6.

Culture 37. A gram positive micrococcus was slightly smaller and spherical in shape. There was no clumping exhibited in broth cultures. The biochemical reactions were similar to 8a, and glucose broth was fermented to a final pH 4.5.

Culture 55. This organism was isolated once and gave rather inconclusive results in the biochemical tests, producing gas in glucose, maltose, sucrose, and xylan and the basal medium with no acid production.

Culture 6ab. A gram positive rod occurring in pairs was isolated two times. This organisms was pleomorphic, occasionally exhibiting large "pear shaped" cells. The fermentation of the carbohydrate was restricted to the six carbon atom configurations and gave a final pH in glucose of 4.2.

Culture 2b. Fermented all of the six carbon atom carbohydrates and arabinose. This was the smallest of the rod form cultured being 0.4μ x 1.0μ.

Culture 6a. A larger gram positive rod, 1.0μ x 3 μ. The colony was larger than 2b. This organism was relatively unreactive fermenting glucose to a final pH of 4.8 and fermenting dextrin and xylose to a final pH of 6.0 - 6.2.

Culture 5b. A large gram positive rod with swollen sporangium and terminal spore was reactive in all of the carbohydrates, producing acid and gas. This organism produced gas in the basal medium.

Culture 60. A gram positive rod, 0.5μ x 3μ was smaller than organism 5b and was not as reactive as organism 5b. This organism also produced gas in the basal medium.

Culture 57. A gram positive rod 1.0μ x 2μ, produced acid in maltose, acid and gas in sucrose and dextrin, and gas but no acid in starch.

Symbiotic Cultures

Two organisms were cultured that grew symbiotically. One was an obligate symbiont, the second organism was successfully cultured separately.

Growth in broth and carbohydrate fermentation. These organisms denoted as 11A and 11B, grew rapidly in broth and produced large amounts of gas. Turbidity was present in 10 hours after transfer in some cases. Microscopic observations of a 24- hour culture usually revealed a gram positive sporeforming rod, 0.5-1μ x 4-6μ, with a spore 1.0μ x 1.5μ and a gram negative non-sporeforming rod 0.5μ x 2μ. In eight and twelve hour cultures vegetative cells predominated. When this mixed culture was inoculated into fermentation tubes containing various carbohydrates all carbohydrates were fermented to acid and gas including the basal medium (Table XI).

Colonial morphology. When shake tubes were prepared and plates poured of this culture very dense growth was present in the first and second plates and very little growth could be seen on the third plate. Characteristic gas formation was found where the organisms grew in contact with the bottom of the petri dish and many small bubbles were present throughout the agar plate. The initial plate, with colonies in very close proximity showed three particular types of colonies; (a) small compact

spindle shaped, (b) filamentous type and (c) combination of "a" and "b". Only mixed colonies were observed on the plates containing well separated colonies.

Bacteria-free filtrates, prepared by filtering a 48- or 72- hour broth culture of these organisms through a ultra fine fritted glass filter, were obtained. These filtrates were used as enrichment in the agar plates and broth in attempts to purify the cultures. In the first trial, 1.0 ml of filtrate was used as enrichment. This stimulated the growth of both organisms but did not allow for the development of well isolated colonies. This was repeated using 2 ml of the filtrate as enrichment. One of the symbiotic organisms was isolated from a plate in this trial. Upon subsequent transfer to broth, it grew very slowly, and after five days it developed a pellicle immediately under the oil film. When transferred to an agar deep it grew exclusively aerobically. While previously, in combination with the smaller rod it grew under anaerobic conditions. This culture (11B) was inoculated into an agar deep simultanously with the symbiotic culture. There was no aerobic growth evident until the seventh day after transfer, while the anerobic growth was present in 24 hours.

Bacteria-free filtrates of the aerobic broth culture of 11B was used as an enrichment in an attempt to isolate 11A. This was not effective. A bacteria-free filtrate of a broth culture of 11B grown anaerobically was obtained. Three ml was used per plate as an enrichment. Shake tubes of the symbiotic culture were prepared and the plates were incubated anaerobically. One isolated colony that contained gram positive and negative rods corresponding to the size of organism 11A was present on

the second plate. Growth did not occur when this colony was transferred to an anaerobic broth tube containing 1 ml of 11B anaerobic filtrate. A duplicate transfer of this colony to a second tube simultaneously inoculated with culture 11B did not produce the characteristic symbiotic growth. There was evidence of growth of organism 11B after four days.

This particular combination of organisms has been seen in five different cultures.

TABLE VIII

THE pH AND SURFACE TENSION OF RUMEN LIQUORS OF CATTLE FED NATURAL RATIONS

Ration Fed	Days on Ration	Sampling Time	pH Steer		Surface Tension* Steer	
		hr	707	741	707	741
	5	4	6.8	6.6		
Corn and Hay	9	4	6.9	7.4		
	12	4	6.9	6.4		
	13	0	7.4	7.4	55.6	55.6
		4	6.8	6.6	56.4	56.4
		9	6.4	6.2	56.9	56.9
	4	4	6.8	6.6	56.6	55.6
	8	4	7.0	6.6	46.3	55.4
Distillers Solubles and Hay	13	0	7.4	7.0	55.7	58.0
		4	6.9	6.4	55.4	53.7
		9	6.4	6.6	62.5	60.3

* Expressed in dynes per sq. cm.

TABLE IX

NUMBERS OF VIABLE ANAEROBIC BACTERIA
IN THE RUMEN OF CATTLE
FED NATURAL RATIONS

Ration	Days on Ration	Sampling Time	Numbers of organisms	
			Steer 707	Steer 741
		hr		
	1	4	100*	100
	5	4	10,000	10,000
Corn and Hay	9	4	100	10
	12	4	100	10
	13	0	100	10
		4	1,000	10
		9	1,000	1,000
	4	4	100	100
	8	4	1,000	1,000
Distillers Solubles and Hay	13	0	1,000	1,000
		4	100	10,000
		9	10,000	1,000

* Millions per gram

TABLE X

CHARACTERISTICS OF ORGANISMS ISOLATED IN PURE CULTURE

No.	Morphology: Microscopic	Morphology: Colonial	Glucose	Maltose	Sucrose	Lactose	Dextrin	Starch	Arabinose	Xylose	Xylan	Base	No. of Isolations
8a	Gram ≠ diplococcus 0.5μ x 1μ	Circular, smooth entire, opaque	A	A	A	A	A	A	A	Sl	Sl	-	5
37	Gram ≠ cocci spherical 0.5μ	Circular, smooth Entire, opalescent	A	A	A	A	A	A	A	Sl	Sl	-	4
55	Gram ≠ diplococcus	Eliptical, opaque	-G	-G	-G	-	-	-	-	-	-G	-G	1
6ab	Gram ≠ rod 1μ x 1.5μ	Irregular, lobate spreading	A	-	A	Sl	A	Sl	-	-	-	-	2
2b	Gram ≠ rod 0.4μ x 1.0μ	Spindle, smooth opaque	A	A	A	A	A	A	A	-	-	-	2
6a	Gram ≠ rod 1μ x 3μ	Circular, 3mm dia. opaque	A	-	-	-	Sl	-	-	Sl	-	-	2
5b	Gram ≠ rod 1.0μ x 3μ	Irregular, lobate opaque	AG	AG	AG	AG	AG	AG	AG	AG	AG	-G	1
60	Gram ≠ rod 0.5μ x 2-3μ	Spreading, opalescent	AG	AG	AG	AG	AG	AG	SlG	AG	SlG	-	1
57	Gram ≠ rod 1.0μ x 2μ	Eliptical, opaque	-	A	AG	-	AG	-G	-	-	-	-	1

A-Acid G-Gas Sl-Slight Acid (-) No reaction

TABLE XI

CULTURAL CHARACTERISTICS OF SYMBIOTIC ORGANISMS

No.	Morphology: Microscopic	Morphology: Colonial	Glucose	Maltose	Sucrose	Lactose	Dextrin	Starch	Arabinose	Xylose	Xylan	Base	Fructose	Galactose	Raffinose	Litmus milk	Citrate	Methyl Red	Voges Proskauer
	Anaerobic growth																		
11 A and B			AG	AG	AG	AG	AG	AG	AG	AG	AG	AG							
11A	Gram - rod 0.5μ x 2μ	Spindle shaped																	
11B	Gram + 0.5-1μ dia. 4-6μ length terminal spore	Filamentous opalescent																	
	Aerobic growth																		
11B	Gram + 1μ x 4-6μ terminal spore	Mucoid spreading opaque	A	A	-		-	-					A	-	-	-	-	-	-

AG - Acid and Gas A - Acid (-) No reaction

DISCUSSION

Passage of Dry Matter and Crude Protein from the Rumen

It is difficult to determine the rate of passage of ingesta from the rumen because it is a moving system, and there is an influx of feed into it over a period of several hours and possibly a near simultaneous passage of ingesta to the omasum, abomasum, and lower digestive tract. The material present in the rumen and the amount fed can be determined and it is assumed the part unaccounted for was absorbed through the rumen wall or passed to the omasum and abomasum. The data presented in Table V show that a high percentage of the dry matter present in the rumen was removed during the first 4- hours after feeding. When the animals were fed hay and corn, this was evident in both animals while the rumen passage during 4- to 9- hours is relatively small. In the case of steer 707, the difference in weights of the 4-9 hour samples on this ration shows an accumulation of 145 gm of dry matter. This is impossible as the animals were not fed during this time and it is thought that these weights are within the ranges of sampling error for this animal. When the steers were fed distillers solubles and hay, they were not in agreement on the rate of passage in 4- hours. Steer 707 had completed 83 percent of the passage in 4- hours while steer 741 had only 57 percent of the passage completed. However, at the 9- hour collection period both animals had passed 98% of the dry matter to the lower digestive tract. In view of the apparent accumulation of dry matter at 0- hour on this ration as compared with the corn and hay ration, this value of 98% passage appears high. Because of the unpalatibility of these solubles noticed in this work and reported

by Huffman, Duncan and Benne (1951), the solubles fed on that day were placed directly into the rumen when the ingesta was replaced. This coupled with a thorough mixing of the ingesta upon two occasions on that day could have stimulated cellulolytic breakdown and subsequent rumen passage.

Although the rate of removal of the crude protein is slightly different than that of the dry matter, it follows the same general pattern. There was an apparent accumulation of protein during the 4-9 hour periods when the steers were fed hay and corn. Some of this could have been caused by sampling error, but some may have resulted from synthesis of bacterial protein from non-protein-nitrogen. When the distillers solubles and hay were fed, there was a continual removal of protein from the rumen during the 0-4 hour and 4-9 hour periods. Again animal 741 demonstrated slower rate of passage than animal 707.

The rate of removal of dry matter in this experiment was considerably faster than the values reported by Chance, Huffman and Duncan (1953) and Hale, Duncan and Huffman (1947). Chance et al. reported removal of 52.6% to 39.2% of the dry matter in six hours, and 69.6% to 60.0% removal in twelve hours. These steers were fed a basal diet of 15 pounds hay and 4 pounds corn. This difference may be reconciled by the following facts: the ration used by Chance et al. contained two pounds more of hay and the hay used was of different analysis. The animal common to both experiments was one year older and the percentage of dry matter in the rumen ingesta of this animal had dropped from a range of 11.9% to 14.1% reported by Chance et al. to a range of 8.82% to 10.37% as noted in this work. Hale et al. investigated the rumen passage and digestion in cattle fed hay alone and the values, when calculated in the same manner, are similar to those of Chance et al. Agrawala et al. (1953) feeding a natural and a

purified diet found a dry matter passage ranging from 54.6% to 63.9%. These animals were under one year of age, but were fed a natural ration of approximately the same composition as was used in this study.

The rate of removal of crude protein was similar in both animals when they were fed corn and hay (Table VI). Steer 707 passed 72% of the crude protein to the lower digestive tract in 4- hours while steer 741 passed 68%. During the 4-9 hour periods, both steers demonstrated an apparent accumulation of crude protein. Comparing this with the data of Table V pretaining to dry matter passage could indicate that these apparent accumulations were the result of error or the end-products of bacterial activity. The latter explanation seems plausible as there was very little dry matter passage in this period, thus allowing the microorganisms ample time to act upon the substrate present. The differences in the rates of removal of the crude protein of the rumen ingesta on the ration of distillers solubles and hay is probably caused by a combination of variation between the animals and microbial activity. On this ration steer 741 showed an accumulation of crude protein and dry matter at 0- hour, indicating a lower coefficient of digestion in the rumen. The rate of removal of crude protein continued at a high level over the entire 9- hour period in both animals. This did not allow for a period of relative quiesence needed to demonstrate an apparent accumulation of crude protein as was shown with the ration of corn and hay during the 4-9 hour period. The rate of removal of crude protein reported in this study is faster than observed by Chance et al. (1953), Hale et al. (1947) and Agrawala et al. (1953).

The Amounts of Methionine and Lysine Present in the Rumen

These data in Table VII show the percentages and grams of methionine and lysine that were present in the rumen ingesta of the steers fed the two rations. There was no significant changes in the amount of methionine present in the rumen ingesta when the steers were on a ration of corn and hay. There was a slight increase during the 4-9 hour period but not sufficient to be significant. There was a slight increase in the amount of lysine present in the rumen ingesta from steer 707 during the 0-4 hour period and a significant increase during the 4-9 hour period. The increase in grams of lysine present in the rumen ingesta of steer 741 was not as marked as for steer 707, but a significant increase was observed. These values correlate well with the apparent accumulation of crude protein that was present during these periods (Table VI).

When the ration was changed to distillers solubles and hay, the continual removal of dry matter did not allow for apparent accumulation of crude protein or amino acids. The rate of removal of methionine paralleled the removal of crude protein in steer 707, but was slightly faster in animal 741. The lysine in the rumen ingesta of steer 707 was again correlated with the removal of crude protein from the rumen. However, during the 0-4 hour period on this ration, steer 741 had less dry matter and crude protein passage than steer 707 and the analysis of the rumen ingesta showed a slight increase in the amount of lysine present. During the 4-9 hour period on this same ration, the rates of removal of dry matter and crude protein were increased and there is a marked decrease in the amount of lysine present in the rumen ingesta. These data are not in agreement with that of Chance, Duncan, Huffman and Luecke (1953b) who

reported a continual decrease in the amounts of both methionine and lysine in the rumen ingesta during a twelve hour period. The apparent synthesis of lysine corresponds with the data of Duncan et al. (1953). However, these authors reported a greater increase in the amount of methionine present in the rumen ingesta in 6- hours than was observed in this study. Block and Stekol (1950) and Block et al. (1951) using radioactive $Na_2S^{35}O_4$ demonstrated the synthesis of methionine in the rumen. McNaught (1951) incubated rumen ingesta in vitro and reported a synthesis of lysine.

The pH and Surface Tension of Rumen Liquors

The pH studies reported in this work are similar to some of those reported by Agrawala et al. (1953). They are higher in some instances than those reported by Smith (1941). This may have been caused by the partial aeration of the sample that occurred during manipulation. Taking the pH readings of samples obtained from the animals on the hay and corn ration at 0-, 4- and 9- hours, as an indication of bacterial activity (accumulation of acids) would tend to indicate that the bacteria were active during the 4-9 hour period. This also correlates with the apparent synthesis of protein and lysine.

The measurements of surface tension were relatively constant with a few exceptions. The reading of 46.3 dynes per sq. cm. obtained from a sample taken from steer 707, on a ration of distillers solubles and hay is low. It is interesting to note that this sample was higher in pH (7.0) and there is a possibility that this sample contained a higher proportion of saliva. The higher readings of 60.3 and 62.5 dynes per sq. cm. were obtained when the pH was lower and could indicate an absence

of saliva or an accumulation of fermentation byproducts that could raise the surface tension. These measurements are subject to slight inaccuracies because of the small amount of debris and combined gas in the rumen liquors. The removal of these would mean that the surface tension measurements were made on a solution that did not represent rumen liquor.

Number of Viable Bacteria Cultured

This particular technique of using a single series of one tube per dilution is subject to a very serious amount of error when one attempts to determine numbers of bacteria present in a given sample. Also the nature of the rumen ingesta did not allow for the procurement of a composite sample. The numbers presented are reported as relative numbers of bacteria that could be cultured and not as the total number of bacteria present or as an exact number of those present that could be cultured. The numbers cultured are lower than reported by Gall et al. (1949) and Huhtanen et al. (1952). An explanation for this may be that these workers express the liquor from the sample until they assume it is about 20 percent dry matter and use this solid material to culture with, while in this work the rumen ingesta was used as it was obtained from the rumen. It is possible, that in some cases, the material cultured contained only one half of the dry matter that Gall et al. used. Throughout this study there did not appear to be a significant difference in the numbers of the organisms cultured.

There were no direct microscopic counts made on these samples because it was felt that none of the techniques published were sufficently accurate or adaptable to warrant their inclusion in any survey work.

Pure Culture Studies

A total of nineteen organisms was isolated in pure culture. These organisms were compared and grouped into nine types of cultures. Organism 8a is similar to organism RO6-TER isolated and reported by Gall and Huhtanen (1951). The remainder of the organisms do not compare with any organisms reported by Gall and co-workers. The biochemical tests have not been sufficiently thorough to allow indentification according to Bergy's Manual. The gas formation that occurs in the basal medium in the absence of added carbohydrate is unexplained. It may be caused by trace amounts of carbohydrate present in the yeast extract or it may be a product of protein decomposition or the combination of both.

It should be stated that after these cultures were subcultured as mixed cultures, the 38 C incubator used to incubate these organisms developed mechanical difficulty and the remainder of the work was carried out in a 35 C incubator. At the time that the incubator developed mechanical difficulty the cultures were at room temperature for 1½ days. Subsequent subculture at 35 C was not successful in several cases.

Symbiotic Cultural Studies

A culture of two symbiotic organisms was studied. One organism 11B was isolated in pure culture and found to grow aerobically and only very poorly anaerobically. When growing anaerobically in symbiosis with culture 11A it was gram positive, and formed terminal spores.

Eight and 12 hour cultures of this organism exhibited larger numbers of vegetative cells than the 24- hour culture. Organism, 11A, was not successfully cultured in pure culture. An organism resembling 11A was cultured on an agar plate when a bacteria-free filtrate of an anaerobic

broth culture of 11B was used as an enrichment. Subsequent subculture was not successful. At this time "organism 11A" was gram positive. It is thought that these organisms stimulate each other by reciprocol symbiosis and they pass the peak of their growth phase quickly.

SUMMARY

Two steers fitted with lucite rumen fistula plugs were used to study the bacterial activity in the rumen. These animals were fed a ration of corn and hay for two weeks and then fed distillers solubles containing 20.5% rye solubles and hay for 2 weeks. The distillers solubles were found to be unpalatable.

The samples taken for chemical analysis were obtained by completely emptying the rumen of the steers at 0-, 4- and 9- hours after feeding. Determinations were made of percent of dry matter, crude protein, methionine and lysine. Samples were taken for bacteriological determinations, pH and surface tension measurement throughout the feeding periods and during the days immediately preceding the sampling for chemical analyses.

The rate of removal of dry matter and crude protein was extremely fast when the ration containing corn and hay was fed. The analysis of the 4-9 hour samples taken during this period showed very little dry matter passage and an apparent accumulation of crude protein. When the steers were fed the ration of hay and distillers solubles, the rates of removal were slower and there was not the apparent accumulation of crude protein during the 4-9 hour period.

An apparent synthesis of lysine occurred in the rumen as shown in the data derived from the amino acid assay of the rumen ingesta on the hay and corn ration. There was very little accumulation of methionine on either ration.

The pH determinations correlate with the accumulation of crude protein and lysine. The measurements of surface tension did not appear to contribute any significant data.

There was not a significant change in the numbers of anaerobic bacteria cultured using the limiting dilution technique. Subculture of these organisms resulted in the isolation of nine different types of organisms of a total of nineteen pure cultures isolated.

A culture of two anaerobic symbiotic organisms was studied. One organism was obtained in pure culture and was found to grow aerobically. Attempts to obtain the second organism in pure culture were unsuccessful.

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